Review

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Bridging the transgenerational gap with epigenetic memory

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It is textbook knowledge that inheritance of traits is governed by genetics, and that the epigenetic modifications an organism acquires are largely reset between generations. Recently, however, transgenerational epigenetic inheritance has emerged as a rapidly growing field, providing evidence suggesting that some epigenetic changes result in persistent phenotypes across generations. Here, we survey some of the most recent examples of transgenerational epigenetic inheritance in animals, ranging from *Caenorhabditis elegans* to humans, and describe approaches and limitations to studying this phenomenon. We also review the current body of evidence implicating chromatin modifications and RNA molecules in mechanisms underlying this unconventional mode of inheritance and discuss its evolutionary implications.

Unconventional mode of inheritance

Heredity is overwhelmingly acknowledged to be governed by the laws of Gregor Mendel, with genes as the primary templates of inherited information. However, an increasing number of exceptions to the rules of genetic inheritance have been reported, suggesting that additional layers of information are also transmitted. During the 1920s, inheritance of mating behavior in toads was reported by Paul Kammerer [1], although this study remains controversial [2]. During the 1940s, Conrad Waddington coined the term 'epigenetic' [3] and described inheritance of wing patterns in response to heat shock in *Drosophila* [4]. Shortly after, Alexander Brink discovered an unconventional mode of inheritance for pigment biosynthesis in plants and termed it 'paramutation' [5]. The discovery of parental imprinting in mammals during the 1980s provided the first indication that epigenetic modifications, such as DNA methylation, were not entirely erased between generations and could underlie transgenerational epigenetic inheritance [6–11]. It was also around this time that several cases of transgenerational epigenetic inheritance were reported in mice, for both exogenous transgenes and endogenous alleles affecting coat color [12,13]. Since these breakthrough reports, additional cases of nongenetic inheritance have been reported in species ranging from worms to mammals, suggesting that this phenomenon is more widespread than originally thought. In parallel, a growing body of work has revealed that exposure of parents to environmental stimuli could affect the phenotype of several generations of descendants, suggesting that changes in epigenetic modifications induced by environmental stimuli might be passed on to descendants via the gametes. Importantly, the field has recently made significant progress in garnering mechanistic insight into the processes underlying transgenerational epigenetic inheritance, by implicating specific chromatin modifications and noncoding RNA molecules.

In this review, we survey the most recent examples of epigenetic inheritance induced by genetic and environmental perturbations in animals (epigenetic inheritance in plants has been reviewed elsewhere [14,15]). We also discuss the current state of progress in elucidating molecular mechanisms of this unconventional mode of inheritance, primarily focusing on chromatin and noncoding RNAs, since the role of DNA methylation has been extensively reviewed elsewhere [16–21]. We mostly focus on instances of nongenetic inheritance that persist for several generations (but that are not permanent), because persistence over multiple generations reduces likelihood of confounding factors, such as maternal effects or direct exposure of the gametes to toxins. We also discuss these important limitations to our understanding of the mechanisms underlying transgenerational epigenetic inheritance. Finally, we reflect on the potential evolutionary implications of this previously underappreciated mode of inheritance.

Epigenetic inheritance induced by alterations of the parental genome

Studying wild type descendants of ancestors with mutations in specific genomic loci has revealed that, in some cases, phenotypes characteristic of the mutant ancestors are still present in wild type descendants (Figure 1).

Transgenerational epigenetic inheritance of color and size in mice

Alterations in the genome, for example transcriptional activation of a chromosomal element and retrotransposon insertion, were shown to lead to transgenerational epigenetic inheritance of Drosophila eye color [22] and mouse coat color [12], respectively. More recently, additional

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Keywords: transgenerational; epigenetic inheritance; chromatin; noncoding RNA; aging; fertility; metabolism; stress stimuli.

  http://dx.doi.org/10.1016/j.tig.2012.12.008 Trends in Genetics xx (2013) 1–11

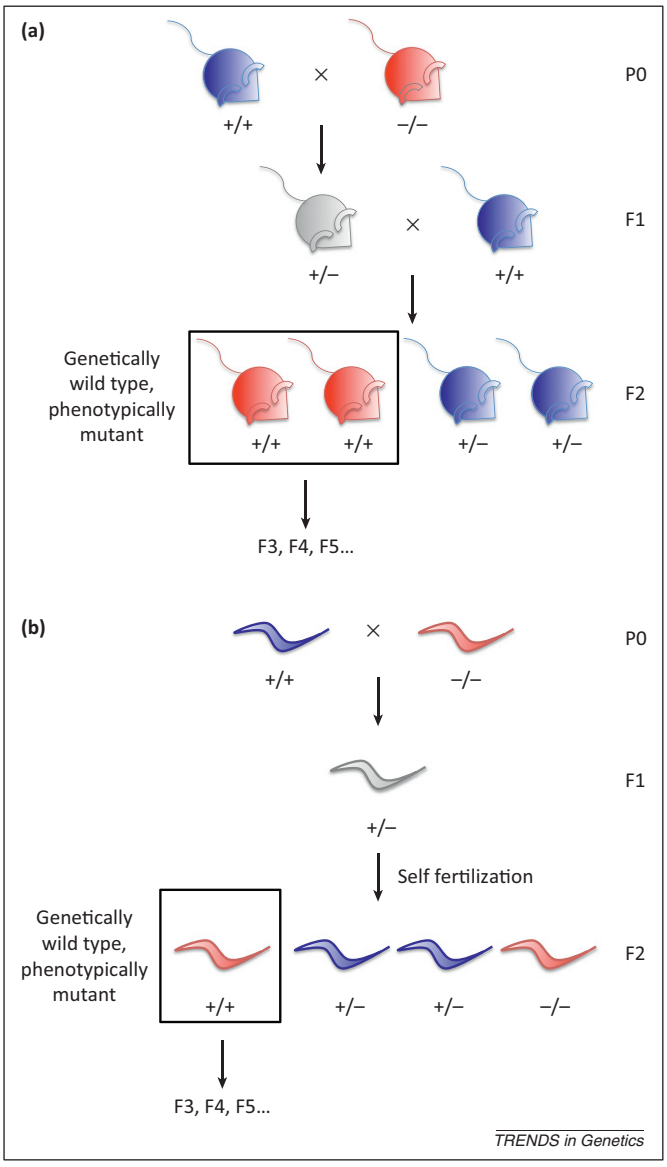

Figure 1. Transgenerational epigenetic inheritance induced by genetic modification in ancestors. **(a)** Crossing scheme used in rodent studies to generate genetically wild type progeny from a mutant ancestor. **(b)** Crossing scheme used in worm studies to generate genetically wild type progeny from a mutant ancestor. The F1 generation arises from mating between a hermaphrodite and male. The F2 generation arises from the self fertilization of F1 hermaphrodites. Key: red, wild type; blue, mutant; gray, unknown phenotype.

examples of transgenerational epigenetic inheritance induced by alterations of the parental genome were described in mice, implicating RNA in this phenomenon [23–25]. In the first study, mice with an insertion of LacZ into the *Kit* gene gave rise to genetically wild type offspring that still exhibited the tail and paw color phenotype characteristic of *Kit* mutants for at least two generations [23]. Genetically wild type descendants of ancestors that had the *Kit* mutant phenotype showed an altered pattern of *Kit* RNA expression, with RNA molecules of shorter size in brain and testis [23]. Microinjection of RNA from heterozygous mutant animals into one-cell embryos was sufficient to recapitulate the mutant phenotype in the following generation [23]. Collectively, these data suggest that disruption of a genomic locus in parents (in this case by LacZ insertion) leads to abnormal RNA production in sperm, which may be transmitted in a transgenerational manner for at least two generations. However, the exact mechanisms underlying this epigenetic memory are still unclear.

The importance of RNA in epigenetic inheritance in mice was bolstered by two examples of transgenerational inheritance of cardiac hypertrophy and organismal growth [24,25]. Injection of fertilized eggs with RNAs and miRNAs targeting cyclin-dependent kinase 9 (*Cdk9*), a regulator of cardiac growth, resulted in cardiac hypertrophy in the next generations [24]. Furthermore, injection of *miR-124*, a miRNA normally expressed in the brain and involved in central nervous system development, into fertilized eggs resulted in progeny that exhibited a 30% larger than normal body size [25]. This 'giant' phenotype persisted until the F2 generation, after which the body size of the descendants reverted back to normal. These studies suggest that RNA-mediated epigenetic phenomena underlie the inheritance of select phenotypes. Why some RNAs have a potential for transgenerational effects whereas others do not is still unclear. Nongenetic inheritance of traits such as organ and animal size may have crucial implications for the etiology of human hypertrophic cardiac myopathy and obesity.

Transgenerational epigenetic inheritance of fertility and longevity in C. elegans

Examples of transgenerational epigenetic inheritance induced by alterations of the parental genome were also recently described in the nematode *Caenorhabditis elegans*. *C. elegans* is a powerful model system for the study of epigenetic memory given its rapid generation time and amenability for controlling many environmental and genetic variables. Transgenerational epigenetic inheritance of sterility and longevity have both been reported, and involve similar histone modifications [26,27]. First, mutants of *spr-5*, one of the worm orthologs of the lysine-specific histone demethylase 1A (LSD1/KDM1), which demethylates the histone 3 lysine 4 dimethyl mark (H3K4me2) [28], exhibit progressive decreased brood size beginning in the first generation and progressive progeny sterility starting around generation 20 [26]. The fertility of severely sterile generations was rescued by introducing one wild type copy of *spr-5* for one generation [26], indicating that this is sufficient to induce epigenetic resetting. Interestingly, accumulation of H3K4me2 and misregulation of spermatogenesis genes is observed in the primordial germ cells (PGC) of *spr-5* mutants [26]. Indeed, it has been recently shown that H3K4me2 increases throughout the entire germline of *spr-5* mutants [28]. Together, these results suggest that failure to reset H3K4me2 marks at select germline genes in the PGCs results in progressive transgenerational sterility.

More recently, the first example of transgenerational epigenetic inheritance of longevity was described [27]. Genetically wild type descendants from ancestors that are mutant for members of the COMPASS H3K4 methylation complex display increased lifespan for up to three generations. The COMPASS complex comprises ASH-2, WD repeat-containing protein 5 (WDR-5) and SET-2 in worms. This complex is conserved across species, and catalyzes the trimethylation of lysine 4 of histone H3 (H3K4me3) [29], a chromatin modification commonly

associated with actively transcribed genes [30]. Deficiency in COMPASS complex members extends lifespan in a manner that depends on the presence of a functional germline [31]. Genetically wild type descendants from a cross between wild type males and hermaphrodites with mutations in the *wdr-5* or *set-2* genes were long lived for up to three generations compared with descendants from pure wild type ancestors [27]. The long lifespan of the descendants is dependent on the H3K4me3 demethylase retinoblastoma binding protein related 2 (RBR-2) and requires a functioning germline [27]. This suggests that longevity of wild type descendants from a cross between mutant ancestors and wild type worms is unlikely to be due to an extraneous mutation present in the initial strain. Transgenerational inheritance of longevity appeared to be relatively specific to members of the COMPASS complex, because manipulation of other longevity-promoting pathways, such as the insulin signaling and mitochondrial pathways, or other chromatin regulators, did not show a transgenerational inheritance of long life [27]. These findings show that manipulation of specific chromatin modifiers in parents can have lasting effects on complex traits of offspring. It is interesting to note that both cases of transgenerational inheritance [26,27] involve the regulation of H3K4 methylation in the germline, suggesting that this histone mark is particularly important for the mechanism of epigenetic memory.

Together, these data describe instances where initial perturbation of the genome of an ancestor can result in transgenerational epigenetic inheritance of a variety of traits. These observations also raise the intriguing possibility that environmental stimuli that perturb RNA or chromatin states could also affect phenotypes of descendants for multiple generations. However, the prevalence and specificity of this epigenetic mode of inheritance is still unknown. Furthermore, the exact sequence and nature of events by which initial mutations in chromatin modifiers in ancestors lead to relatively persistent phenotype changes in wild type descendants is not known, and is discussed further below, in the mechanism section.

Epigenetic inheritance induced by metabolic changes in parents

Interestingly, epigenetic inheritance can also be induced by environmental changes in parents. The past few years have seen a flurry of reports on the inheritance of acquired metabolic phenotypes resulting from over- or undernutrition in parents. To avoid potential confounds of *in utero* and altered maternal caretaking, studies have investigated transgenerational inheritance of metabolic physiology through the paternal (Figure 2a) or the maternal (Figure 2b) lineage. In such schemes, the gametes of the P0 father or the F1 mother are directly exposed to the environmental stimulus, which may alter the genome in ways that could be transmitted to the F1 or F2 offspring, respectively. Thus, studying the F2 generation from males or F3 generation from females provides a way to distinguish direct exposure from epigenetic inheritance (Figure 2).

Overfeeding

Exposure to a chronic high-fat diet in rat fathers resulted in impaired insulin metabolism and pancreatic cell gene expression in female F1 offspring [32]. Female offspring from fathers fed a high fat diet mated with mothers fed a control diet exhibited an increase in blood glucose in the glucose tolerance test and a decrease in insulin secretion compared with offspring with both parents fed a control diet [32]. Microarray analysis of islet cells from female offspring of fathers fed a high-fat diet uncovered genes involved in a range of pathways, including insulin and glucose metabolism as well as mitogen-activated protein

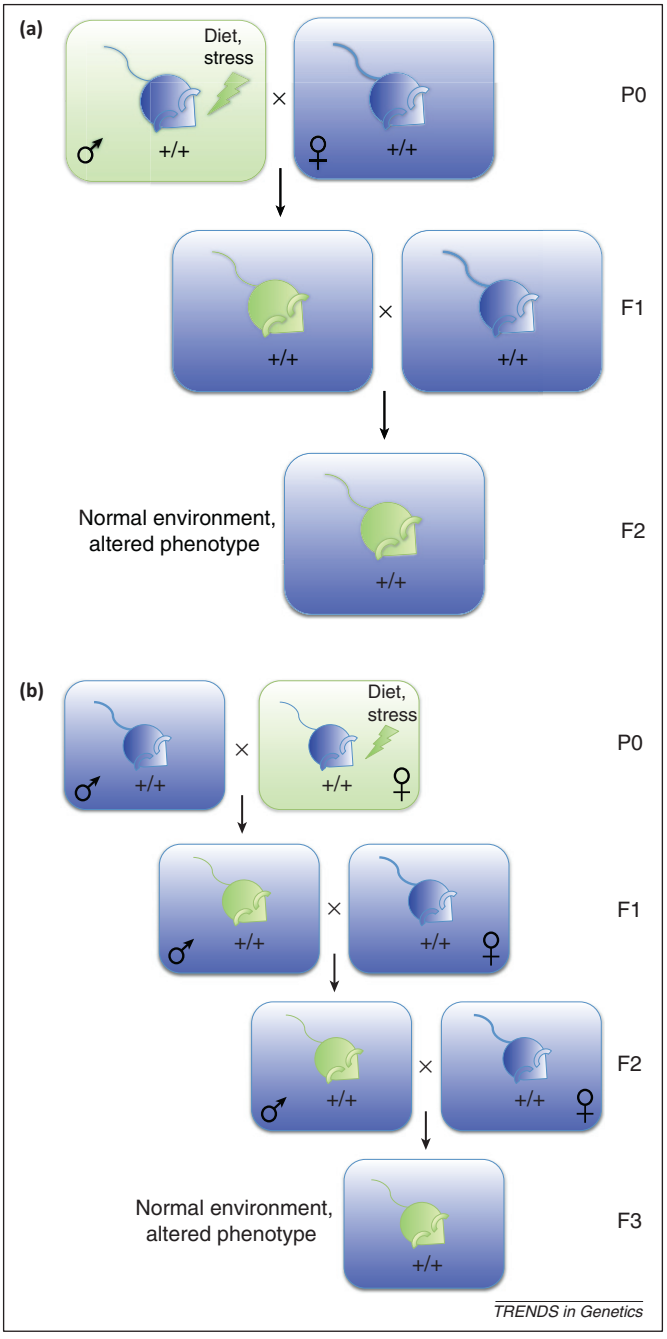

Figure 2. Transgenerational epigenetic inheritance induced by environmental manipulations in ancestors. **(a)** Strategy used to generate progeny from a paternal ancestor subject to an environmental perturbation. This scheme minimizes maternal effects in organisms that require significant maternal care during development. **(b)** Scheme used to generate progeny from a maternal ancestor subject to an environmental perturbation. This scheme allows for the study of transgenerational effects induced by an initial change in maternal environment, but minimizes cryptic maternal effects. Key: blue, normal phenotype; green, altered phenotype resulting from a perturbation to the ancestral environment.

(MAP) kinase and Wnt signaling [32]. The greatest difference observed was an increase in expression of the gene encoding interleukin 13, *Il13ra2*, a cytokine involved in Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling [32]. It will be interesting to understand the extent to which the dysfunctional β cell phenotype is mediated by differential expression of *Il13ra2*. It will also be important to test whether this phenotype can persist for more than one generation to distinguish direct exposure from *bona fide* transgenerational inheritance. Interestingly, another study reported that overfeeding of male mice, via culling of litter size, resulted in altered insulin and glucose metabolism of two subsequent generations of male offspring [33]. The observation that F2 male offspring of overfed fathers also exhibited altered metabolic phenotypes supports the notion that this phenomenon is mediated via an epigenetic mechanism rather than by permanent damage to the genome induced by changes in metabolism.

The observation that metabolic traits can be inherited in a transgenerational epigenetic manner was further bolstered by studies of three generations of offspring from mothers fed a high-fat diet [34,35]. Both the F1 and F2 generations inherited an increase in body length and a decrease in insulin sensitivity, through both maternal and paternal lineages [34]. Studying the inheritance of phenotypes to the F3 generation in this type of scheme is crucial to minimize the impacts of *in utero* exposure to environmental stimuli [35]. Only F3 females, but not males, inherited the increase in body length, and this was passed on only via the paternal lineage. These observations raise the possibility that imprinted genes are involved in this inheritance of body size in parents. Consistent with this possibility, paternally expressed genes showed a greater change in the magnitude of expression versus maternally expressed genes [35].

It will be important to explore further the mechanisms of inheritance of overfeeding-induced phenotypes. One hypothesis is that the contents of sperm and seminal fluid (e.g., chromatin, RNA, and/or metabolites) from fathers are influenced by diet and these changes are sufficient to be transferred to descendants, in some cases over several generations. Such changes may be mediated by relatively stable, yet reversible, molecular entities, and perhaps via amplification loops.

Dietary restriction

Dietary restriction has also been found to result in heritability of various metabolic phenotypes. For example, offspring of male parents fed a low-protein diet exhibited differential metabolic gene expression and changes in lipid metabolism [36]. Male mice fed a low-protein diet and subsequently mated to naïve females gave rise to offspring of both sexes that exhibited increased expression of genes involved in fat and cholesterol biosynthesis, as well as genes important for DNA replication [36], corresponding to altered lipid metabolism and a hyperproliferative state, respectively. Metabolic analysis of the liver showed that offspring from males on a low-protein diet had lowered hepatic cholesterol and other changes in lipid metabolism [36]. Whether these diet-induced phenotypes can be inherited for more than one generation is currently unknown. Importantly, this study investigated DNA methylation and histone modifications in sperm of males subjected to a low-protein diet and showed that the epigenome of sperm can be altered by paternal diet [36]. However, which of these (or other) changes are causative of the transgenerational inheritance of metabolic phenotype remains an open question.

Human cohorts of food scarcity

The potential impact of ancestral diet on metabolic phenotypes of descendants has started to be examined in humans via two retrospective cohort studies, the Dutch Hunger Winter Families Study in the western Netherlands [37,38] and the Overkalix cohort in Sweden [39]. The Dutch Hunger Winter famine occurred at the end of World War II from October 1944 to May 1945, when official rations, comprising mostly bread and potatoes, fell as low as 500 kcal/day. One study found that female, but not male, offspring from mothers exposed to famine during pregnancy displayed increased total cholesterol, increased triglycerides, and increased low-density lipoprotein (LDL) cholesterol, compared with unexposed same sex siblings [37]. Interestingly, second-generation offspring exhibited higher neonatal adiposity and were subject to metabolic disease 1.8 times more frequently than were unexposed controls [38]. Together, these studies suggest transgenerational effects of prenatal famine exposure on birth weight and metabolic disease rates. It would be important to study the F3 generation to assess more thoroughly transgenerational inheritance and to determine the propensity of these cohorts to develop age-related diseases, such as cardiovascular disease, cancer, diabetes, and hypertension.

The other cohort of humans in which differences in metabolism were studied transgenerationally is the Overkalix cohort, a three-generation group of families from northern Sweden from the late 1800s through to the early 1900s who experienced a fluctuating food supply. Studies in this cohort revealed that low food intake during a critical period in the early adolescence of grandparents correlated with survival of grandchildren [39]. These results suggest that early adolescence is an important period for the response to the nutrient environment in ways that potentially affect future generations. This period could correspond to a critical window in gamete maturation in which metabolic environment may be an important determinant of gamete quality. However, a confound of the Overkalix cohort studies is that the nature of the environmental trigger of these survival phenotypes remains unclear, because the available information is on global food availability, not the specific nutritional intake of parents.

Epigenetic inheritance of phenotypes induced by environmental stress

In *Drosophila*, heat shock and osmotic stress induce phenotypes, including wing changes and the disruption of heterochromatin, that are transgenerationally heritable for multiple generations [4,40]. The epigenetic inheritance of heat shock-induced chromatin disruption occurs via drosophila activation transcription factor 2 (dATF-2) [40], a transcription factor that functions in heterochromatin nucleation [41]. Cellular stress induces the phosphorylation of

dATF-2 and, thus, its release from chromatin, resulting in disrupted heterochromatin [40]. Stress induction over several consecutive generations of ancestors caused inheritance of a defective heterochromatin state in multiple generations of descendants, but the chromatin state eventually returned to normal. Thus, although the epigenome may be significantly changed upon environmental intervention, the resulting chromatin state retains the capacity to be reset once the insult is no longer present. This reversal also indicates that this stress induces *bona fide* epigenetic changes, rather than genetic mutations. Finally, this study suggests that transgenerational epigenetic inheritance is more evident upon a persistent, rather than temporary, environmental stimulus. In line with the idea that stress stimuli can induce heritable epigenetic changes, a recent study in *Drosophila* showed that developmental alterations induced by toxic challenges can be inherited in an epigenetic manner, which is mediated at least in part by the repression of chromatin regulators of the Polycomb family [42].

Epigenetic inheritance of RNAs derived from a viral sequence

A recent study in *C. elegans* reported transgenerational inheritance of small RNAs derived from a virus [43]. The authors generated a worm transgenic line expressing the Flock House virus, which results in the production of small-interfering RNAs derived from the virus (viRNAs). These viRNAs serve to silence the viral genome. Interestingly, the silencing effect of viRNAs is transmitted in an epigenetic manner to several generations of descendants [43]. The viRNAs themselves are transmitted in a manner that is independent of the template that generated them [43]. What is particularly intriguing is that both short-term (approximately three generations) and long-term (>50 generations) silencing occurs, but only long-term silencing requires the RNA-dependent RNA polymerase *rrf-1* [43]. It will be interesting to understand further the mechanisms underlying the difference between worms that lose inherited silencing of viRNAs early on and worms that exhibit stable silencing of viRNAs. One could speculate that in an organism with a short life cycle, such as the worm, the epigenetic inheritance of a response to pathogens may have beneficial effects for the offspring.

Epigenetic inheritance of behaviors

Depressive-like behaviors

Exposure to prenatal psychological stress in mice appears to impact F2 generation offspring in an epigenetic manner [44]. F2 males from fathers that were exposed to prenatal stress exhibited a brain gene expression profile that was more similar to that of control females than of control males (termed 'dysmasculinization') [44]. In particular, three miRNAs targeting *β-glycan*, a member of the transforming growth factor (TGF) β family known to regulate release of gonadal hormones, were significantly reduced in the brains of F2 males from fathers that were exposed to prenatal stress [44]. In addition, these males had decreased anogenital distance and testis weight, another characteristic of dysmasculinization [44]. The observation that prenatal stress of fathers results in dysmasculinization of male offspring indicates potential epigenetic transfer of neurodevelopmental phenotypes. It will be important to test whether any of the observed phenotypes can be passed on to descendants via *in vitro* fertilization (IVF), which would test whether the contents of sperm are sufficient to induce transgenerational inheritance, thereby implicating an epigenetic mode of transfer.

Studies examining how postnatal exposure to stress in parents affects the behavior and physiology of subsequent generations have reported heritable effects of chronic, unpredictable maternal separation and unpredictable maternal stress (MSUS) on mouse behavior [45,46]. Depressive-like behaviors were observed in two generations of offspring from mice exposed to MSUS during the first 2 weeks after birth [45]. Males, but not females, exposed to MSUS exhibited more depressive-like behaviors. They also consumed less sucrose, a model of anhedonia, or lack of enjoyment in normally rewarding tasks [45]. Similarly, maternal separation results in heritable increased risk-taking behavior, which is exacerbated when mothers are also subjected to unpredictable stress [46]. Some behavioral changes were inherited via the male germline through multiple generations [45], suggesting an epigenetic mode of inheritance. Consistently, the sperm of males subject to MSUS showed a significant increase in DNA methylation of the chromatin regulator methyl CpG binding protein 2 (MeCP2), along with a decrease in DNA methylation of the stress hormone receptor corticotropin-releasing factor receptor 2 (CRFR2) [45]. It will be important to test the F3 generation to determine whether the observed inheritance is truly transgenerational and not due to multigenerational exposure to stressful stimuli.

These findings suggest that unpredictable postnatal stress leads to the inheritance of increased depressive-like behaviors, impaired social interactions, and increased risky or reckless behaviors. The presence in descendants of less anxious behaviors, decreased anhedonia, and decreased fear of aggressor-specific odors, however, points to the possibility that early stress could blunt subsequent normal responses to stress. Thus, it remains unclear whether these inherited behaviors play a beneficial or detrimental role in the life of animals.

It is important to note that several confounding factors might affect the interpretation of behavioral experiments (Box 1). For example, mothers may not invest as much energy in raising offspring from males they perceive as deficient, a confound to epigenetic inheritance called 'maternal provisioning' [47]. Using IVF with sperm from experimental fathers should help rule out such confounds. For example, offspring of socially defeated males mated with control females displayed increase depressive and anxiety-like behaviors [48]. However, when IVF with sperm from socially defeated males was conducted, the only significantly altered behavioral phenotype observed in F1 offspring was a decreased latency to immobility in the forced swim test, indicating that either few or subtle changes are passed on to offspring via the sperm epigenome alone.

Olfactory imprinting behavior

Although most studies on behaviors have been performed in rodents and were done mostly in response to stressful

Box 1. Caveats underlying studies of transgenerational epigenetic inheritance

Although many of the studies discussed in this review describe compelling and well-controlled examples of transgenerational epigenetic inheritance of acquired traits, it is important to consider the potential confounds that exist in the design and interpretation of such experiments.

In studies involving genetic crosses (Figure 1, main text), there is the possibility that the parents are not completely isogenic, thereby carrying extraneous mutations and potentially creating 'hybrid vigor' effects. Using highly backcrossed strains can minimize these effects, but it is almost impossible to eliminate differences in genetic backgrounds of ancestor strains. Moreover, initial changes in the genomes of the ancestors may affect chromosomal structure and result in defects in meiosis that could in turn affect the genotype or phenotype of subsequent generations. For example, meiotic silencing of gene expression by unpaired DNA has been observed in fungi, worms, and flies [84]. This potential confound has been ruled out in one study of transgenerational inheritance [43], but it remains unclear whether it could affect others.

In cases of transgenerational inheritance induced by nutrient changes, environmental stress, or psychological stress (Figure 2, main text), it is possible that such stimuli may result in genetic mutations in the germline of parents that are subsequently passed on to offspring. Deciphering whether epigenetic marks are directly inherited or just downstream consequences of another genetic event will be instrumental in providing further insight into the mechanism underlying transgenerational epigenetic effects.

Other more complex confounds also exist. It is also possible that uncontrolled alterations in the microbiome, the intestinal flora of the organism, could underlie some of the observed nongenetic transmission of metabolic and other phenotypes [85,86]. Cryptic behavioral phenomena, including maternal provisioning, whereby mothers allocate more or fewer resources to progeny depending on the quality of their mate [47], could also be a confound in the interpretation of epigenetic inheritance of behavioral and/or metabolic phenotypes.

Thus, although great care and experimental rigor is usually taken in studies of transgenerational epigenetic inheritance, it is important to note that confounding genetic and environmental caveats may be difficult to eliminate completely. The use of *in vitro* fertilization (IVF) could provide an alternative, and perhaps more direct, way of testing transgenerational inheritance. However, IVF itself also increases the risk of aberrant genomic imprinting [87] and might bring a set of epigenetic issues of its own. Discovering the specific molecules involved in the mechanism of transgenerational inheritance, including DNA methylation, histone modifications, and noncoding RNAs, should be a fundamental step in identifying which instances of nongenetic inheritance are truly epigenetic.

stimuli, a recent study described the inheritance of an acquired olfactory imprinting behavior in *C. elegans* [49]. Olfactory imprinting is a process whereby exposure to an olfactory cue early in development affects the behavioral response of an animal, in this case positive chemotaxis, upon encountering this chemical in adulthood [49]. In worms, olfactory imprinting requires the presence of food [49]. Worms that have been imprinted not only exhibit a more robust ability to migrate toward the chemical, but also lay significantly more eggs, suggesting that olfactory imprinting provides a memory of a favorable environment [50]. Strikingly, inducing imprinting over multiple consecutive generations of worms resulted in a stable inheritance of the positive chemotaxis behavior in subsequently naïve descendants, in some cases for over 40 generations [49]. This study suggests that acquired behavioral plasticity can be inherited for many generations when ancestors were exposed to a persistent stimulus. One could speculate that specific mechanisms of transgenerational epigenetic inheritance may have been selected for, possibly to allow organisms with a short life cycle to pass on information to their offspring about previous favorable environments.

Molecular mechanisms of epigenetic inheritance

DNA methylation

DNA methylation can be transmitted across generations, for example in genomic imprinting, and could thus underlie transgenerational inheritance of specific traits [16–19,21] (Figure 3, Table 1). Indeed, several studies discussed here have observed heritable changes in DNA methylation at specific loci, for example in response to a high-fat diet and maternal and postnatal stress [32,34,45]. By contrast, other studies have found DNA methylation to be minimally affected, for example after maternal caloric restriction or a paternal low-protein diet [36,51]. It is also noteworthy that worms display transgenerational epigenetic inheritance, yet do not appear to have classical DNA methylation enzymes. Thus, DNA methylation may play a direct mechanistic role in some, but not all, examples of transgenerational epigenetic inheritance. Given that DNA methylation has been extensively reviewed elsewhere [16–19,21], we focus here on other epigenetic mechanisms.

Histone modifications

Histone modifiers, in particular those affecting H3K4 methylation, have been implicated in transgenerational epigenetic inheritance of sterility and longevity in *C. elegans* [26,27]. In flies, environmental stress induces disruption of heterochromatin over several generations [40]. Furthermore, epigenetically heritable alterations of fly development in response to toxic challenges are mediated, at least in part, by suppression of genes encoding the Polycomb group family of H3K27me3 regulators [42]. In mice, levels of trimethylated histone 3 lysine 27 (H3K27me3), a repressive histone mark, are lower at specific loci in the sperm of offspring from males fed a low-protein diet [36]. Furthermore, chromatin remodelers have previously been identified in a dominant screen in mice associated with epigenetic reprogramming [52]. These observations suggest that histone modifications, and possibly chromatin states, are central to mechanisms of transgenerational epigenetic inheritance in many organisms (Figure 3, Table 1).

The exact sequence of events in which a change in histone marks could lead to transgenerational inheritance of traits is not yet known, but potential scenarios are emerging (Figure 3). The initial depletion of a histone mark (for example H3K4me3) may not be entirely replenished at specific loci in the next generations, perhaps due to an amplification loop involving other epigenetic mechanisms, or a blockage by antagonistic marks at these loci. For example, transgenerational inheritance of fertility or longevity due to deficiencies in H3K4me3 regulators, such as the COMPASS complex (WDR-5, ASH-2, SET-2) or SPR-5, could be relayed by changes in H3K36me3 [53]. Indeed, the H3K36 methyltransferase maternal effect sterile (MES)-4 has been shown to be involved in transgenerational maintenance of chromatin states [53,54]. In early embryos, MES-4 is bound to genes previously expressed in the maternal germline [54]. MES-4 plays a role in the

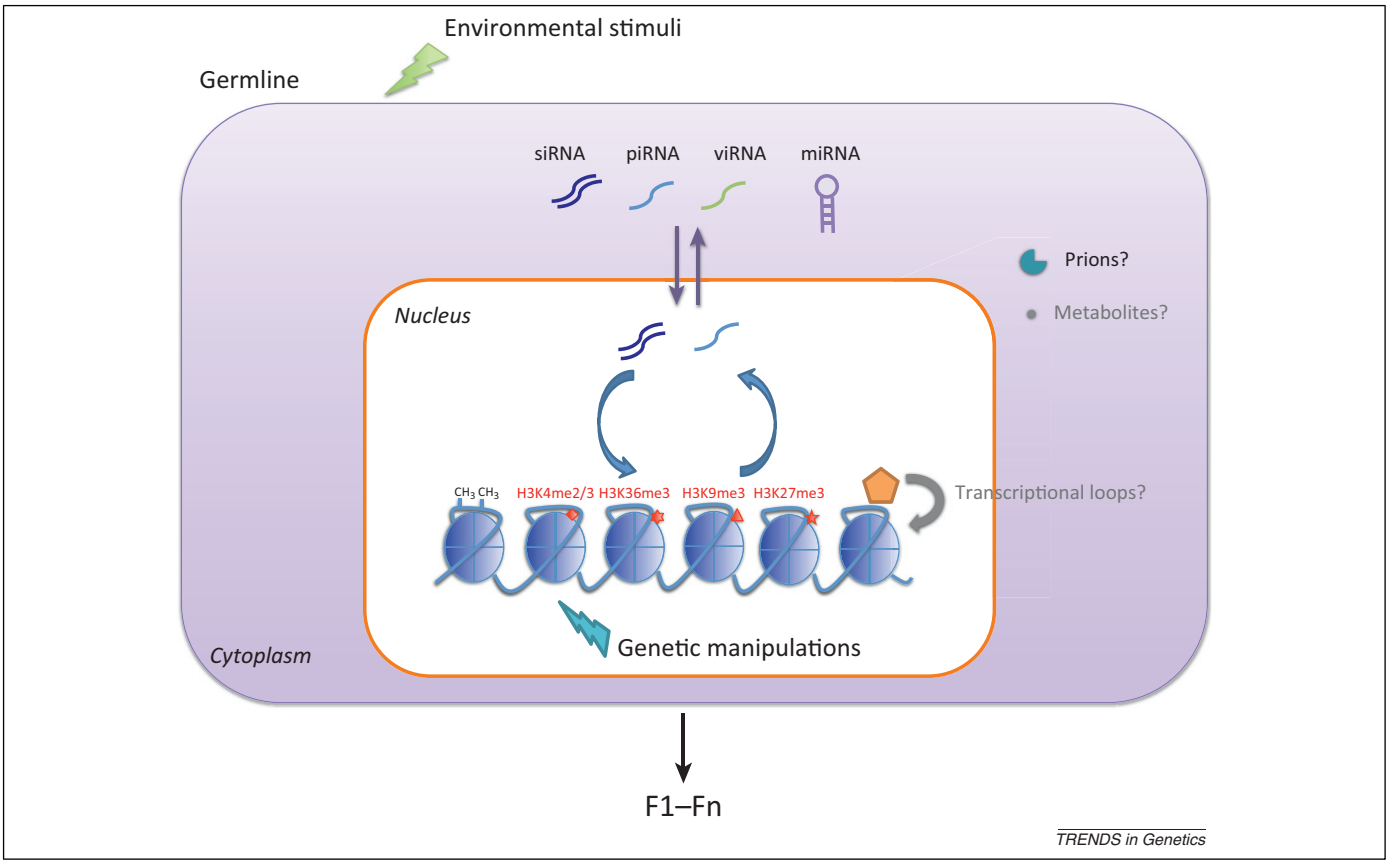

Figure 3. Schematic of molecular players involved in transgenerational epigenetic inheritance. Alterations to the parental genome (aqua lightning bolt) or environmental stimuli (green lightning bolt) could trigger epigenetic changes in the germline of the organism that could be transmitted to the next generation. Such epigenetic changes could possibly be relayed or amplified in the germline of subsequent generations, and persist for several generations. Eventually, epigenetic changes would be reset to a basal state. So far, the epigenetic mechanisms that have been described include changes to chromatin (e.g., histone marks and DNA methylation) and changes to noncoding RNAs, in particular those involving the nuclear RNAi pathway. An amplification loop could be initiated by alteration in chromatin marks (e.g. H3K9me3) at a genomic locus, followed by the generation of noncoding RNAs at this particular locus, which would then be transmitted via the germline, and in turn guide H3K9me3 deposition at that same genomic locus in the germline of the next generation. Curved arrows indicate known molecular interaction. Straight arrows indicate nucleo-cytoplasmic exchange. In gray are additional potential mechanisms that remain to be investigated (Box 2). Abbreviations: piRNA, Piwi-interacting RNAs; siRNA, small interfering RNA; viRNA, small-interfering RNAs derived from the virus.

maintenance of H3K36me3 at germline genes in a manner that is largely independent of their transcriptional status [53]. Thus, an initial depletion in H3K4me3 might result in the depletion of MES-4 and/or H3K36m3 at germline genes, which may underlie transgenerational inheritance of longevity and fertility in *C. elegans*, in a transcription-independent manner.

However, in some other cases, the transcriptional status of specific genes may actually play a role in transgenerational epigenetic inheritance [55]. Chromatin modifications and histone variants established in the germline can be retained in mature gametes and even in the early embryo, specifically H3K4me2, a mark associated with active transcription, and H3.3, an alternative histone associated with memory of an active transcriptional state [56]. Heritable epigenetic information appears to be influenced by parental germline transcription [55]. Active gene expression in adult germ cells in the parent correlated with more robust expression of the same gene in the somatic cells of offspring [55]. In addition, active transcriptional states were found to be strongly inherited in *Dictyostelium*, and this phenomenon required H3K4 methylation [57].

Table 1. Summary of molecular players involved in transgenerational epigenetic inheritance

Epigenetic molecule			Species	Refs
Histone modifications	Active	H3K4me2/3	Worm	[26,27,55]
		H3K36me3	Worm	[53,54]
	Repressive	H3K9me2/3	Worm, fly	[40,58,59,66,72]
		H3K27me3	Mouse, human	[36,60,61]
DNA methylation			Mouse, rat	[32,34,45]
Noncoding RNAs		viRNA	Worm	[43]
		siRNA	Worm	[58,59,69–72]
		piRNA	Worm, fly	[63–66,73,76]
		miRNA	Mouse	[23–25,36,44]
Prions			Yeast	[83]

Thus, transgenerational inheritance could be mediated by transcription-dependent mechanisms, in line with the observation that gene expression can also be altered in a transgenerational manner [27,55].

It is also possible that depletion of an activating mark at specific loci may allow a repressive mark to expand at these loci. In turn, this repressive mark could itself be inherited, thereby preventing the replenishment of the activating mark over several generations. For example, the repressive mark histone H3 trimethyl Lys9 (H3K9me3) has been shown to be involved in transgenerational epigenetic inheritance of genomic loci targeted by noncoding RNAs in *C. elegans* [58,59], and this could mediate the transgenerational inheritance due to changes in H3K4me3 [26,27].

Environmental stimuli that trigger transgenerational phenotypes could also do so by modifying specific chromatin marks in egg and/or sperm. Even though most histones in sperm are replaced by protamines, the remaining histones and DNA still retain extensive epigenetic modifications [60,61]. In addition, protamines themselves are known to be modified post-translationally [62]. Protamine modifications could be influenced by the modification of the histones they are replacing, and vice versa, thereby providing a way of transferring epigenetic information at specific loci. Interestingly, genomic loci encoding genes that are crucial for early development, miRNA promoters, and imprinted gene clusters are enriched for specific epigenetic modifications, for example H3K27me3, in human sperm [60]. In mature human spermatozoa, genes with higher H3K27me3 at their transcriptional start sites are more likely to be repressed during gametogenesis and early embryogenesis [61], suggesting that this mark facilitates memory of a transcriptional state across generations. Because H3K27me3 at specific loci can be affected by paternal diet [36], one could speculate that certain environmentally induced chromatin modifications may be selectively retained across generations, leading to phenotypic changes in descendants.

Noncoding RNAs

Mounting evidence indicates that a variety of noncoding RNAs, including small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), and miRNAs, are involved in transgenerational epigenetic inheritance in worms, flies, and mice [23–25,43,63–66] (Figure 3, Table 1). Given that noncoding RNAs can also influence epigenetic marks and chromatin states, it is tempting to speculate that these RNAs also underlie instances of transgenerational inheritance that are initiated by changes in chromatin states.

The RNAi machinery, chromatin states, and heritability

The RNAi machinery appears to be important for both maintenance of chromatin state and heritability of noncoding RNAs. In the yeast *Schizosaccharomyces pombe*, the RNAi machinery is necessary for initiation and maintenance of centromeric heterochromatin, and double-stranded (ds) RNA-triggered release of RNA polymerase II (Pol II) at the replication fork induces heterochromatin spreading [67,68]. In *C. elegans*, several siRNAs are known to be inherited for several generations [69–71]. The RNAi machinery is necessary for the maintenance of heritable expression of dsRNAs and deposition of the heterochromatic mark histone 3 lysine 9 methylation (H3K9me) [58,59]. dsRNA accumulation precedes the appearance of the H3K9me3 chromatin mark [58,59], and the spread of this repressive mark can extend for several kilobases around the trigger region targeted by dsRNAs [59]. The H3K9me3 mark can in turn be maintained for at least two generations in the absence of the initial dsRNA trigger [59]. Thus, small noncoding RNAs appear to be necessary for the deposition of repressive chromatin modification on genomic regions with sequence complementarity in *C. elegans*. An intriguing possibility is that small noncoding RNAs induce transgenerational changes in selective gene expression by directing deposition of histone modifications in a sequence-specific manner.

The nuclear RNAi pathway appears to be particularly important for the transgenerational inheritance of small RNA and the maintenance of the chromatin state. Indeed, the gene *heritable RNAi defective-1* (*hrde-1*; also known as *wago-9*) was recently identified as a gene encoding a nuclear argonaute involved in transgenerational inheritance of germline RNAi in worms [65,66,72]. In *hrde-1* worm mutants, H3K9me3 at germline target genes is progressively lost over generations, which correlates with overexpression of target genes that are normally repressed in the germline [65]. Correspondingly, *hrde-1* mutants become progressively sterile over generations [65]. Thus, the nuclear argonaute protein HRDE-1 is required to transmit epigenetic information to future generations in *C. elegans*. It will be interesting to determine whether nuclear argonaute proteins play a more general role in transgenerational inheritance of traits in different species.

piRNAs

In *C. elegans*, piRNAs can trigger stable, multigenerational silencing of transgenes [63–66]. This silencing activity involves imperfect base pairing of transcripts and is independent of the Piwi endonuclease slicer activity [63,64]. piRNA silencing of transcripts occurs in *trans* by triggering the secondary siRNA response [63–66]. Initiation of silencing requires the Piwi argonaute piwi-related gene *prg-1*, and maintenance of this response requires RNAi-related factors, such as the germline-specific nuclear argonaute *hrde-1*/*wago-9*, *nrde-2*, *rde-1*, *rde-3*, *rde-4*, and *mut-7* [65,66]. Additionally, maintenance of piRNA-induced silencing requires components of chromatin modifiers, such as the heterochromatin protein 1 (HP1) ortholog HPL-2 [65,66], and putative H3K9 methyltransferases SET-25 and SET-32 [65]. Consistent with the involvement of H3K9 regulators, the transgene sequences silenced by piRNAs were enriched for H3K9me3 [66]. Thus, as was the case for siRNA inheritance [58,59], these studies provide support for the interplay between noncoding RNA molecules and chromatin in establishing transgenerational epigenetic inheritance. It is tempting to speculate that this small RNA–chromatin pathway could underlie transgenerational inheritance of other traits that do not involve transgene silencing (for instance, those induced by deficiencies in endogenous genes or environmental stresses).

In flies, piRNAs have also been shown to underlie transgenerational inheritance of germline gene silencing [73,74]. piRNAs can protect the germline against transposable elements (TEs) by triggering heterochromatin in these regions [75]. In flies, TE silencing is achieved via an amplification loop involving the generation of primary and secondary piRNAs [74]. Interestingly, the ability to produce secondary, but not primary, piRNAs in fly ovaries can be inherited in a transgenerational epigenetic manner [73]. Recently, clusters of transgenes have been found to induce strong *trans*-silencing effects on homologous clusters of transgenes and convert them into strong silencers themselves [76]. Interestingly, this epigenetic phenomenon involves piRNAs that are maternally inherited through the cytoplasm [76]. This transgenerational silencing of transgenes lasts for approximately 50 generations and is reminiscent of the plant 'paramutation' phenomenon [14,15]. Together, these results suggest that piRNAs are also involved in transgenerational inheritance in flies. As many different classes of piRNAs have been observed in different stages of mouse spermatogenesis [77], piRNAs might also play a role in epigenetic inheritance in mammals.

miRNAs

Injection of specific miRNAs into fertilized eggs in mice resulted in transgenerational inheritance of cardiac hypertrophy and large body size [24,25]. Furthermore, prenatal exposure to stress in male mice was associated with the reduction of three miRNAs that target regulation of gonadal hormone release [44]. These results suggest that miRNAs contribute to transgenerational inheritance of some traits. However, changes in miRNA expression did not correlate with opposing changes in the expression of potential RNA targets for these miRNAs in livers from offspring of fathers fed a low protein diet [36]. Thus, whether miRNAs are key mediators of the epigenetic memory of metabolic traits, or merely are susceptible to the heritable effect, remains to be determined.

Although most mechanistic work to date has largely focused on chromatin- and nucleic acid-based mechanisms, it is also important to note that other molecules, including proteins and metabolites, may play an important role in certain cases of transgenerational inheritance (Box 2).

Box 2. Other possible mechanisms for transgenerational inheritance: prions, metabolites, and transcriptional loops

Although chromatin modifications and noncoding RNAs have received most of the attention for mediating transgenerational inheritance mechanisms, other nongenetic mechanisms could be at play, including prions, metabolites, and transcriptional loops.

Protein-based mechanisms could serve as an alternate means of transmitting adaptive phenotypes across generations [88]. Many wild yeast strains harbor a variety of proteins that behave as prions and confer adaptive phenotypes to the responses of different yeast strains to a range of environmental stresses such as osmotic, oxidative, pH, ethanol as well as stress from antifungal drugs and DNA-damaging agents [83]. These observations raise the possibility that endogenous prions are transmitted through meiosis and fertilization and perhaps underlie certain modes of transgenerational inheritance.

Another potential mechanism for transgenerational epigenetic inheritance that remains to be explored is the possibility of metabolites or other small molecules serving as the transmitted signal. Changes in metabolite levels can affect the activity of various chromatin-modifying enzymes [89,90], which could result in altered chromatin states that would then be inherited over generations. Alternatively, small amounts of metabolites present in the cytoplasm of oocytes could be directly transmitted to the zygote, which could either interact with chromatin or affect cellular physiology directly. This model would require the existence of bioenergetic loops that, when triggered, would be capable of changing metabolite profiles for several generations before decaying.

Finally, it is also possible that the mechanisms underlying transgenerational inheritance involve altered transcriptional feedback loops that do not directly require any histone modifications or other epigenetic players [91]. Such self-propagating gene regulation governed by transcriptional machinery has been observed in the fungus *Candida albicans* [92]. In this example, the changes in cellular state occurred stochastically, but it is possible that, in other instances, an extracellular signal could activate a transcription factor that would upregulate transcription of its own gene. These feedback loops, once activated, could cause changes in gene expression that persist in the absence of the original activator. For example, an initial genomic mutation in the ancestor could cause a change in the DNA-binding affinity of a transcription factor, which could in turn initiate a transcriptional loop.

Concluding remarks

Transgenerational epigenetic inheritance has been observed for a series of different phenotypes and can result from a variety of genetic or environmental manipulations in either parent. Many recent studies aimed at elucidating the mechanism of this unconventional mode of inheritance have identified promising candidates for mediating these lasting epigenetic effects, including histone modifications and noncoding RNAs. It is possible that different instances of transgenerational epigenetic inheritance have distinct underlying mechanisms. Alternatively, transgenerational inheritance may be mediated by a few major players involved in selective retention of key epigenomic molecules between generations.

Many important questions remain, including how epigenomic changes at key loci are maintained via the germline, how the duration of maintenance is determined, and how the marks or signals are eventually erased and reset. It would be interesting to explore how changes in the strength or duration of stimuli affect the number of generations an epigenetically inherited phenotype lasts.

Transgenerational epigenetic inheritance has been observed in several species. Thus, mechanisms of epigenetic inheritance are likely to have been selected for during evolution. The ability to pass on information about one's environment to descendants could be evolutionarily advantageous. For example, the transmission of some viral RNAs through the worm germline could provide viral immunity to several generations of progeny [43]. However, it is also possible that some cases of transgenerational epigenetic inheritance are not selected for, and only represent erroneous transmission of epigenetic marks, which would normally be erased and replenished.

Could transgenerational epigenetic inheritance itself increase the evolvability, or rate of evolution, of an organism? Although evolvability is itself a controversial notion [78], one could speculate that inheritance of epigenetic marks might affect chromatin states at specific loci, which could in turn make these regions more or less accessible to DNA repair enzymes, and thereby more or less prone to

mutations [79,80]. Whether transgenerational epigenetic inheritance potentially impacts evolvability has never been tested, but model systems with short generation times, such as worms or flies, would be particularly well suited for these studies.

Although genetic information is clearly the main mediator of inheritance, it is becoming increasingly evident that epigenetic mechanisms can also play a role in transmission of several traits. An important next step is to decipher the exact mechanisms of epigenetic inheritance, and identify the molecular entities that are either transmitted or reset between generations. It will be interesting to test whether the mechanisms of transgenerational inheritance are similar or different between organisms with different generation times, such as *C. elegans* and mammals. It will also be important to test whether other phenotypes, and possibly diseases, can be inherited in an epigenetic manner [81,82]. Indeed, epigenetic memory may fill some gaps in the 'missing heritability' that human genome-wide association studies have been unable to reconcile for complex traits, such as longevity, and disorders, such as obesity and schizophrenia.

Acknowledgments

We would like to thank E. Greer, S. Han, B. Benayoun, and D. Valenzano for critical reading of this manuscript, and G. Sherlock and A. Sidow for advice on potential evolutionary implications. Supported by NIH DP1-AG044848, NIH R01-AG031198, and the Glenn Laboratories for the Biology of Aging at Stanford University. J.P.L. is supported by NIH T32-MH020016 and a NSF Graduate Research Fellowship.

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