Title: Intergenerational and transgenerational epigenetic inheritance in animals

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Abstract:

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

Main text:

Introduction

DNA is a reliable information transfer system because of the accuracy of DNA replication. Humans, for example, copy 6 billion bits of information to their offspring with an error rate of approximately 2 bits per 100 million[1]. However, eggs and sperm contain more than DNA, and it has become increasingly apparent in recent years that other molecules beyond the genome sequence transfer information between generations. Moreover, there are mounting examples in which this information is altered depending upon the physiological and environmental conditions of previous generations. Multiple mechanisms have been proposed to underlie non-DNA sequence-based inheritance and these can be either genome-associated (e.g. covalent modifications of DNA and histones or transfer of small RNAs complementary to genomic sequences) or genome-independent (e.g. microbiome transfer). They also vary in their generational duration, with inheritance spanning one generation to a seemingly indefinite number.

The terms 'intergenerational' and 'transgenerational' are often used to describe such effects and require clarification. Transgenerational effects refer exclusively to phenomena that could not be ascribed to direct effects of a particular trigger on the affected organism. For instance, an environmental stimulus can directly affect a gestating embryo (and the already-formed oocytes within a female embryo in mammals[2, 3]). As such, only altered phenotypes occurring in the second or third generation after a trigger can truly be described as transgenerational for male and female transmission, respectively. Effects spanning shorter timescales are described as parental or intergenerational. Nonetheless, many described intergenerational effects share established mechanisms with transgenerational ones. Another term that warrants discussion is epigenetic, whose once broader meanings[4] have narrowed in recent years, not without objections[5], to most commonly refer only to genome-associated mechanisms of non-DNA sequence-based inheritance - chiefly DNA methylation, histone modifications and inherited RNAs[6]. These specific 'epigenetic mechanisms' underlie some, but not all, characterised examples of

intergenerational and transgenerational inheritance.

A key difference between DNA sequence-based and other mechanisms of inheritance is the fidelity of information transfer. Whilst DNA-based information transfer is extremely high-fidelity, other mechanisms are normally far less robust. Consequently, the timescales of reliable information transfer by DNA sequence-based and other mechanisms are usually very different[7]. One point of confusion concerns two separate distinctions that are often conflated: firstly, genetic (i.e. DNA-based) vs epigenetic mechanisms of inheritance, and secondly, environmentally-responsive vs unresponsive phenomena. Inheritance of environmentally-acquired traits can also be mediated through genetic inheritance, as occurs in the CRISPR innate immunity system of prokaryotes[8]. Conversely, stable long-term transcriptional repression is often achieved by an inherited epigenetic memory, but one that is largely unresponsive to environment and physiology. It is the question of whether epigenetic mechanisms can provide a heritable (and potentially adaptive) memory of ancestral environmental exposure that has proven most controversial[3].

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

Parental effects

Examples of parental genotype or environment affecting progeny phenotype independent of inherited DNA ('parental effects') are numerous. However, with direct contact between the individuals exposed to a trigger and their immediate progeny (or their mate), many potential mechanisms can be involved. To confidently implicate specific mechanisms of inheritance, careful experimental design and interpretation are required[3]. Particular research effort has been directed at paternal effects[6], with the expectation that limiting a male´s interactions with partner and progeny to the act of mating alone will narrow potential mechanisms down to those transmitted via gametes. Even so, genome-independent mechanisms may still affect progeny phenotypes **(Figure 1).** For example, microbiome transfer from father to mother can rescue the intergenerational effects of maternal antibiotic use in *Drosophila melanogaster*[9], and apparent paternal effects may in fact be cryptic maternal effects, when paternal condition, such as depression-like states in mice[10], influences maternal investment or care.

The parental effects of diet and obesity is a well-studied paradigm (reviewed in [11]), with obvious potential relevance to health given the rise in obesity rates in Western countries in the past few decades[12]. Intergenerational effects of parental nutrition have been suggested in humans[13, 14] and demonstrated in rodents[15-22], *D. melanogaster*[23] and *C. elegans*[24, 25]. In mammals, for example, under- or over-nutrition in either parent commonly impacts offspring glucose metabolism[11]. Counterintuitively, the effects of maternal and paternal diet are often qualitatively and quantitatively similar[21, 22, 26, 27]. However, such effects are often non-monotonic[23, 24] and can be dependent on the developmental context of parental or grandparental exposure[13, 15] and on progeny sex[13-15, 17] and diet[23]. For instance, both low- and high-sugar paternal diets increased offspring adiposity in *D. melanogaster*, but only when offspring were themselves challenged with a high-sugar diet[23].

Maternal provisioning and metabolism

Maternal provisioning to offspring may mediate effects of maternal diet[28, 29] or other physiological factors. For example, we recently found that increased provisioning of a lipoprotein yolk complex to offspring with advancing maternal age has a major impact on progeny growth rates and starvation resistance in *C. elegans*[30]. Offspring phenotypes may also be affected by provisioning of specific regulatory products such as mRNAs[31, 32] or essential micronutrients such as zinc[33]. Physiological alterations in maternally supplied organelles, particularly mitochondria, could also underlie parental effects of diet, as a maternal high-fat diet impairs fetal mitochondrial function in mice[21]. Perturbation of maternal metabolism genetically[34] or by dietary intake of specific metabolites can influence epigenomic regulation in progeny and even further generations (reviewed in [35]). For instance, progeny DNA methylation can be influenced by maternal dietary intake of methyl donors in mice[36] with striking heritable effects on coat colour. Similar effects have also been suggested in humans, where seasonal changes in dietary intake of methyl donors around conception in rural mothers correlate with alterations in DNA methylation in children[37].

Microbiome transfer

Non-DNA-based inheritance may also act via transfer of an altered parental microbiome[9]. Bacterial strains can be inherited maternally in humans[38], although the mechanisms- whether by breast milk, birth canal or even placental transfer - remain unclear[39]. In mice, diet-induced microbiome changes, specifically a progressive loss of taxonomic diversity due to a Western-style low-fibre diet, are cumulative over generations and eventually irreversible via extinction of specific microbiotic subpopulations[40]. This suggests that multigenerational environmental exposure could cause a stable transgenerational alteration of progeny physiology via the microbiome.

DNA methylation in sperm

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

inherited from the male or female germline – is associated with differences in DNA methylation and demonstrates that DNA methylation states *can* be transmitted between generations in mammals[41]. The sperm methylome is reportedly altered by various severe interventions which produce intergenerational or transgenerational effects, such as *in utero* malnutrition[42, 43], early-life overnutrition[44] and diabetes[45] in mice and by obesity in humans[46]. However, the mechanisms by which sperm methylation could be modified at specific sites are unclear. Moreover, methylation is largely erased upon fertilisation[47] and it is not obvious how alterations could affect gene expression in progeny with high penetrance[11]. It was also reported that sperm methylation was unaffected by several diets that induce phenotypic effects in progeny[48].

Although cytosine methylation is virtually absent from many organisms such as *D. melanogaster*[49] and *C. elegans*[50], it is now apparent that DNA methylation can also occur at adenosine residues, although the functional significance of this mark, and whether it carries information across generations[51], is unclear[52].

Small noncoding RNAs in sperm

Small noncoding RNAs (sncRNAs), particularly tRNA-derived small RNAs (tsRNAs) and microRNAs (miRNAs), are emerging as possible mediators of environmental information transmission through sperm in mammals (reviewed by [53]). Derived from precursor or mature tRNAs, tsRNAs are of diverse size and biogenesis[54] and have in the last decade been implicated in a range of cellular processes, including repression of transposable elements[54-56]. Like miRNAs[57], tsRNAs can interact with small RNA-binding proteins of the Argonaute family to induce post-transcriptional gene silencing[54, 58] via sequence complementarity to the 3'UTRs of target mRNAs[58, 59].

tsRNAs comprise most of the sncRNA pool in mature mammalian sperm [60], with miRNAs a distant second[55, 61]. Sperm tsRNAs are reportedly altered by diet[61] or exposure to an endocrine disruptor[62] in rodents and by obesity in humans[46], while sperm miRNAs are altered by psychological stress in mice[63, 64] and men[65], and by parental genotype[66], diet[67-69] and environmental deprivation[70] in mice, all conditions associated with paternally-acquired

disorders. Crucially, in several cases zygotic injection of total sperm RNA[64, 66, 69, 70], sncRNA fractions[61, 69] or specific sncRNAs[55, 66, 68, 71] could partially or fully recapitulate these paternally acquired phenotypes[11]. In mice, inheritance of sncRNA-mediated phenotypes has been reported to rely on the activity of the RNA methyltransferase *Dnmt2*[69, 72], indicating that RNA modifications may constitute an additional layer of regulation important for transmission of acquired phenotypes through sperm[61]. In keeping with a role in repressing transposons, which often use conserved tRNAs as primers for replication[56], a specific sperm-borne tsRNA influenced by paternal diet was found to specifically regulate genes governed by the pluripotency-promoting endogenous retroviral element MERVL in the mouse zygote[55]. Remarkably, it was shown that sperm tsRNAs do not originate from sperm tRNAs but rather are acquired via transfer of extracellular vesicles from the epididymis[55], offering a tantalising hint of soma-to-germline transmission of information. Recent results indicate that sperm miRNAs similarly acquired during epididymal transit could be essential for embryonic development[73].

Histone modifications

There is some[21, 23, 25], but little, evidence for covalent modifications of histones mediating parental effects. However, histone modifications are certainly transmitted between generations at some loci in mammals[74], fish[75] and worms[76] and they have been implicated in longer-lasting transgenerational phenomena. It is plausible, therefore, that they could also underlie some parental effects. In *C. elegans* an epigenetic memory of germline transcription, mediated by deposition of H3K36me3 on active genes[77, 78] and H3K27me3 on repressed genes[76], is passed from each generation to the next and is essential for germline viability[77, 78], representing an example of non-environmentally-responsive epigenetic inheritance that is critical for normal development and physiology.

<u>Multi-generation epigenetic inheritance</u>

Documented examples of true transgenerational epigenetic inheritance

(TEI) induced by parental genotype, physiology or environment are becoming increasingly numerous in model invertebrates. In most cases, however, the effects described have a limited duration, for example typically spanning 3-4 generations in *C. elegans*, before reversion to the baseline phenotype[79-82]. Characterised mechanisms commonly involve inheritance via gametes of genome-associated epigenetic information, such as histone modifications or small RNAs. The likely reasons for the limited lifetime of many transgenerational effects can be found in the passive and active mechanisms that underlie changes in small RNA populations and histone modifications from generation to generation[83].

Inheritance of RNAi in C. elegans

Although occurring in artificial laboratory conditions, the inheritance of gene silencing induced by ancestral RNAi interference (RNAi) in *C. elegans* has provided the most incontrovertible demonstration of TEI and has proven invaluable in dissecting the mechanisms involved. Worms supplied with exogenous double-stranded RNA (dsRNA), usually by feeding, employ an amplification machinery which results in systemic silencing of complementary genes in almost all tissues, including the germline. dsRNA is processed by Dicer and accessory proteins to form primary short interfering RNAs (siRNAs). Primary siRNAs bind to a member of the Argonaute family of small RNA-binding proteins and guide them to complementary mRNA transcripts. RNA-dependent RNA polymerases (RdRPs) are then recruited to produce abundant secondary siRNAs (otherwise known as 22G RNAs for their length and 5' guanosine bias). RdRP-associated silencing mechanisms are found in diverse taxa, although not in vertebrates. In turn, these 22G RNAs engage a variety of Argonautes to destroy complementary mRNAs, inhibit transcription[84] and deposit the repressive chromatin marks H3K9me3 and H3K27me3 at the target locus[84-86].

Gene silencing induced by dsRNA can be inherited[87, 88], typically for up to 3 generations[80] but sometimes as long as 80 generations when selecting for the resulting phenotype[88]. The nuclear Argonaute *hrde-1* (heritable RNAi defective) is dispensable for gene silencing in exposed worms but is necessary for its inheritance in subsequent generations[89], demonstrating that *C. elegans*

possesses cellular machinery dedicated to the information transmission over generations. The nuclear RNAi pathway, which shuttles 22G RNAs into the nucleus[90], is required for the maintenance of inherited silencing in progeny[91]. The limited typical duration of the silencing response may be due to dilution of siRNAs over generations[85]. Unlike primary siRNAs, secondary siRNAs rarely serve as templates for further amplification of the gene silencing response induced by dsRNA, which is therefore limited[92, 93]. The repressive H3K9me3 and H3K27me3 footprints triggered by secondary siRNAs also persist in the absence of the dsRNA trigger for at least 2 generations[85, 86], although H3K9me3 deposition is dispensable for heritable silencing at some loci[94, 95]. Interestingly, the H3K9 methylase *met-2*, responsible for H3K9me1/2, conversely limits the generational duration of some dsRNA-induced silencing by altering siRNA inheritance[96]. Application of additional dsRNA triggers unrelated to the original target in subsequent generations can extend the duration of inherited silencing, suggesting that negative feedback by downregulation of the RNAi machinery may act to limit the duration of a heritable response[97].

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

Small RNAs and histone modifications in TEI

The importance of small RNAs for the inheritance of RNAi-triggered repression in *C. elegans* underscores mobile RNAs as an attractive candidate for mediating transgenerational inheritance in multiple species (reviewed in [103]). dsRNA produced in somatic tissues, including neurons, can be inherited in *C. elegans*[104] and reports indicate transfer of somatic RNAs to gametes in mice[55, 73, 105]. The RNAi pathway in *C. elegans* was found to also target endogenous genes, utilising a similar amplification mechanism as exogenous RNAi[106, 107]. Indeed, endogenous RNAi is necessary for transgenerationally-inherited gene regulatory and physiological changes in response to ancestral starvation[108] and

heat stress[82].

Histone modifications are important in the inheritance of RNAi in *C. elegans*, and a variety of histone modifications have been implicated in other cases of transgenerational inheritance, including methylation of H3K4 in mice[109] and *C. elegans*[51, 79, 110, 111], H3K9 in *C. elegans*[51, 112-115] and H3K27 in *C. elegans* and *D. melanogaster*[114, 116, 117]. Stress-induced perturbations to histone modifications may revert slowly over generations[115], leaving a gradually fading transgenerational memory. In some cases global levels of histone modifications remain modified in later generations[115, 116] although in others global levels are unchanged[25, 79], implying differential regulation of specific loci[118]. In *C. elegans*, transgenerational expression of longevity phenotypes caused by ancestral mutations in the conserved COMPASS H3K4 methylases is dependent on the corresponding demethylase[79], demonstrating that alterations in the antagonistic activity of chromatin-modifying enzymes over generations can induce transgenerational phenotypes[51].

TEI to pre-adapt progeny to environmental conditions

Despite the increasing popularity of research into TEI, the evidence for adaptive, environmentally-responsive transgenerational inheritance, whereby ancestral experience equips progeny to better withstand environmental challenges, remains scant. At the time of writing most documented cases of inheritance of environmental experience occur in artificial contexts[110, 115], even when those experiments attempt to mimic naturally occurring challenges[81], and the relationship of ancestral environment to alterations in progeny gene regulation or physiology in terms of fitness is often far from clear[81, 82, 108, 116]. Nonetheless, a few reports suggest the possibility of adaptive TEI. A recent study reports that exposure of *C. elegans* to a heavy metals leads to increased resistance to the same stresses in future generations, what the authors call transgenerational hormesis[111]. Likewise, ancestral starvation in *C. elegans* induces transgenerational resistance to starvation, by unknown mechanisms[119, 120]. Despite most described TEI effects occurring in *C. elegans*, the most striking case of potentially adaptive TEI involving soma-to-germline communication is found in mice, where a conditioned fear response to a specific

odour in male mice can be inherited for two generations[121]. In this case, the effect was associated with enlargement of neuroanatomical structures in progeny, and with differential methylation of the locus encoding the corresponding odour receptor in the sperm of exposed males (though not their sons). Still, at present it seems that adaptive, environmentally-responsive TEI, if it exists, is the exception rather than the rule. Nonetheless, it is clear that epigenetic mechanisms can transfer information about ancestral state between generations, and although the extent of this transfer is typically limited to a few generations, some specific cases – arising from a loss of gene repression – can lead to longer-lasting memories.

Long-lasting TEI

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

For example, growth at elevated temperature[115] or impaired DNA replication during embryogenesis[122] can result in a loss of repression of heterochromatic transgene arrays in *C. elegans* that can take more than 10 generations to fully re-establish **(Figure 1b)**. Importantly, expression of a subset of endogenous repetitive elements repressed by H3K9me3 also heritably increased at elevated temperature, albeit for fewer generations[115]. Heat can also derepress pericentromeric heterochromatin in *D. melanogaster*[123], leading to a long transgenerational epigenetic memory of ancestral environment. In both *C. elegans* and *D. melanogaster*, multiple generations of heat exposure and consequent de-repression were required to maximise the generational duration of the resulting memory[115, 123]. These results are consistent with the gradual

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

The mortal germline of C. elegans

A reciprocal phenomenon to this slow recovery following chromatin perturbation is found in the mortal germline (Mrt) phenotypes of *C. elegans* mutants (and some naturally-occurring strains [124]), which display a progressive reduction in fertility, often temperature-sensitive, that accumulates over generations and ultimately results in sterility **(Figure 1c)**. While Mrt phenotypes of some mutations result from genetic changes such as telomere loss[125, 126], many genes with a mutant Mrt phenotype are involved in histone modifications[51, 89, 96, 127-130] or small RNA pathways[89, 129, 131, 132] and the phenotype can be rapidly reverted by returning animals to the permissive temperature[118, 124, 129], altering diet[133], re-introducing functional gene copies[127] or introducing downstream mutations[96], demonstrating that these transgenerational phenotypes are epigenetic in nature. Interestingly, a recent study found that the Mrt phenotype of *C. elegans* Piwi mutants results not from a profound loss of germline totipotency but rather from the aberrant (and reversible) induction of reproductive quiescence, normally induced under stress, as a consequence of transcriptional dysregulation in the germline[133]. If this finding is generally applicable it suggests why the reversion of accumulated Mrt phenotypes can be achieved so rapidly.

Stable TEI: enjoy the silence

Small-RNA-triggered stable silencing

The inherited repression of transposons and foreign DNA is essential for 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 sequences[136].
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 in [137]). 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 cycle[137]. 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 *1*[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 memory[82].
418 Recent work in *C. elegans* has elucidated how primary piRNAs provide
419 surveillance over germline transcription[138-141]. While piRNAs in mammals and *D.*
420 *melanogaster* exhibit near-perfect complementary base pairing with targets[137],
421 *C. elegans* piRNAs, like miRNAs[57], tolerate significant mismatches outside of a
422 5' seed region[141]. In this way, thousands of piRNAs can engage the entire
423 germline mRNA transcriptome[138]. 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 genes[142] 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

silencing[139]. A third mechanism is associated with the Argonaute CSR-1, whose bound 22G RNAs display complementarity to almost all germline-expressed genes[144] and which has been proposed to license gene expression[145, 146] by protecting mRNAs from piRNA targeting and subsequent siRNA generation[138]. Interestingly, both CSR-1 and the *C. elegans* Piwi orthologue PRG-1, along with newly discovered proteins that seem to have a role in transgenerational epigenetic inheritance[147, 148], reside in perinuclear phase-separated liquid-like granules[144, 149] with a defined spatial organisation[147], suggesting that the temporal order of transit through this system of granules of mRNAs exiting the nucleus may be important for RNA-directed silencing and licensing mechanisms[147, 148]. However, this hypothesis awaits experimental verification.

Mechanisms of stable silencing

In *C. elegans*, once silencing has been initiated by piRNAs, target sequences can remain stably repressed for many generations even in the absence of the triggering piRNA-Piwi complex[112, 113, 150], although in some cases Piwi may still act to maintain silencing[139]. The maternal transmission of tertiary 22G RNAs, downstream of secondary 22G RNAs and the germline nuclear RNAi pathway including *hrde-1*, is sufficient for inherited piRNA-initiated silencing, indicating that a feed-forward amplification loop maintains high levels of siRNAs in the absence of both the trigger and the initially silenced locus[93]. Mutually reinforcing feedback between small RNAi pathways and repressive chromatin, such as those demonstrated in *Schizosaccharomyces pombe* and *Arabidopsis thaliana* (reviewed in [151]), would explain the extraordinary stability of this silencing[83]. An analogous mechanism has been proposed in *D. melanogaster* (reviewed in [152]), although to date such a feedback has not been convincingly demonstrated in animals. Nonetheless, it is clear that stable gene silencing generally involves multiple epigenetic pathways. In *C. elegans*, the multigenerational stability of piRNA-initiated silencing requires both the RNAi pathway and chromatin modifiers, especially H3K9 methyltransferases[113, 135]. Secondary piRNAs also guide DNA methylation at the targeted locus in mice[153, 154] and the formation of heterochromatin at the targeted locus in *D. melanogaster*[155-158] **(Figure 2)**.

Conclusions and outlook

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

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

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

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

Conflict of interest statement

The authors report no conflict of interest.

Figure/Table Legends

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

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

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

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

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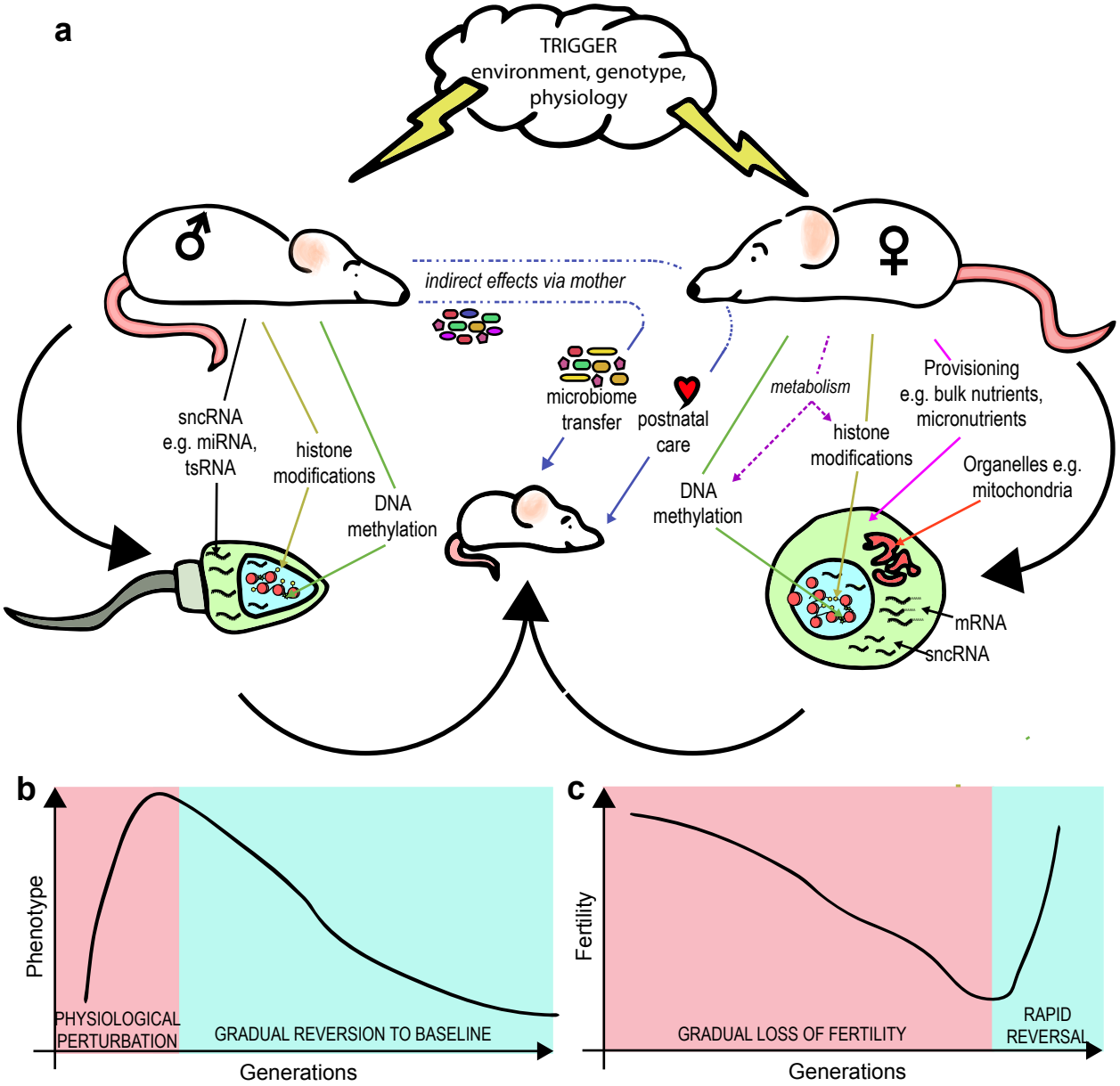
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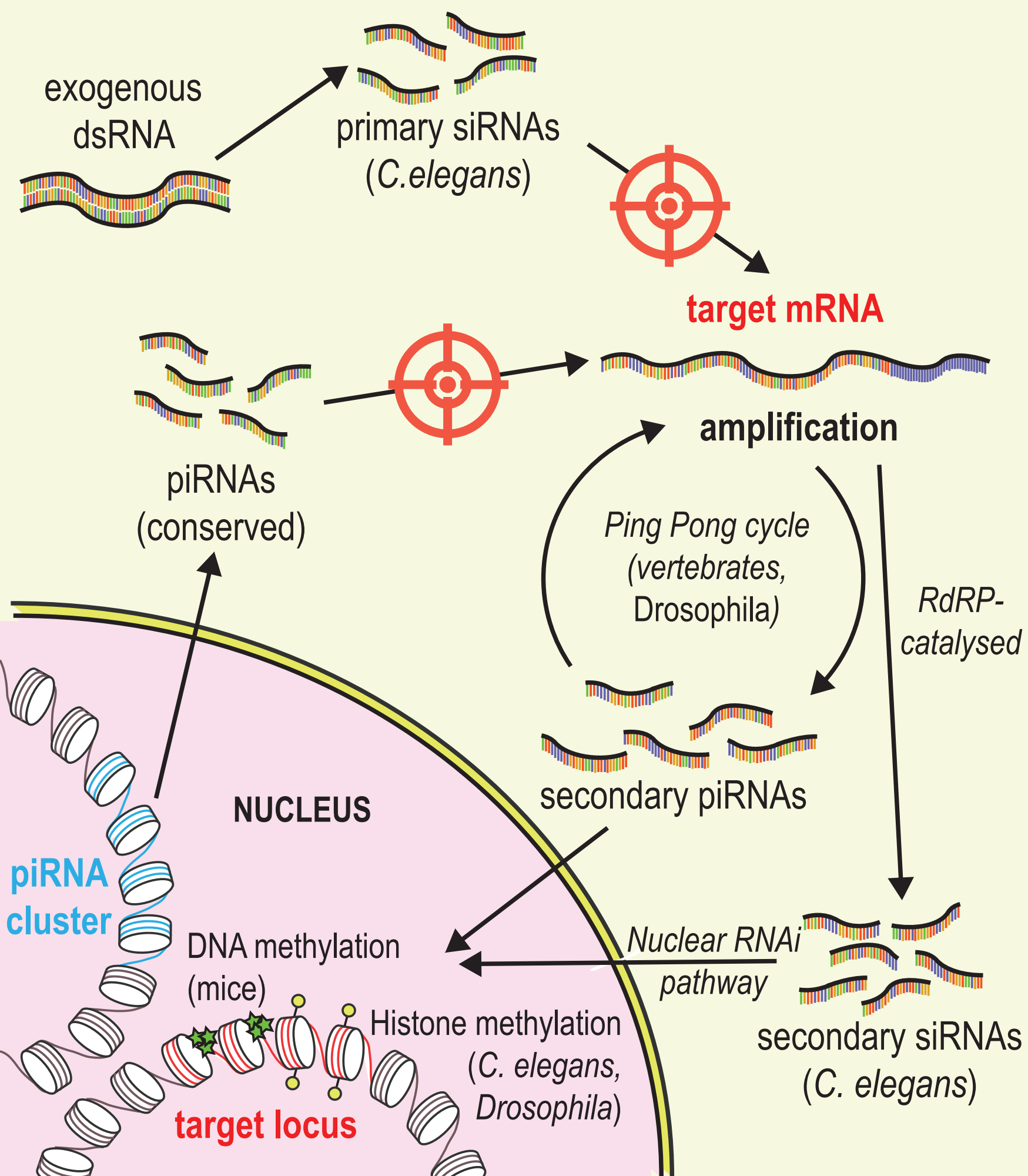
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Nuclear RNAi
pathway
Histone methylation
(C. elegans,
Drosophila)
target locus
secondary siRNAs
(C. elegans)

Duration	Trigger	Species	Effects on progeny	Proposed mechanism of inheritance	Ref
1 generation	Paternal high-sugar diet	*D. melanogaster*	High triglyceride levels (on high-sugar diet)	Chromatin modifications in sperm (H3K9me3, H3K27me3)	23
1 generation	Young mother	*C. elegans*	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	*Mus musculus, Rattus norvegicus*	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	*D. melanogaster*	Delayed development	Heritable depletion of riboflavin-producing commensal bacteria	9
1-2 generations	Ancestral high glucose diet	*C. elegans*	Reduced fecundity, resistance to oxidative stress	COMPASS H3K4 methylases required for inheritance of stress resistance	25
2 generations	Maternal dietary supplementation with methyl donors	*M. musculus*	Alterations in coat colour	Increased DNA methylation at the agouti locus caused by retrotransposon insertion	36
2 generations	Undernourishment during pregnancy	*M. musculus*	Metabolic alterations	Hypomethylation of specific loci in F1 males	17, 43
2 generations	Paternal odour-conditioned fear response	*M. musculus*	Inherited fear response to specific odour	Neuroanatomical changes in progeny, locus-specific hypomethylation in sperm	121
2-3 generations	Exposure to various mild stresses	*C. elegans*	Increased stress resistance and proteostasis	Somatic insulin signaling, COMPASS H3K4 methylases in germline	111
3 generations	Ancestral mutation in COMPASS H3K4 methyltransferases	*C. elegans*	Increased longevity	Altered histone methylation, longevity phenotypes due to possible alteration in lipid metabolism	79; 159
3 generations	Overexpression of H3K4 demethylase in sperm	*M. musculus*	Reduced survival, developmental abnormalities	Alterations in sperm-borne RNA	109
3 generations	Ancestral development at elevated temperature	*C. elegans*	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	*C. elegans*	Inherited gene repression	Secondary siRNAs; histone methylation	80, 88, 89
3 generations	Ancestral starvation during larval stage in wildtype worms	*C. elegans*	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)	*D. melanogaster*	Alterations in eye colour	Disruption of heterochromatin by phosphorylation of ATF-2	123
3-9 generations	Ancestral starvation during larval stage in AMPK mutants	*C. elegans*	Reduced fecundity	Abnormal methylation of H3K4 by COMPASS histone methylases	110
14 generations	Growth at elevated temperature (multiple generations)	*C. elegans*	Increased expression from repetitive transgene array	Loss of H3K9me3-mediated repression	115
Indefinite	Spontaneous transgene silencing in the germline	*C. elegans*	Stable gene silencing with no reversion	piRNA-targeting induced nuclear RNAi guided by secondary siRNAs; histone methylation	112, 113, 134, 135

Table 1.