Review

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Transgenerational epigenetic inheritance: resolving uncertainty and evolving biology

Abstract: Transgenerational epigenetic inheritance in animals has increasingly been reported in recent years. Controversies, however, surround this unconventional mode of heredity, especially in mammals, for several reasons. First, its existence itself has been questioned due to perceived insufficiency of available evidence. Second, it potentially implies transfer of hereditary information from soma to germline, against the established principle in biology. Third, it inherently requires survival of epigenetic memory across reprogramming, posing another fundamental challenge in biology. Fourth, evolutionary significance of epigenetic inheritance has also been under debate. This article pointwise addresses all these concerns on the basis of recent empirical, theoretical and conceptual advances. 1) Described here in detail are the key experimental findings demonstrating the occurrence of germline epigenetic inheritance in mammals. 2) Newly emerging evidence supporting soma to germline communication in transgenerational inheritance in mammals, and a role of exosome and extracellular microRNA in this transmission, is thoroughly discussed. 3) The plausibility of epigenetic information propagation across reprogramming is highlighted. 4) Analyses supporting evolutionary significance of epigenetic inheritance are briefly mentioned. Finally, an integrative model of 'evolutionary transgenerational systems biology' is proposed to provide a framework to guide future advancements in epigenetic inheritance.

Keywords: epigenetic memory; exosome; microRNA; soma to germline communication; transgenerational epigenetic inheritance.

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Introduction

Despite the fallacy of Lamarck's theory of evolution and its two hundred years of discredit, discourse on inheritance of acquired characteristics has staged a surprise entry in mainstream biology. In its modern avatar, this hitherto improbable mode of heredity has arrived in the garb of transgenerational epigenetic inheritance. At the core is experimental evidence suggesting germline inheritance of environmentally induced phenotypes across generations in animals including mammals (1–11). In transgenerational inheritance, the epigenetic basis is theoretically inferred from an inability to explain the transmission based on known features of DNA mutation and genetic inheritance (9, 11–13), with practical demonstration that primary DNA sequence changes do not indeed underlie the reported heritability still remaining (11, 14). Overall, the transmission is considered to be mediated not by genetic mutations but by other factors in the germ cells such as the usual epigenetic marks, namely, DNA methylation and histone modifications, and non-chromatin factors like RNA that can influence gene expression and epigenetic state (2, 14–20). Epigenetic inheritance is consistent with emerging evidence supporting the post-fertilization presence and propagation of gametic DNA methylation (2, 9, 14, 21–28) and histone modifications (1, 2, 6, 7, 9, 14, 15, 22, 27–33), and a role of noncoding RNAs (ncRNAs) in epigenetic regulation and transmission of epigenetic information across generations (1, 2, 6, 7, 9, 14, 22, 27, 28, 34–42). Although it has been argued that DNA methylation and histone modifications are not self-perpetuating and lack target specificity, as opposed to RNA that can be contributed by the gametes and bear base sequence specificities, and therefore do not truly represent 'epigenetic' that implies memory (43, 44), emerging evidence does suggest that these marks can be directly or indirectly inherited across generations (15, 17).

Differences in the mechanism and course of germline epigenetic modification and reprogramming render epigenetic memory survival across generations more likely in plants than animals (14, 34, 45–47). For example, unlike

mammals, plant DNA methyltransferases act during gametogenesis and embryogenesis, thereby allowing propagation of DNA methylation marks from parent to progeny (45). In mammals, DNA methylation and histone marks are efficiently reset during reprogramming both in the germline and in the zygote immediately after fertilization, leaving little chance for inheritance of epigenetic modifications (14). However, evidence suggests that certain marks do escape from these reprogramming events (28, 48). Regarding RNA-mediated epigenetic information transfer, the presence of RNA-dependent RNA ploymerases in plants, as also in the worm *Caenorhabditis elegans* and yeast, can allow amplification of inherited small RNAs and perpetuation of epigenetic effects (38, 39). Besides post-transcriptional regulation, small RNAs can also regulate gene expression at the transcriptional level by interacting with RNA binding proteins to trigger DNA methylation in plants, yeast and mice, and to induce histone modifications in plants, yeast, worm and the fruit fly *Drosophila melanogaster* (35, 39, 49).

Germline inheritance signifies epigenetic transmission via gametes, a mode that is distinct from context-dependent transmission wherein somatic epigenetic modifications can be imposed in each generation due to persistence of inducing factors in the environment (14–16, 22, 50–52). Theoretically, environmental exposure may induce epigenetic modifications in the germline either directly or through affecting somatic cells (53–55). The former possibility is consistent with the fundamental principle which states that hereditary information flows from germline to soma, not in reverse. The latter however poses a fundamental challenge in biology as it envisages information transfer in the reverse direction (15). In plants, the germline is formed from somatic cells following exposure of developmental and environmental cues, is poorly defined and is subjected to somatic modification (14, 45), attributes that are permissive for inheritance of acquired traits. Moreover, in plants, as also in *C. elegans*, small RNAs move systemically, and evidence suggests that these molecules can cross from somatic cells to germ cells and mediate transgenerational epigenetic inheritance (38, 39, 55–58). In *C. elegans*, for example, exogenous dsRNA induces a systemic RNAi response wherein small RNA movement from soma to germline can trigger gene silencing across generations (38, 58). Moreover, neuron to germline transmission of dsRNA leading to transgenerational silencing of a gene of matching sequence in worm has also been demonstrated recently (59). Extracellular RNAs also exist in mammals, largely contained within exosomes, and these RNAs show several similarities with mobile RNAs in plants and worm in terms of intercellular communication potential (57, 58). Interestingly, bioinformatic analyses have shown an association between circulating miRNAs and gene expression in transgenerational inheritance in mammals (60, 61). Consistent with this, a concept of exosome-mediated soma to germline information transfer in epigenetic inheritance has been advanced (10, 61–63). Remarkably, supporting experimental evidence has recently been produced in mouse (4).

In mammals, studies that report a phenotype at least in F3 generation, if not beyond, following exposure of F0 gestating female, or F2 generation following F0 male exposure, are considered transgenerational, providing evidence for epigenetic germline inheritance. This is because an environmental factor can be in direct contact with F1 and, through its germline, F2 generations in female exposure example, and with F1 in the case of male exposure. Therefore, to exclude the possibility that the phenotype observed is not caused by direct exposure, it is required that a phenotype is demonstrated in the first unexposed generation, which is F3 in the case of female exposure and F2 in the case of male exposure. Unfortunately, studies falling short of this standard have often been inappropriately termed transgenerational, creating confusion (8). Another controversy is with regard to social transmission wherein a phenotype can appear due to either a direct interaction between the ancestral and descendant generation or an indirect interaction through maternal rearing conditions that can influence descendant biology (3). Studies involving *in vitro* fertilization, cross-fostering and multiple descendant generations far removed from the exposed generation are therefore required to exclude possible confounding by social transmission (3). A caveat here is that *in vitro* fertilization and cross-fostering may themselves cause an effect and complicate analysis (64), and also, such measures would not exclude other confounders like cryptic genetic variation (15). Nevertheless, paucity of such studies has been one of the reasons for considering the existence of transgenerational epigenetic inheritance in mammals as uncertain (15). It is however notable that certain studies do have confirmed inheritance following *in vitro* fertilization (22, 65), cross-fostering (62, 66, 67) and analysis of multiple generations (12, 68–72).

Most of the reported examples of germline epigenetic inheritance in mammals relate to maternal exposure in the founding generation (51, 52). However, inferring germline epigenetic inheritance in experiments describing maternal exposure encounters greater difficulties due to, as mentioned above, potential confounds including effects of *in utero* environment and somatic components of oocytes, maternal care and social and behavioral transfer (14, 15, 22, 31, 52). In contrast, male contribution to

offspring is supposed to be largely limited to sperm, and hence discerning germline inheritance faces lesser complications in schemes employing paternal exposure and male lineage (1, 11, 27, 31, 52, 73). A caveat here is that non-gametic ejaculate-borne information carriers may also influence the offspring phenotype following male exposure (20, 27, 74) and, in mammals, fathers can influence offspring development through direct paternal care or through affecting quality of mother-infant interactions (75). Nevertheless, reports of paternal exposure-induced inheritance via the male line have been scant, causing serious concern about existential evidence of transgenerational epigenetic inheritance (52). However, a few studies do have indeed produced evidence of paternal exposure-induced transmission in animals including mammals. Besides the first report in *Drosophila* (76), these studies describe male exposure-induced transgenerational epigenetic inheritance via paternal lineage in rats (73) and mice (22, 52, 62, 66, 77–79).

Cumulatively, germline epigenetic inheritance in animals, especially mammals, has been controversial, with not only perceived implausibility of DNA methylation and histone marks surviving reprogramming, and of soma to germline communication posing fundamental impediments in its acceptability, but also seeming deficiencies in its demonstrated occurrence causing existential dilemma (2, 3, 8, 14–16, 43, 44, 62, 80, 81). This article counters these disputes by highlighting key experimental and conceptual advances. Evolutionary significance of epigenetic inheritance is another area of debate (82–88) that this article discusses. Subsequently, an integrative model of transgenerational epigenetic inheritance based on supporting evidence is presented.

Existential evidence

As mentioned, inheritance via paternal lineage following male exposure provides stronger evidence of transgenerational epigenetic inheritance. Experiments demonstrating that are reviewed here in detail. In the *Drosophila* example referred above, Sharma and Singh examined the transgenerational effect of the neuroactive drug pentylenetetrazole, a γ-aminobutyric acid receptor antagonist, in a freshly generated isogenic line (76). Of note, demonstration of epigenetic inheritance in animals using isogenic strains safeguards against potential confounding by genetic variations (80). In the fly study, the F1 and F2 generations were produced via the male line following F0 paternal exposure, and microarray-based gene expression profiling across generations was carried out to investigate if drug-induced transcriptomic changes are inherited. Interestingly, the drug was found to induce transcriptomic alterations not only in the founder males' central nervous system (CNS) and testis, but also in the F1 CNS and testis, and the F2 CNS. In microarray clustering, the F0 male CNS closely resembled the F2 male CNS, and the F0 and F1 testis resembled the F1 and F2 CNS, in that order. This suggested that the transcriptomic effect of pentylenetetrazole is inherited through the germline. In *Drosophila*, epigenome reorganization occurs during both gamete differentiation and early embryogensis (89). The above fly study thus implied that environmentally induced epigenetic changes are propagated across reprogramming.

In another example, it was reported that subjecting primiparous female mice (F0) and their litters (F1) to unpredictable maternal separation combined with maternal stress (MSUS) results in inheritance of altered behavioral responses to aversive conditions in paternal line-derived F2 and F3 generations (77). The MSUS paradigm was characterized by maternal care deprivation only in F1, not F2 and F3, generation. Interestingly, Franklin et al. observed depressive-like behaviors not only in F1 males but also in F2 females and F3 males (77). Altered social exploration, on the other hand, characterized F2 and F3, not F1, males (78). As the females bred to F1 and F2 males showed normal maternal behaviors, the transmission was considered to represent epigenetic rather than social inheritance. Confounding effects of physiological and developmental factors were also considered unlikely because the males did not have any contact with their pups in the MSUS paradigm (78). Next, Franklin et al. found in F1 MSUS sperm increased DNA methylation in the CpG island surrounding the transcription initiation site of two candidate genes: *Mecp2* encoding a transcriptional regulator that binds methylated DNA, and *Cnr1* encoding the cannabinoid receptor-1 that is associated with emotionality in rodents (77). In contrast, a decreased methylation was observed in the CpG island located 5′ of the transcription initiation site of the *Crhr2* gene that encodes corticotrophin-releasing factor receptor 2, a stress hormone receptor. Strikingly, these methylation changes were also observed in the brain of F2 females. The changes were associated with expected gene expression levels in the F2 female brain, confirming functional relevance of altered DNA methylation. Besides, like F1 sperm, F2 sperm also showed *Mecp2* hypermethylation and *Crhr2* hypomethylation. Males exposed to MSUS, when adult, exhibit altered behavioral responses along with changes in histone post-translational modifications at the mineralocorticoid receptor (MR) gene and

decreased expression levels of MR in the hippocampus (4). The behavioral characteristics were reproduced by mimicking these molecular changes *in vivo* through pharmacological manipulation. Both F1 MSUS males and the F2 offspring exhibited impaired long-term memory when adult. In the hippocampus of F1 and F2 individuals, the long-term potentiation (LTP) was abolished, and cross-fostering experiments provided evidence supporting male germline, not maternal care, mediated transmission of the LTP phenotype (66). Further, decreased levels of DNA methylation at the promoter of the *Prkcc* gene encoding brain-specific γ isoform of protein kinase C were observed both in the hippocampus of the offspring and in the sperm of fathers, with the former also showing altered levels of *Prkcc* expression (66). Together, these findings strengthened the epigenetic basis of MSUS-induced phenotypes. Second, like F1 MSUS males, F1 females also transmit behavioral alterations to their offspring (64). The known normal maternal behavior of these females, together with the finding that control pups cross-fostered to these females do not show altered behavioral responses, supported a germline-based inheritance rather than social transmission. In a separate study on transgenerational inheritance of chronic social instability-induced behaviors in mice, cross-fostering experiments did not provide evidence for maternal care as an underlying factor (67). Third, consistent with the understanding that stress in early life can be a metabolic dysregulator, MSUS was also found to cause altered glucose metabolism across generations (90). The F1 MSUS sperm, and the brain structures associated with stress response, hippocampus and hypothalamus, showed altered expression of miRNA including miR-375. It is notable here that in a separate study, upregulation of several miRNAs including miR-375 was independently shown in sperm of chronically and variably stressed male mice offspring of which exhibited altered stress responsivity along with gene expression changes in stress regulating brain regions (91). In the MSUS model, F2 hippocampus also exhibited abnormal miRNA levels. This suggested that MSUS-induced transmission originates from changes in F1 sperm miRNAs. Notably, injection of sperm RNA isolated from MSUS males into fertilized mouse oocytes from naive females resulted in offspring with altered behavioral, metabolic and molecular phenotypes, as observed in the offspring of MSUS-exposed males. The *in vitro* fertilization experiment therefore strongly supported germ cell-mediated nongenetic transmission of MSUS-induced characteristics. Notably, *in vitro* fertilization experiments have also provided evidence for sperm-mediated transmission in a mouse model of chronic social defeat stress-induced phenotypes (65).

In one study, epigenetic inheritance of chemical-induced hepatic fibrosis was investigated in rats (73). Zeybel et al. treated F0 adult male rats with the hepatotoxin carbon tetrachloride to induce chronic wound healing leading to liver fibrosis, allowed for injury cessation and resolution of fibrosis, and then used the rats to obtain future generations via male line. Outbred rats were used in the experiment to reduce the potential confounding influence of wound healing-related genetic traits. Notably, following carbon tetrachloride treatment, the F2 males showed, compared to control, a significantly decreased amount of fibrotic collagens and a significantly reduced number of smooth muscle α-actin positive myofibroblasts, the major cellular drivers of hepatic fibrosis, in the liver. At the molecular epigenetic level, Zeybel et al. found in the liver of F2 males, compared to control, decreased DNA methylation at specific CpG sites in the promoter region of the gene PPAR-γ, methylation-dependent repression of which is known to be critical in generation of the myofibroblast phenotype. Zeybel et al. also found higher levels of the histone modification H3K27me3 and the histone variant H2A.Z in the chromatin at the PPAR-γ promoter in the sperm of F0 founder rats with carbon tetrachloride-induced fibrosis.

In a different example, the transgenerational consequence of diet-induced paternal obesity was investigated in mouse (79). The F0 male mice were fed with a high fat diet and future generations of these mice obtained through paternal line. The metabolic health of experimental and control mice in F0, F1 and F2 was examined. In F0 males, high fat diet caused increased adiposity and hyperlipidemia, but not altered glucose homeostasis, fasting insulin levels and insulin sensitivity. The F1 offspring showed increased body weight, with males showing increased plasma leptin levels, not obesity, and females showing obesity as well as increased circulating lipids. Both males and females exhibited impaired glucose tolerance and insulin resistance. In the F2 offspring, the males showed reduced levels of plasma leptin and the females exhibited obesity, increased pancreas and liver weight, and impaired insulin sensitivity. The testis and sperm of high-fat diet fed F0 males showed increased levels of several miRNAs. In the testis, expression levels of mRNAs that are predicted targets of these miRNAs showed downregulation, as expected. These genes enriched several processes including metabolic disease, production of reactive oxygen species, lipid metabolism, spermatogenesis and embryonic development. Global DNA methylation analysis revealed hypomethylation in F0 founder males' testis and late elongated spermatids.

In an elegant study, Dias and Ressler investigated if olfactory experience influences future generations in mice (62). They conditioned F0 male mice with the odorant acetophenone and used them to obtain future generations via male line. Strikingly, an increased behavioral sensitivity to acetophenone was observed in the F1 and F2 offspring of acetophenone-conditioned male mice, compared to control. This increased behavioral sensitivity was found to correlate with an increased neuroanatomical representation of the olfactory receptor pathway activated by acetophenone. Dias and Ressler then collected sperm of conditioned F0 males after a washout period and performed *in vitro* fertilization to produce F1 offspring. Importantly, these offspring also showed an enhanced neuroanatomical representation of the acetophenone receptor pathway. To examine the possibility of maternal transmission arising due to some influence of conditioned F0 males on maternal behavior toward F1 offspring, Dias and Ressler conducted a cross-fostering experiment. In the experiment, sexually naive females with or without acetophenone conditioning were mated with acetophenone naive male mice and the resulting F1 offspring were divided into four groups: offspring of control mothers, offspring of conditioned mothers, offspring of control mothers cross-fostered by mothers conditioned to acetophenone and offspring of conditioned mothers cross-fostered by mothers not conditioned to acetophenone. As such, offspring in none of these groups were directly exposed to odor-related behavior and intrauterine learning. Interestingly, an increased behavioral sensitivity to acetophenone was observed in the second group in comparison to the first, and in the fourth group in comparison to the third. The increase was also found to correlate with an enhanced neuroanatomical representation of the acetophenone receptor pathway. These results provided strong evidence for germline inheritance of parental olfactory experience. In order to identify epigenetic modifications underlying transgenerational inheritance, Dias and Ressler examined the levels of DNA methylation in the acetophenone receptor gene in sperm of conditioned F0 male mice and their F1 offspring. Consistent with an enhanced neuroanatomical representation of the receptor pathway in F1 and F2 offspring, they found that the receptor gene was hypomethylated in both F0 and F1 sperm.

In another study, inheritance of diet- and drug-induced metabolic changes in male mice was investigated (52). Wei et al. induced insulin resistance and impaired glucose tolerance in F0 male mice by feeding a high-fat diet and injecting streptozotocin, in that order. The founder males were then used to produce subsequent generations via paternal line. Remarkably, impaired glucose tolerance and reduced insulin sensitivity were found to characterize both F1 and F2 offspring. Microarray gene expression profiling revealed differential expression of hundreds of genes in the pancreatic islets of F1 offspring, compared to control. Consistent with metabolic changes observed in offspring, these genes showed overrepresentation of several processes including insulin and glucose metabolism. Further, genome-wide DNA methylation analysis revealed thousands of differentially methylated loci including regions spanning several insulin signaling genes in F1 islets, compared to control. Notably, several of the differentially methylated loci in F1 exhibited a similar pattern in F2 islets. Additional genome-wide cytosine methylation analysis revealed thousands of differentially methylated regions in F0 sperm. The methylation pattern in F0 sperm and F1 pancreatic islets was globally correlated, suggesting that epigenetic status in the germline strongly predicts the same in the soma. Together, the experimental evidence produced in the above studies clearly suggested that non-genetic environmental factor-induced phenotypic effects can be inherited through the germline in mammals (Figure 1).

Soma to germline communication

Evidence of soma to germline communication in transgenerational epigenetic inheritance in mammals, with miRNA as its potential mediator, is discussed here in detail. It has been suggested that extracellular miRNAs in mammals are exchanged between cells *in vitro*, based on the demonstration of functional effects of miRNAs in the recipients (58). However, evidence has been lacking for mammalian circulating miRNA-mediated cell-cell communication *in vivo*. Notwithstanding, newer findings do suggest that extracellular miRNAs in mammals possibly play a role in soma to germline information transfer, and thereby may mediate inheritance of acquired characters. Experimental evidence for soma to germline communication in epigenetic inheritance as such was first obtained in the rat model of hepatic injury discussed above (73). As surgical liver injury, like carbon tetrachloride treatment, was also found to induce hepatic fibrosis, Zeybel et al. hypothesized that liver damage results in accumulation of a soluble factor in the serum that leads to modification of the chromatin structure in the germ stem cells and/or mature sperm. To test the hypothesis, Zeybel et al. injured rats with carbon tetrachloride, and after a washout period, transferred the serum from these animals to uninjured rats, and then examined the levels of H3K27me3 and H2A.Z at the PPAR-γ

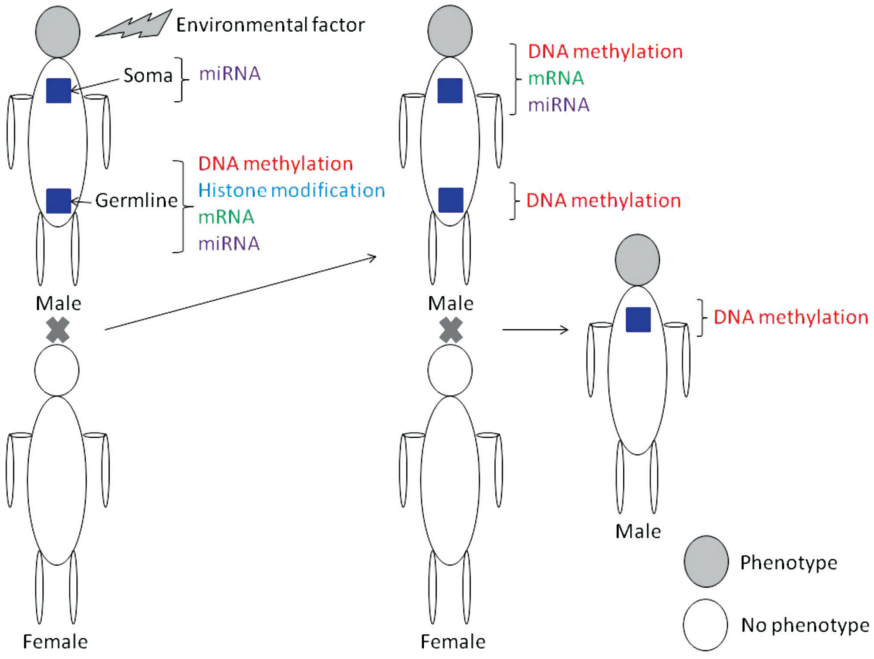

Figure 1: Existential evidence of transgenerational inheritance.
Cumulative findings of various studies reported so far involving paternal exposure and inheritance via male line are illustrated. Note inheritance of the phenotypic effect and its epigenetic correlates.

promoter in sperm of uninjured rats. Interestingly, higher levels of these chromatin marks were observed in sperm. Subsequently, Zeybel et al. hypothesized that the serum factor which mediates PPAR-γ chromatin remodeling may originate from myofibroblasts derived from hepatic stellate cells. To test this, they added media conditioned by cultured, activated rat hepatic stellate cells to rat bone marrow-derived mesenchymal stem cells and examined chromatin in the latter. Strikingly, an increased recruitment of H3K27me3 and H2A.Z was observed at the PPAR-γ promoter. These results provided evidence for a role of soma to germline communication in epigenetic inheritance, challenging the inviolability of the Weismann principle which prohibits hereditary information flow from somatic cells to germ cells (60, 92).

What could be the circulating factors that mediate soma to germline communication in inheritance of induced traits in mammals? Circulating microvesicles like exosomes, which contain miRNAs, mRNAs, proteins and lipids, have recently emerged as important mediators of intercellular communication that provide autocrine, paracrine and endocrine signals to cells by transferring their contents (93–122). Could exosomal communication be involved in epigenetic inheritance in mammals? The question seems promising in view of the following. Gamete borne miRNAs, mRNAs, proteins and lipids are all considered to potentially play regulatory and epigenetic roles in fertilization and embryonic development (40, 50, 122–135). Available evidence supports direct regulatory function of gamete borne RNAs including miRNAs in fertilization, and zygotic and embryonic development in mammals (50, 128, 130, 134). Further, sperm RNAs show potential for mediating epigenetic modifications including histone modifications and DNA methylation (128), and functional relevance of sperm borne miRNAs in epigenetic inheritance in mice has been demonstrated (4, 133, 135). Together, available evidence supports sperm RNA as a potential mediator of epigenetic inheritance across generations (136).

As certain RNA sequences are preferentially sorted into extracellular vesicles like exosomes (137, 138), a bioinformatic analysis was carried out to examine if mRNA or miRNAs that have been identified as differentially expressed after environmental exposure in the exposed generation or in the unexposed future generations overrepresent circulating miRNAs (60). The overrepresentation was examined either directly or indirectly by identifying mRNA targets of miRNAs. Similar enrichment analysis was also carried out for exosomal mRNAs and proteins (61). In the analyses of data pertaining to several mammalian species, environmental factors, life cycle stages, tissues, and generations, and both the genders, a statistically significant overrepresentation was observed across studies. These results led to the suggestion that circulating miRNAs and extracellular vesicles may possibly mediate soma to germline communication in inheritance of acquired traits in mammals (61).

Direct experimental evidence suggesting a potential role of circulating miRNAs in epigenetic inheritance in mammals was first obtained in the MSUS mouse model discussed above (4). Besides sperm and brain, the serum of F1 MSUS males was also found to exhibit altered levels of miRNAs (4). One of the miRNAs, miR-375-3p, was particularly notable in that it showed upregulation in F1 hippocampus, serum and sperm as well as in F2 hippocampus. Although the mechanisms underlying MSUS-induced alterations in sperm miRNA are unknown, one possibility could be that the stress hormones glucocorticoids reach testes through circulating blood, bind to their receptors that are expressed on sperm and somehow induce changes in miRNA expression (139). Another possible explanation however could be that miRNAs released by brain structures involved in stress response, miR-375 released by hippocampus, for example, reach testes through circulation and trigger altered miRNA expression in sperm through some mechanism. Interestingly, the hypothetical existence of an axis connecting the brain and germline was speculated previously to explain transgenerational spermatogenic inheritance of neuroactive drug-induced transcriptomic changes in the aforementioned *Drosophila* model (76).

Hormone or miRNA-mediated communication has also been proposed to explain odorant-induced transgenerational inheritance in mice described above (62). Although the mechanisms underlying olfactory stimulation-induced epigenetic modification in sperm remain unknown, it has been speculated that acetophenone may enter the circulation, bind to its receptor expressed on sperm and somehow affect DNA methylation in the receptor encoding gene (62). Alternatively, it has been suggested that odorant-induced fear conditioning causes release of glucocorticoids or miRNAs in the circulation, and these molecules then act on spermatogonia and direct changes in the levels of DNA methylation (10).

As mentioned above, it has been reported that male mice exposed to chronic variable stress produce offspring with altered stress response (91). This alteration is due to reduced hypothalamic-pituitary adrenal (HPA) axis stress responsivity. Global analyses of sperm miRNAs in exposed mice and brain mRNAs in offspring have implicated epigenetic reprogramming in transmission of stress-induced phenotypes. Interestingly, consistent with the aforementioned hypothetical brain-germline axis mediating soma to germline communication (76), it has been speculated that activation of the HPA axis may cause exosomes containing stress evoked miRNAs to shuttle from epididymis to sperm, and ultimately to ovum (63). Subsequently, in the developing embryo, the miRNAs may influence formation of the HPA axis through epigenetic mechanisms. An altered HPA axis responsivity in offspring would again result in miRNA-mediated information transfer as above, thus setting off a transgenerational cascade of altered stress responsivity (63). The hypothesis is consistent with the demonstrated role of exosomes in sperm epididymal maturation, a process necessary for sperm production (140). The view that exosomal miRNA can potentially mediate soma to germline information transfer in epigenetic inheritance (60–63) is also supported by evidence suggesting involvement of miRNAs in germ cell differentiation, post-meiotic male germ cell function and growth, and development and maturation of oocytes (141). In another example, based on the observation that ethanol exposure results in persistent changes in plasma miRNAs and is associated with transgenerational inheritance of behavioral and neuronal phenotypes, the possibility has been raised that soma to germline transfer of miRNAs may underlie inheritance of ethanol-induced characters (142).

It was previously demonstrated that miRNA secreted through exosomes in a culture medium can be taken up by cells that do not express the miRNA, with downregulation of miRNA target genes shown in the recipient cells (143). However, in the absence of evidence of the cellular uptake of extracellular endogenous miRNAs contained within or outside of exosomes, the possibility that circulating miRNAs can mediate intercellular communication *in vivo* in mammals has been questioned (58). Interestingly, newer studies provide evidence supporting such a role for these miRNAs (101). For example, it has been shown that mice injected with atherosclerosis patients' plasma-derived microvesicles, that show miR-150 enrichment, exhibit elevated plasma levels of miR-150, known to promote angiogenesis *in vitro*, and increased amount of erythrocyte-filled blood vessels (144). Similarly, given that patients with type 2 diabetes and various types of cancers also show elevated plasma levels of miR-150, both mice implanted with human tumor and ob/ob mice with a diabetic phenotype have been found to exhibit increased miR-150 plasma levels as well as elevated angiogenesis. The potential role of miR-150 in intercellular communication *in vivo* was further confirmed by using miR-150 inhibitor in mice experiments (144). In another example, the hypothesis was tested that multipotent mesenchymal stromal cells (MSCs) promote neurological recovery from stroke in rats by transferring exosomal miR-133b *in vivo* (145). In this, knockin and knockdown technologies were used to up- or downregulate miR-133b levels in MSCs and their corresponding exosomes, respectively, and cerebral artery occlusion was used to model stroke. Following intravenous

injection of MSCs in the animals, an increased expression of miR-133b in MSCs and in the exosomes released by these cells was found to correlate with enhanced functional recovery and, in the ischemic boundary zone (IBZ), cortical axonal density and neurite remodeling. Interestingly, by tagging exosomes with a green fluorescent protein, it was demonstrated that exosomes are released from MSCs in the IBZ and transferred to adjacent astrocytes and neurons. Importantly, decreased expression of selective targets for miR-133b in the IBZ was also demonstrated. In a separate study on the murine model of *in vivo* bone metastasis, treatment with miR-192 enriched exosome like vesicles has been found to precondition osseous milieu, impair tumor-induced angiogenesis and reduce metastatic burden (146). Combined with the finding that miR-192 inhibits tumor-induced angiogenesis and osseous metastasis *in vivo*, and *in vivo* infusion of fluorescent labeled exosome like vesicles leads to accumulation of the label in cells of the osseous compartment, this study supported the view that exosomal miRNAs can be transferred *in vivo* and mediate intercellular communication. In another example, it has been found that direct intramyocardial transplantation of mesenchymal stem cell-derived exosomes, known to protect cardiomyocytes from hypoxia-induced apoptosis *in vitro*, at the border of an ischemic region in the rat heart, in which ischemia was triggered through ligation of the left anterior descending coronary artery, restores cardiac contractile function and reduces infarct size (147). Evidence suggests that the observed protection of damaged tissue was mediated by transfer of miR-19a from exosomes to cardiomyocytes, with subsequent reduction in the expression of the miRNA target PTEN and activation of the cell survival-related Akt and ERK signaling pathways in the recipient cells. Recently, in a study investigating the potential of exosomes in therapeutically blocking inflammation in the CNS, de Rivero Vaccari et al. isolated exosomes from embryonic cortical neuronal cultures, loaded them with short-interfering RNA (siRNA) against an apoptosis speck-like protein containing a caspase recruitment domain (ASC), a protein that is elevated in spinal cord motor neurons and cortical neurons after CNS trauma, and administered the loaded exosomes to spinal cord-injured animals (148). Remarkably, the exosomes were found to cross the injured blood-spinal cord barrier and deliver their cargo *in vivo*, with ASC protein levels showing a decline.

Importantly, the potential for exosomal RNA-mediated soma to germline communication in transgenerational epigenetic inheritance in mammals has been demonstrated recently (149). Cossetti et al. subcutaneously injected human melanoma cells stably expressing an enhanced green fluorescent protein (EGFP) in male mice and, after tumor growth, collected samples of plasma and epididymal spermatozoa from the xenografted animals. Interestingly, Cossetti et al. found that EGFP RNA is present not only in the circulating exosomes but also in sperm heads. Given that all possible sources of cell contamination and experimental artefacts were addressed in the study, these results strongly supported the possibility that RNA expressed in somatic cells can be transferred to male germline cells through circulating extracellular vesicles. Cumulatively, the evidence obtained in the above studies is consistent with the idea that exosomes and extracellular RNAs may potentially mediate soma to germline information transfer in inheritance of acquired characters in mammals (Figure 2).

Epigenetic memory

In a *Drosophila* model of paternal sugar-induced offspring obesity, germline transmission involving heterochromatin embedded gene expression has been shown to associate with H3K9me3- and H3K27me3-dependent reprogramming of metabolic genes in two distinct germline and zygotic windows (31). Evidence suggests that chromatin-dependent signatures in this model are forecast in the paternal germline, providing an example of epigenetic memory across generations. In the vertebrate model organism zebrafish, inheritance of DNA methylome has been demonstrated, with the early embryos displaying a sperm methylome pattern (21). In mice, genome-wide analysis suggests that rare but functionally relevant methylation epialleles could survive reprogramming and be inherited transgenerationally (48). Single loci DNA methylation marks have been found to resist demethylation in both male and female primordial germ cells in mice. Notably, it has been observed that reprogramming resistant single loci typically also escape erasure in the early embryo, providing potential substrates for epigenetic inheritance (28).

Evidence for survival of histone modifications across reprogramming has also been obtained, even in sperm where histones are largely replaced by protamines (28). Remarkably, reprogramming resistant sperm nucleosomes show enrichment for H2K27me3, suggesting that this mark may represent an inherited signal (28). In a study on the formation of constitutive heterochromatin in human preimplantation embryos, it has been found that canonical histone modifications are retained in this region in sperm chromatin, transmitted to the oocyte, incorporated in paternal embryonic constitutive heterochromatin, and recognized by H3K9me3/HP1 pathway maternal chromatin modifiers and propagated over the embryonic cleavage divisions (33). These findings support the occurrence of

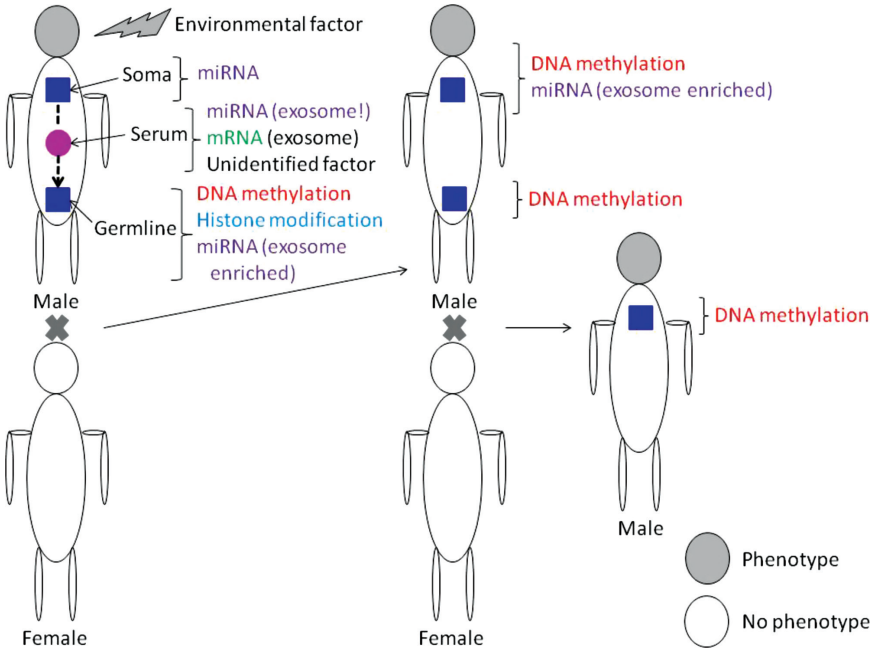

Figure 2: Soma to germline communication.
Combined evidence supporting information transfer from somatic cells to germ cells in transgenerational inheritance. Note that circulating exosomal miRNA is the potential mediator.

transgenerational epigenetic memory in human. Regarding the underlying mechanisms, evidence suggests involvement of the maintenance DNA methyltransferase DNMT1 that restores DNA methylation on the newly synthesized DNA after replication in mammals (15, 150, 151). For histone modifications, either the marks themselves may remain associated with the daughter chromatin after replication, as shown in *C. elegans*, or histone modifying complexes that remain anchored to the daughter DNA reestablish the marks after replication, as suggested by investigations in *Drosophila* (29, 30, 32). Interestingly, theoretical modeling based on experimental data related to nearest-neighbor lateral enzyme interactions and nucleosome modification associated enzyme recruitment has revealed that histone marks can be profoundly inheritable despite interference of stochastic cellular processes (152).

The widely considered view of extensive reprogramming in mammals notwithstanding, available evidence suggests that a significant amount of epigenetic information is transmitted across generations, and the message that is passed on may potentially affect early mammalian embryogenesis (153). Experimental findings show that mammalian germ cells *in vivo* maintain bivalent histone modifications, associated with both gene activation and repression, at promoters of several genes involved in somatic development, with the marks retained from developmental stages through meiosis and gametogenesis (154). A conceptual model of intrinsic transgenerational inheritance has been proposed in which hypothetical bivalent histone modifications in the germ cells are speculated to regulate somatic development in the next generation (152, 154). Also, the known influence of histone modifications on DNA methylation raises further possibilities for epigenetic inheritance. Although regulation of genomic DNA methylation patterns and the mechanisms underlying recruitment and activity of DNA methyltransferases *in vivo* are unclear, recent experimental findings do establish a role of sequence and histone marks in directing *de novo* enzyme activity and methylome integrity (19, 155).

As regards RNA-mediated epigenetic information transfer, the presence of RNA-dependent RNA polymerases in plants, as also in the worm *C. elegans* and yeast, can allow amplification of inherited small RNAs and perpetuation of epigenetic effects (38, 39). Besides post-transcriptional regulation, evidence suggests that small RNAs can also regulate gene expression at transcriptional level by interacting with RNA binding proteins to trigger DNA methylation in plants, yeast and mice, and histone modifications in plants, yeast, worm and the fruit fly *D. melanogaster* (35, 39, 49). The concept is emerging that small RNAs, in their cell of origin or in the host cell upon short- or long-distance transfer, bind to proteins that act in the nucleus and the resulting complex together regulates gene expression, with the small RNAs base pairing with partially or fully transcribed nascent mRNAs and the proteins directing methyltransferases and histone modifiers

at the target site through interactions (39). The RNA-mediated epigenetic inheritance has been demonstrated in mice, wherein injection of sperm RNA of a mutant, as also of mutant gene-specific miRNAs, into the pronuclei of fertilized mouse eggs was shown to cause appearance of the mutant phenotype (133). In this model, miRNA injection was associated with inheritance of a distinct histone modification in the promoter region of a target gene (156). Given the presence of a complex and diverse set of RNAs in spermatozoa, ova and early embryos, and the evidence that fertilized eggs possess a reservoir of RNAs contributed by germ cells, the concept has emerged that inherited RNAs influence embryonic development through various gene regulatory mechanisms (37). These mechanisms may involve transcription, translation, transcript stability or other yet unknown regulatory pathways (37). As gametes are considered transcriptionally quiescent, demonstration of transcript stabilizing post-transcriptional RNA modifications in spermatozoa provides support to RNA-mediated inheritance (37, 157). Also, sequence-based mechanisms involving RNA binding proteins and antisense RNAs have been suggested to stabilize RNAs in oocytes and zygotes (37, 158). Altogether, evidence obtained so far supports the view that epigenetic information can be transmitted across generations without being dissolved during reprogramming in animals including mammals (Figure 3).

Evolutionary significance

With increasing appreciation of its potential evolutionary significance, epigenetic inheritance challenges the established neo-Darwinian dogma that evolution is driven exclusively by random mutational events in the germline followed independently by natural selection (84–86, 159–167). For example, epigenetic inheritance of DNA methylation associated gene expression and phenotypic variations is considered to potentially play an evolutionary significant role. Evidence supporting a role of epialleles in evolution has been obtained from genome-wide signatures of DNA methylation in plant, avian and mammalian species (159–176). It has been suggested that in evolutionary time course mutations may arise that fix the epialleles in a genetic context (81, 177). The 5-methylcytosine is an unstable DNA modification, with mCpG transitions from C to T occurring due to a variety of processes including spontaneous and enzymatic deamination (13, 178). It therefore seems plausible that mCpG is fixed as T following error-prone replication repair (179).

Besides empirical evidence, theoretical considerations and modeling also suggest that epigenetic inheritance can contribute to and accelerate evolutionary processes (5, 53, 160, 165, 180–190). The idea has therefore emerged that modern synthesis, the contemporary theory of evolution based exclusively on Darwinism and principles in genetics, needs to include non-genetic inheritance and lead to a new extended evolutionary synthesis (88, 187, 191–193). Notably, given a role of DNA methylation in ncRNA-mediated regulation of gene expression, and a propensity of methylated cytosine to change to thymine, a theory of 'RNA-mediated gene evolution' has recently been proposed to suggest that RNA may possibly participate in the natural selective process to drive not only cellular but also organismal evolution (194). Cumulatively, it is increasingly being recognized that epigenetic inheritance possibly plays a significant role in evolution (Figure 4).

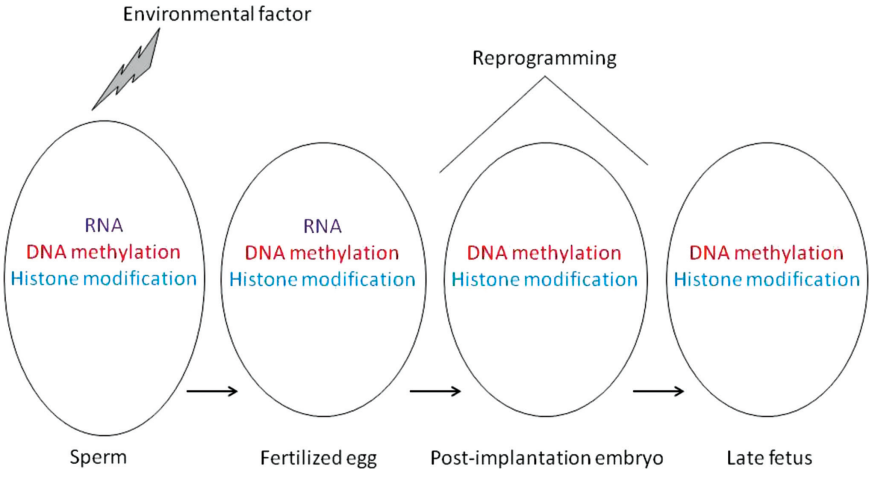

Figure 3: Epigenetic information propagation.
Accumulated evidence of epigenetic information propagation across generations is depicted. Note survival of marks across both zygotic and early embryonic reprogramming.

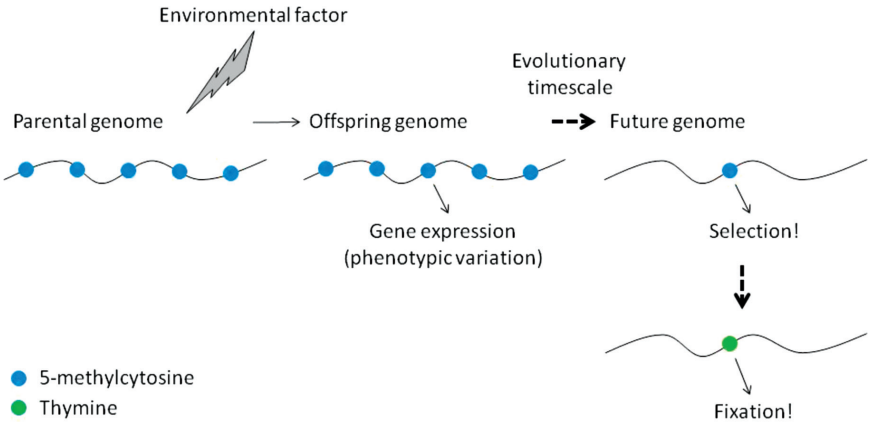

Figure 4: Evolutionary significance of epigenetic inheritance.
Available empirical evidence and theoretical considerations for potential implication of epigenetic inheritance in evolution are outlined. Note that epigenetic modification may lead to genetic variation in due course.

Integrative model

Recent advances discussed in the above sections provide enough evidence to conceptualize an integrative model of transgenerational epigenetic inheritance (Figure 5). In this model, an environmental factor can affect the exposed generations differently, with epigenetic modification in the germline being the common effect. This modification can be caused by the environmental factor either directly or through somatic alterations. In the latter scenario, the hereditary information can be transmitted from the soma to the germline through exosomes and circulating RNA-mediated intercellular communication system. Once established in the germline, the epigenetic modification can be transmitted to the next generation by escaping erasure during epigenome reorganization and reprogramming. This may lead to a self-perpetuating cycle of soft inheritance. In evolutionary time course, the epigenetic modification may disappear or continue to persist as such or transform into a genetic mutation and become a part of

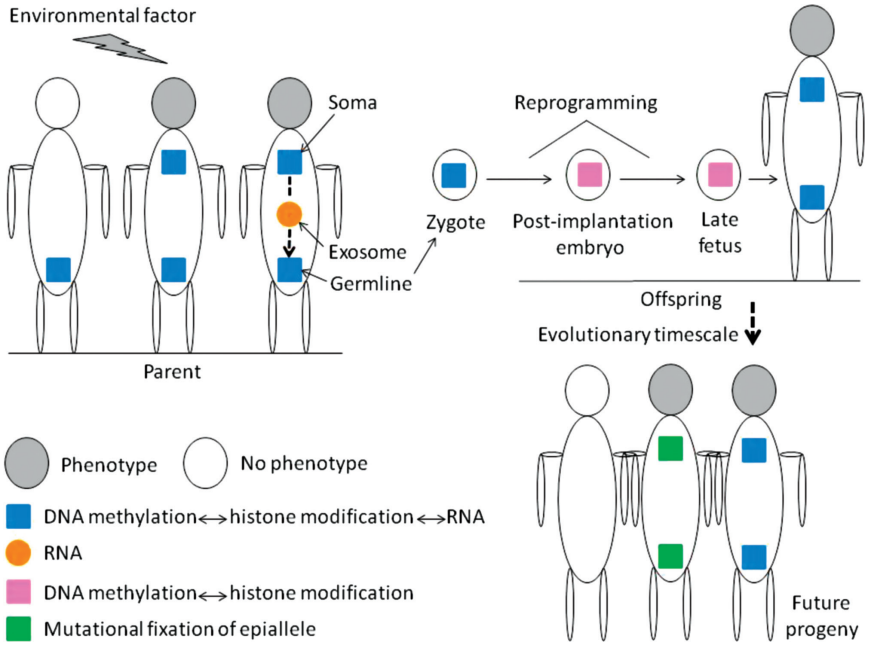

Figure 5: An integrative model of transgenerational epigenetic inheritance.
The model is based on available and suggestive evidence presented in Figures 1–4. Note integration of gene-environment interaction, systems biology and evolution.

hard inheritance. This evolutionary systems biology perspective of transgenerational epigenetic inheritance provides a broad framework that may guide future course of experiments and theoretical discourse. It is notable here that a concept of 'transgenerational systems biology' was recently proposed based on the bioinformatic prediction that exosomal contents may mediate soma to germline communication in inheritance of acquired traits (61, 195). This model conceptualizes that environmental exposure sequentially leads to alterations in the systems biology of somatic cells, release of circulating factors, interactomic perturbation-induced epigenetic modifications in the germline, transmission of epigenetic factors to the oocyte, gene network alterations in the embryo and phenotype appearance in the adult. Recent experimental demonstration of circulating miRNA association in transgenerational inheritance (4, 22) and transfer of exosomal RNA from soma to germline (149) in mice indeed provided evidence supporting that concept.

Conclusion

The skepticisms surrounding the existence of epigenetic germline inheritance in mammals are increasingly being resolved. Studies have been addressing the possible confounding effects and providing credible evidence for the occurrence of transgenerational epigenetic inheritance in rats and mice. Regarding seemingly implausible soma to germline communication, newer findings support the idea that exosome and circulating miRNA may mediate intercellular communication in epigenetic inheritance in mammals. As regards epigenetic memory, available evidence suggests that information in the form of DNA methylation, histone modifications and RNA can be propagated across generations in animals including mammals. Besides, evidence favoring a role of epigenetic inheritance in evolution is accumulating. Together, emerging advances in epigenetic inheritance are expanding the frontiers in biology.

Note added in proof: Two recently published papers separately report the inheritance of ectopically induced domains of the histone modification H3K9me through many mitotic and meiotic cell divisions in the absence of DNA sequence-specific initiator, in the fission yeast *Schizosaccharomyces pombe* (Ragunathan et al., Science 2015, doi: 10.1126/science.1258699; Audergon et al., Science 2015, doi: 10.1126/science.1260638). The reported studies demonstrate that a direct read-write mechanism involving H3K9 methyltransferase can stably copy and propagate H3K9me across generations. This evidence for sequence-independent transgenerational memory survival immensely supports the mechanistic plausibility of nongenetic inheritance.

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