Molecular mechanisms of transgenerational epigenetic inheritance

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Abstract | Increasing evidence indicates that non-DNA sequence-based epigenetic information can be inherited across several generations in organisms ranging from yeast to plants to humans. This raises the possibility of heritable 'epimutations' contributing to heritable phenotypic variation and thus to evolution. Recent work has shed light on both the signals that underpin these epimutations, including DNA methylation, histone modifications and non-coding RNAs, and the mechanisms by which they are transmitted across generations at the molecular level. These mechanisms can vary greatly among species and have a more limited effect in mammals than in plants and other animal species. Nevertheless, common principles are emerging, with transmission occurring either via direct replicative mechanisms or indirect reconstruction of the signal in subsequent generations. As these processes become clearer we continue to improve our understanding of the distinctive features and relative contribution of DNA sequence and epigenetic variation to heritable differences in phenotype.

Epigenetic
Mitotically or meiotically heritable gene regulatory information that is independent of changes in DNA sequence.

Intergenerational epigenetic inheritance
Transmission of epigenetic information from parent to offspring to the F1 or F2 generations when the signal originated in males or females, respectively.

Transgenerational epigenetic inheritance
(TEI). Transmission of epigenetic information across generations beyond the limit of intergenerational epigenetic inheritance.

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As the basis of genetic information, DNA sequence is the foundation of life on Earth. However, its proper implementation requires another level of information, in the form of regulatory signals. The study of these signals is known as 'epigenetics', a term originally coined by Conrad Waddington in 1942 that has since undergone several redefinitions. In this Review we consider epigenetics to denote "the study of molecules and mechanisms that can perpetuate alternative gene activity states in the context of the same DNA sequence"[1]. This definition encompasses those molecular signals peripheral to the DNA that are generally referred to as epigenetic, such as DNA methylation or modification of histone proteins, as well as more recently discovered gene regulatory signals such as 3D genome organization. It also includes both mitotic inheritance of these signals and inheritance across generations, which is the focus of this Review.

Examples of epigenetic inheritance across generations were reported in fungi and animals decades ago[2–5], but whether this type of inheritance was widespread, particularly in mammals, remained unclear. When discussing epigenetic inheritance, it is important to distinguish between 'intergenerational' and 'transgenerational' inheritance[6] (FIG. 1). Intergenerational inheritance occurs when an organism in which a change is triggered, for instance by an environmental stimulus, passes this change on to its immediate offspring. However, apparent observations of such short-term inheritance do not necessarily entail the transmission of an epigenetic signal. In a first instance, an epigenetic change may be triggered in the parent only, having a different effect on its germline, which then manifests in the offspring but is subsequently lost. Alternatively, the change may be triggered directly but separately in both parent and offspring. Indeed, exposure of the parent entails exposure of the parent's germline, which includes the cells that will go on to produce the next generation. Thus, in a sense, the offspring were directly exposed to the stimulus as well. For an epigenetic change to be transgenerational it must be inherited past the point at which the individual that carries it had direct contact with the environmental cue, demonstrating that the epigenetic signal is being maintained in the absence of this stimulus. In mammals, this means inheritance starting from the F2 generation if the male parent was exposed, or the F3 if the female parent was exposed (given that exposure of a pregnant female results in exposure of the germline of her fetus also).

There is abundant evidence for intergenerational epigenetic inheritance[7,8], but the relevance of transgenerational epigenetic inheritance (TEI), although proposed for a long time, has been more controversial, particularly in mammals[9,10]. This is in part due to the difficulty in proving that phenotypic differences are epigenetic in origin, not genetic. It is only relatively recently that technical advances coupled with carefully controlled studies, primarily in model organisms, have provided

Epimutations
Heritable epigenetic changes, usually causing an observable phenotype.

robust evidence for TEI and insight into its molecular mechanisms. While such comprehensive evidence remains scarce with regard to mammalian TEI, correlational studies continue to raise the possibility of an epigenetic component in several inherited phenotypes. The strong case for TEI in some other organisms may also open the door for a comparative approach, by which our newfound understanding of TEI in species such as *Caenorhabditis elegans* and *Drosophila melanogaster* may be brought to bear on possible cases of TEI in mammals. However, care must be taken in such an approach not to draw too many similarities between such disparate organisms, as an increasing body of evidence shows that TEI mechanisms can vary greatly and, in many cases, are likely to have evolved independently.

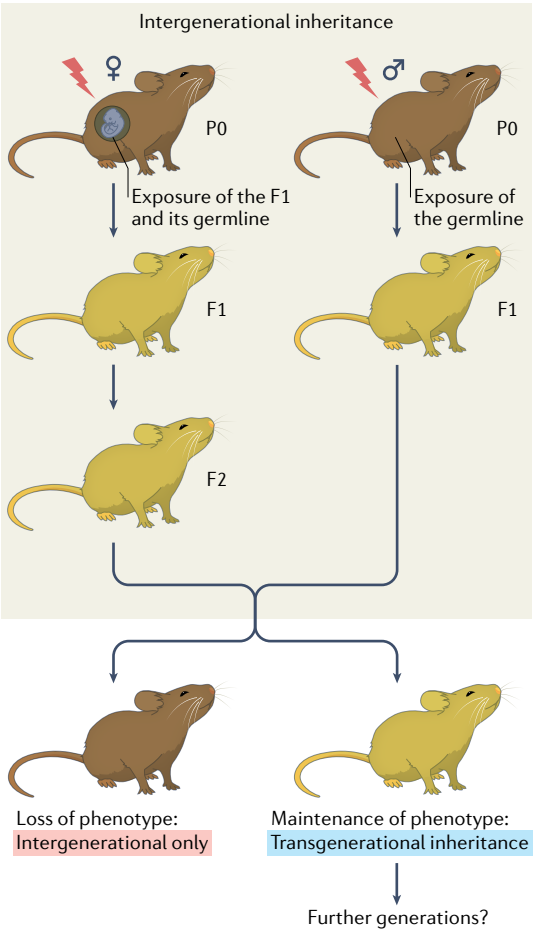


Fig. 1 | **Intergenerational and transgenerational epigenetic inheritance.** Epigenetic change can arise in an individual sporadically or by exposure to some environmental stimulus. If this change is passed on to the next generation, it becomes a heritable epigenetic mark. Inheritance in the immediate offspring of the individual in which the change arose is termed 'intergenerational'. In mice this corresponds to inheritance in the F1 generation for an exposed male parent or in the F1 and F2 generations for an exposed female. This is due to the exposure not only of the individual mouse but also its germline and potentially, in the case of the female, of its unborn offspring's germline. Beyond these first generations many epigenetic signals are lost, and inheritance does not proceed past the intergenerational stage. In some cases, however, the signal is maintained in the F2/F3 generations and beyond. Past this point, it is termed 'transgenerational' epigenetic inheritance, because the epigenetic signal is maintained even in the absence of the initial stimulus or epigenetic trigger.

The implications of these findings are wide-ranging, and call for a re-evaluation of the relative contribution of epigenetics and genetics to phenotype. To this end, some studies have already revealed heritable epigenetic factors in diseases such as cancer[11] and obesity[12]. A major tenet of evolutionary biology is that genetic information is the ultimate basis for selection. However, it has been proposed that 'epimutations' may contribute to short-term survival in a changing environment, or represent precursors to later genetic mutations[13]. These factors have been reviewed elsewhere and will not be the focus of our discussion. Several other reviews give an excellent overview of the field and the evidence for TEI in various organisms[1,6,8,14].

In this Review, we discuss our current understanding of the molecular underpinning of TEI: the mechanistic basis that makes all these implications possible. We first describe the molecular signals — that is, the non-DNA sequence-based carriers of epigenetic information that underpin epimutations — citing examples of TEI across many organisms in which such signals are implicated. We then look at the mechanisms by which these different signals are transmitted from one generation to the next. We see how knowledge of these molecular processes helps to bridge the gap between environment and phenotypic response, and also provides a better understanding of the role that epigenetic information may play in inheritance and fitness, areas long thought to be the sole domain of genetics.

Molecular signals of TEI

Before we discuss the mechanisms by which epigenetic information is transmitted across generations, we must first define the molecular signals that underpin this information. Although we will primarily deal with these signals individually, it is important to note that they by no means exist in isolation, and are indeed frequently in cooperation with each other within the context of the cell. Whenever possible or most relevant we will make every effort to highlight the interaction between these molecular signals. However, for the sake of clarity, we will frequently consider them independently to focus on one particular aspect of TEI.

DNA methylation

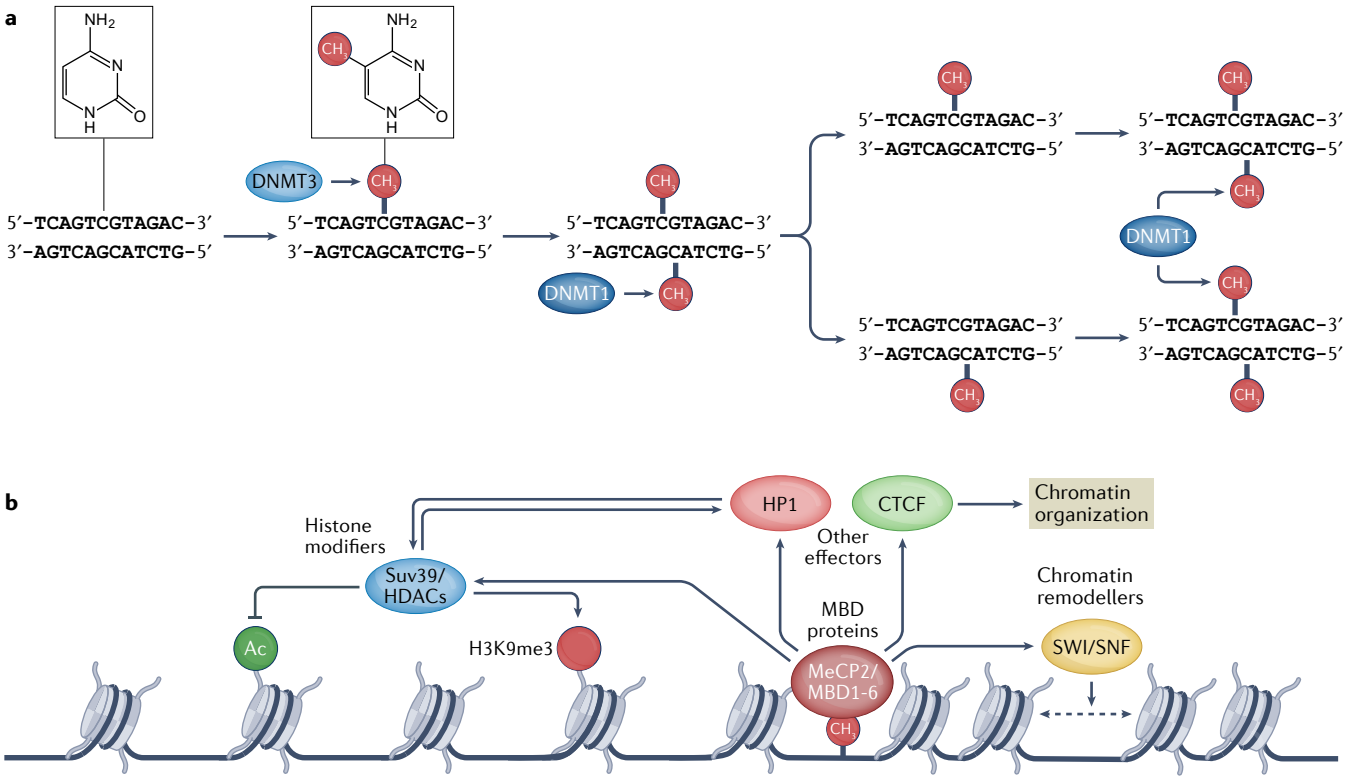
The covalent addition of a methyl group to a DNA nucleotide is a well known epigenetic mark. It can occur in several forms and in a variety of organisms, but the best studied, and most relevant to TEI owing to its mechanism of transmission, is the methylation of cytosine in CpG dinucleotides (BOX 1). Although it is present in invertebrate and fungal species, it is in vertebrates and plants that DNA methylation has the most prominent role in transcriptional regulation and thus the greatest potential to contribute to transgenerationally inherited phenotypes.

Box 1 | **DNA methylation deposition and inheritance**

DNA is primarily methylated on the cytosines of CpG dinucleotides in most organisms, although methylation of CHG and CHH (where H is A, T or C) also occurs, most notably in plants[152,153]. This modification is initially catalysed by a 'de novo methyltransferase', which adds a methyl group to the cytosine at the 5′ position of the pyrimidine ring[85] (see the figure, part **a**). How and when this de novo methylation is targeted varies between organisms. In vertebrates the de novo DNA methyltransferase 3 (DNMT3) enzymes are most active during two key stages of development, gametogenesis and the pre-implantation stage[152], to establish new patterns of methylation following reprogramming. In plants, de novo methylation occurs by an RNA-directed DNA methylation pathway in concert with the small interfering RNA (siRNA) pathway (see BOX 3). Its timing in development is more complex and occurs at a variety of stages[153].

The symmetrical nature of the CpG dinucleotide provides a simple mechanism by which its methylation can be maintained across cell division. Upon replication the methylated parental strands are separated and new unmethylated daughter strands are synthesized from their template, such that the resultant double-stranded DNA molecules are asymmetrically methylated. A 'maintenance methyltransferase' (DNMT1 in vertebrates, MET1 in plants) recognizes this hemi-methylated DNA and methylates the unmodified cytosine[85] (see the figure, part **a**). This mechanism makes CpG methylation the ideal candidate for a 'true' epigenetic mark, with the potential to maintain itself indefinitely unless interfered with. Methylation at non-CG sites is dependent on other maintenance methyltransferases, which rely more on factors external to the DNA methylation itself, including the local chromatin landscape[153].

In both plants and animals the most relevant function of DNA methylation to gene expression is to repress transcription initiation, which it achieves by blocking or promoting the binding of effector proteins to gene promoters[152,153]. Key among these are the methyl-binding domain (MBD) family of proteins, which bind to methylated CpG and recruit an array of histone modifiers (including the histone H3 lysine 9 (H3K9) methyltransferase Suv39 and several histone deacetylases (HDACs)), chromatin remodellers (primarily of the SWI/SNF family such as Mi-2, BRM and ATRX) and more[154] (see the figure, part **b**). CpG methylation is thus a key component of the transcriptional regulation machinery with a clear mechanism of transmission, and is therefore an attractive candidate for the underlying cause of epialleles.



CTCF, CCCTC binding factor; HP1, heterochromatin protein 1; MeCP2, methyl-CpG-binding protein 2.

Because the mechanisms of deposition, transmission and action of CpG methylation are very well understood, it is often proposed to be the molecular basis for observed inheritance of epigenetic phenotypes in vertebrates. In particular, many studies have investigated changes in DNA methylation triggered by ancestral exposure to some environmental insult. However, these studies frequently limit themselves to parent-to-offspring transmission and proof of the inheritance of the phenotype to the F3 or beyond is rare. In TABLE 1 we present some of the more recent or compelling examples of TEI, for which a molecular mechanism is at least suggested. These include DNA methylation-associated cases of transgenerational adaptations of behaviour in birds[15], sex determination in fish[16] and pesticide-triggered pathologies in mammals[17].

Two classic examples of epigenetic inheritance in mice are the *Agouti viable yellow* (A^{vy}) and *Axin fused* ($Axin^{Fu}$) alleles. Both are so-called metastable epialleles: alleles that are variably expressed in genetically identical individuals (reviewed in REF.[18]). The variable expression of both alleles results from the insertion of retrotransposons of the intracisternal A-particle (IAP) family adjacent to a gene, resulting in CpG methylation levels that vary considerably between individuals, while remaining relatively constant between tissues of a single

Metastable epialleles
Genetically identical alleles that are variably expressed owing to epigenetic factors in genetically identical individuals.

Table 1 | **Example cases of TEI with molecular mechanisms**

Organism	Observed phenotype	Generations inherited	Epigenetic signals	Refs
Plants				
A. thaliana	Pathogen resistance	9	DNA methylation	29
A. thaliana	Gene expression changes	8	DNA methylation	148
A. thaliana	Flowering time	Naturally occurring	DNA methylation	26
S. lycopersicum	Fruit ripening	Naturally occurring	DNA methylation	25
L. vulgaris	Floral symmetry	Naturally occurring	DNA methylation	24
H. foetidus	Plant size and fecundity	Naturally occurring	DNA methylation	149
Fungi				
S. pombe	Caffeine resistance	Many (mitotic)	H3K9me	35
S. pombe	Metabolic gene silencing	32 (mitotic) 5 (meiotic)	siRNA H3K9me	36
M. circinelloides	Anti-fungal resistance	>3	ncRNA	46,47
Vertebrates				
R. norvegicus	Obesity and testis disease	3	DNA methylation, ncRNA	17,150
M. musculus	Traumatic stress behaviour	3	ncRNA	80
M. musculus	Developmental defects	3	H3K4me3	40,41
M. musculus	Testis and kidney disease	3	DNA methylation	147
M. musculus	Obesity	6	DNA methylation	132
D. rerio	Sex ratio	3	DNA methylation	16
C. japonica	Egg-laying, social behaviour	3	DNA methylation	15
Insects				
D. melanogaster	Eye colour	>50	H3K27me3	32
D. melanogaster	Eye colour	5	H3K9me3	37
Nematodes				
C. elegans	Longevity	3	H3K4me3	151
C. elegans	Pathogen avoidance	4	siRNA, piRNA	71
C. elegans	*daf-21* gene expression	14	H3K9me3	38
C. elegans	Chemotaxis	3	siRNA	72
C. elegans	Longevity	3	siRNA	69
C. elegans	Gene expression changes	4	siRNA	70
C. elegans	Gene expression changes	4	siRNA, H3K23me3	62

A. thaliana, *Arabidopsis thaliana*; *C. elegans*, *Caenorhabditis elegans*; *C. japonica*, *Camellia japonica*; *D. melanogaster*, *Drosophila melanogaster*; *D. rerio*, *Danio rerio*; H3K, lysine residues of histone H3; *H. foetidus*, *Helleborus foetidus*; *L. vulgaris*, *Lysimachia vulgaris*; *M. circinelloides*, *Mucor circinelloides*; me, (di- or tri-) methylation; me3, trimethylation; *M. musculus*, *Mus musculus*; ncRNA, non-coding RNA; piRNA, PIWI-interacting RNA; *R. norvegicus*, *Rattus norvegicus*; siRNA, small interfering RNA; *S. lycopersicum*, *Solanum lycopersicum*; *S. pombe*, *Schizosaccharomyces pombe*; TEI, transgenerational epigenetic inheritance.

individual. This variation leads to a coat colour phenotype in A^{vy} mice and a 'tail-kink' phenotype in $Axin^{Fu}$ mice, both of which can be inherited across generations. A comprehensive screen for other metastable epialleles similar to A^{vy} and $Axin^{Fu}$ identified 87 candidate variably methylated IAPs[19]. However, only one of the six experimentally validated candidates showed parental inheritance of the methylation pattern, raising the question of how widespread this phenomenon actually is. It is interesting to note that although these phenotypic differences result from epigenetic variation, this variation is dependent on a particular 'permissive' genetic framework, namely an IAP retrotransposon. Further work on the aforementioned library of variably methylated IAPs also demonstrated that their methylation state was highly susceptible to genetic background effects, linked to a cluster of Krüppel-associated box (KRAB) zinc finger proteins[20]. These findings highlight the difficulty of separating genetic from epigenetic factors in inheritance.

In contrast to the few confirmed examples in mammals, the involvement of CpG methylation in epigenetic inheritance in plants is more pervasive, and is in fact one of the clearest instances of TEI. Natural populations of *Arabidopsis thaliana* for instance have been found to vary greatly in their cytosine methylation, with any two accessions differing from each other by between 90,000 and 500,000 differentially methylated positions[21,22]. Much of this natural variation is expected to be due to changes in the underlying DNA sequence

Epigenetic recombinant inbred lines
(epiRILs). Inbred plant strains with different DNA methylation profiles obtained from a cross between two parents from the same genetic background of whom one bears a mutation in a DNA methylation gene.

Quantitative trait loci
(QTLs). Loci where genetic variation correlates with variation in a quantitative, non-discrete phenotype.

variation, although some is thought to be truly epiallelic. In addition, artificially induced methylation differences have also been found to be maintained for many generations. Epigenetic recombinant inbred lines (epiRILs) have been generated by breeding parents from the same genetic background, one with a mutation in a DNA methylation gene and the other with the wild-type version of the gene. From this initial cross, further offspring are produced that recapitulate the parental wild-type background, eliminating the mutation. In this way, many genetically identical wild-type individuals are produced from a common ancestor that had DNA methylation defects. Interestingly, these individuals have drastically different methylation profiles, which can be maintained until at least the F8 generation[23].

Many striking instances of phenotypic variation in various plant species have been ascribed to heritable DNA methylation. Natural DNA methylation variation at the *Lcyc* locus in the toadflax species *Linaria vulgaris* was found to directly correlate with flower morphology, resulting in either bilateral or radial symmetry[24]. In tomatoes, methylation of the promoter of the *LeSPL-CNR* gene results in reduced expression and colourless, non-ripening fruits[25]. Additionally, methylation at the *FWA* promoter in *A. thaliana* results in a late-flowering phenotype[26]. Other examples are included in TABLE 1. However, owing to the complex and often redundant nature of plant genomes, the widespread but small methylation differences evoked above are more likely to produce frequent but subtle changes in gene expression patterns[27]. Thus, rather than underlying switch-like differences in phenotype at a single locus, the most important role of DNA methylation-based epialleles in plants is probably as a contributing factor to the broad spectrum of natural variation in quantitative trait loci (QTLs), governing such complex and frequently polygenic traits as plant size or flowering time. Indeed, DNA methylation was found to underlie several 'epiQTLs' in *A. thaliana*[28], and four such loci were independently shown to confer partial resistance to a fungal pathogen[29]. In this way, DNA methylation and DNA sequence polymorphisms can accumulate and combine at several loci to produce variation in a single complex trait.

This complexity once again raises an important question about considering the relative contribution of both genetic and epigenetic factors to phenotype, especially with regard to QTLs and polygenic phenotypes. While it is certainly true that epigenetics underlies more phenotypic variation than previously thought, it is also the case that studies on epigenetic responses to environmental stimuli rarely assess the potential impact of genetic variation on the observed phenotypes. Even environmental stimuli that are not historically thought to induce DNA mutations may in fact be triggering genomic instability and genetic changes that can be selected for. This has been found, for instance, in what has long been held up as a classic example of TEI, the so-called 'canalization' experiments performed by Conrad Waddington in *D. melanogaster*[30]. This type of analysis should be included more generally in TEI papers in order to fully parse the contribution of genetic and epigenetic factors to environmental responses.

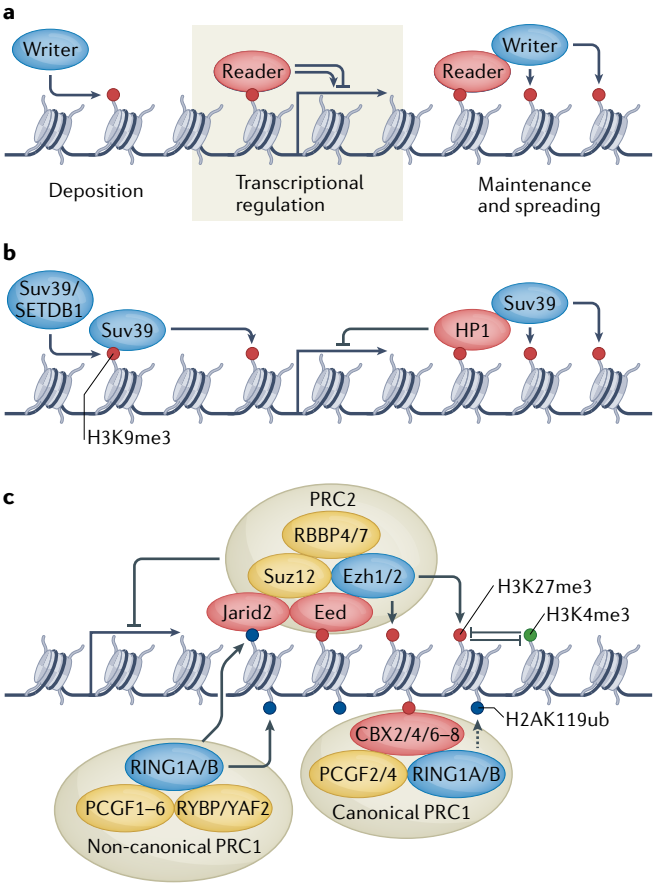
The examples provided by mouse metastable epialleles also illustrate the importance that genetic background can have on epigenetic variation[20]. Similarly, the formation of plant epiRILs shows that the initiation of an epigenetic change may require a genetic mutation, albeit a temporary one. These are themes that are relevant to cases of TEI in other organisms (see below)[31,32]. Examples like these raise the question of whether or not these phenomena can be said to be truly 'epigenetic' if underlying them are fundamentally genetic processes. Indeed, proteins, non-coding RNAs (ncRNAs) and transposons are all encoded in the DNA sequence, which also contributes much of the regulatory information for genome function. Thus, many phenomena that appear epigenetic at first glance might ultimately be genetically determined. Nevertheless, claims for epigenetic inheritance can be made by demonstrating that other molecules carry heritable information to subsequent generations in addition to that carried by the DNA sequence. This is the case in several systems, including mouse IAPs and plant epiRILs, which represent cases of non-DNA sequence-based variation between genetically identical individuals across several generations, fitting comfortably within the definition of TEI.

Histone modifications

Histone proteins form the core of the nucleosome, which is the functional unit of chromatin around which DNA is wrapped. The chemical modifications that can be applied to histones are many and varied, as are their potential effects on the underlying DNA. In addition to their well known role in regulating the expression of genes, many histone modifications have properties that lend themselves well to TEI. These include the potential for self-propagation and spreading of the epigenetic signal by coupling 'reader' and 'writer' functions (BOX 2).

Two modifications frequently involved in TEI are the repressive marks histone H3 lysine 9 trimethylation (H3K9me3) and H3K27me3. In many cases these marks are inextricably linked to other epigenetic signals. H3K9me3, for instance, is strongly associated with DNA methylation in vertebrates[33], and can thus be considered a factor in many of the examples alluded to in the previous section[17,18,34]. On the other hand, there are examples of TEI in which histone modifications appear to be the sole or primary epigenetic signal. In the fission yeast *Schizosaccharomyces pombe*, where DNA methylation is absent, H3K9me has been shown to be heritable under certain conditions when deposited ectopically[31] (where H3K9me refers collectively to H3K9me2 and H3K9me3). In addition to such artificial instances of H3K9me TEI, exposure of *S. pombe* to environmental insults such as caffeine and oxidative stress can also induce epimutations that can be inherited across generations[35]. In the case of caffeine exposure, several epimutant strains were generated, many of which were genetically identical to the wild type, in which caffeine resistance had arisen from heritable silencing of key genes by H3K9me. These epimutations were not tracked through meiosis, although in another study similar H3K9me-based epimutants were inherited through both mitosis and meiosis[36]. In this case, however, the

Box 2 | **Histone modifications**



Histone proteins, around which DNA wraps to form the nucleosome (the basic structural unit of chromatin), are one of the main carriers of epigenetic information in eukaryotes. In a first instance, this is achieved by the placement of variant histones, which can carry information beyond that of canonical histones. Most prominent among these are CENP-A, the histone H3 variant that marks centromeric domains, H3.3, associated with active regulatory elements such as enhancers and promoters, and H2A.Z, which has several roles that may include acting as an epigenetic 'placeholder' in transgenerational epigenetic inheritance (TEI)[155], although many more exist[156].

The most important carrier of epigenetic information, however, is the post-translational modification of histones, both canonical and variant, which have emerged as one of the prime constituents of the transcription regulatory machinery[157]. These modifications usually affect the long lysine-rich amino-terminal tails of histones, H3 being the most studied. The most common and well known modifications are the methylation and acetylation of lysine residues, although lysine ubiquitylation and serine/threonine/tyrosine phosphorylation also have important roles.

In broad terms histone modifications are deposited by a 'writer' protein or complex that catalyses the chemical modification of the target amino acid, and can be selectively removed by an 'eraser'. Once deposited, the modification can be specifically recognized by a 'reader'[158] (see the figure, part **a**). Readers influence the underlying chromatin both by direct action and by recruitment of secondary effectors such as transcription factors, chromatin remodellers and other chromatin modifiers, mediating downstream effects that may have an impact on the expression of underlying genes.

Additionally, coupling of a reader and a writer is a common mechanism by which histone modifications may reinforce themselves after their initial deposition. With such a read–write mechanism, certain modifications ensure their maintenance over a particular locus both within a single cell and across cell division, and in some cases may facilitate the spreading of the modification from a small seed over a larger domain[158]. These principles all apply to the two repressive histone modifications most relevant to TEI: trimethylation of lysine 9 and lysine 27 of histone H3 (H3K9me3 and H3K27me3). Other histone marks can contribute to the transgenerational inheritance of epigenetic states[40,41], and in-depth characterization of the components involved in their maintenance will be required to find out if they also use combinations of writer–reader factors.

H3K9me3 is a widespread modification in eukaryotes catalysed by SET domain histone methyltransferases (HMTs), including suppressor of variegation 3–9 (referred to generally as Suv39 and including Su(var)3–9 in *Drosophila melanogaster*, SUV39H1 and SUV39H2 in mammals, Clr4 in fission yeast and some homology to SET-25 in *Caenorhabditis elegans*) and SET-domain bifurcated 1 (SETDB1 in mammals, MET-2 in *C. elegans*)[159] (see the figure, part **b**). The most important reader of H3K9me3 is heterochromatin protein 1 (HP1, Swi6 in fission yeast, HPL-1 and HPL-2 in *C. elegans*). HP1 recruits an array of secondary partners leading to transcriptional silencing and chromatin compaction, and thus inaccessibility. Binding of Suv39 both directly to H3K9me3 and indirectly via HP1 ensures the coupling of writer and reader functions and thus the maintenance and propagation of the chromatin signal[158]. H3K9me3 domains are often termed 'constitutive heterochromatin' owing to their role at perpetually silenced regions such as centromeres and telomeres. However, H3K9me3 is also important in the targeted silencing of other regions, notably repetitive elements. In many organisms, including mammals, H3K9me3 is also often strongly associated with DNA methylation[33].

H3K27me3 is another widespread repressive mark that is most important in metazoans and plants. H3K27me3 is deposited by the Polycomb repressive complex 2 (PRC2), which integrates a number of proteins that together mediate the writer (Ezh1/2), reader (Eed being the most important) and other functions[129] (see the figure, part **c**). H3K27me3 is a hallmark of so-called 'facultative heterochromatin', which silences genes during development, acting in opposition to the activating H3K4me3 mark. It is often associated in a complex relationship with H2AK119ub, deposited by the PRC1 complex. PRC1 exists in a variety of so-called 'canonical' and 'non-canonical' forms that vary in composition, although all include one of six PCGF proteins, and the writer E3 ubiquitin ligase RING1A/B (see the figure, part **c**). Whether independent of PRC2 or highly associated, each PRC1 complex has its own pattern of recruitment, and the relationship between the two is still being elucidated[160].

Small RNA
Technically refers to RNA molecules under 200 nucleotides in length. More commonly refers to a diverse set of 19–36-nucleotide RNAs implicated in gene regulation, including small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs) and microRNAs (miRNAs).

modifications were coupled with small RNA silencing mechanisms, which will be discussed in other contexts below. Conversely, stress-induced loss of H3K9me3 has been found to be heritable over several generations in both *D. melanogaster*[37] and *C. elegans*[38], resulting in derepression of certain genes for several generations. These events were not linked to any other epigenetic signal and seem to arise more from the failure of the self-maintenance system of H3K9me3 to re-establish the lost histone mark than from any mechanism actively maintaining a derepressed state. Whether this lag in recovery is an evolutionary adaptation for plasticity or simply a flaw in the machinery is an interesting question with implications for the role of TEI in response to environmental stress.

H3K27me3 has also been found to mediate an intriguing case of selectable TEI at a transgene in *D. melanogaster*. In this study the expression level of the mini-white marker, regulating eye colour, could be selected over several generations to generate flies with either fully white or fully red eyes from a parental line with mosaic eye colour[32]. These phenotypic differences arose from differences in H3K27me3 levels at the transgene (known as Fab2L), which could gradually be increased or decreased over generations. Flies maintained an epigenetic memory of the H3K27me3 levels over the transgene, but the transgene could nonetheless be reset to a 'naive' state in a single generation.

Although H3K9me3 and H3K27me3 are the most frequently found histone modifications in TEI, other marks are known to contribute to TEI (TABLE 1) and it is likely that others have gone unnoticed thus far. In *C. elegans*, mutations in the H3K4 histone methyltransferase complex lead to decreased H3K4me and increased longevity. Even after restoring the wild-type genotype, this longevity is heritable for three generations in wild-type progeny of mutant ancestors[39]. Similarly, overexpression of the KDM1A demethylase in the mouse germline led to decreased sperm H3K4me and gene expression changes that were heritable for several generations after normal KDM1A expression levels were restored[40,41]. Interestingly, the authors found no change in DNA methylation compared to control mice, casting doubt on a potential contribution from this more clearly heritable mark. As with all histone modifications, the contribution of different epigenetic marks to TEI cannot always be parsed owing to their interdependence, and attempts to do so may not be fruitful. Nonetheless, whether alone or in combination with each other or with other signals, histone modifications play an important part in defining the epigenetic states underpinning many instances of TEI across diverse organisms.

Non-coding RNAs

Small non-coding RNAs are a major regulator of gene expression at both the transcriptional and post-transcriptional level. For transcriptional regulation, several pathways exist by which small RNAs can alter the epigenetic state of a gene to silence it, namely the small interfering RNA (siRNA)[42] and PIWI-interacting RNA (piRNA) pathways[43,44] (BOX 3). In this capacity they are thus not an epigenetic mark in isolation, but act through chromatin modifications, including DNA methylation. On the other hand, they have clear mechanisms of propagation, and are thus candidates for a heritable signal in TEI. For post-transcriptional regulation, small RNAs can act by mRNA degradation[44,45]. Despite not affecting chromatin, these processes can alter gene activity in a DNA sequence-independent manner which may be considered 'epigenetic' (a point to which we will return later). An interesting example can be found in the pathogenic fungus *Mucor circinelloides*, in which exposure to antifungal drugs results in the appearance of several resistant epimutations[46], a result that is highly reminiscent of the previously cited work in fission yeast[35]. Investigation of the mechanism revealed that these epimutations were dependent only on the Dicer-independent RNA interference (RNAi) silencing pathway[47], and thus appear to derive solely from the post-transcriptional silencing of key genes, in contrast to the caffeine resistance in *S. pombe*.

In plants, the role of small RNAs in TEI is difficult to separate from that of DNA methylation as, unlike in animals, all de novo DNA methylation is RNA-directed. Once established, however, CpG methylation can be faithfully transmitted in the absence of an RNA signal by methylation maintenance mechanisms[48] (BOX 1). RNA itself does not therefore act as the heritable epigenetic signal, although it is crucial in the establishment of epialleles. One exception is methylation at CHH rather than CG sites (where H is any A, C or T nucleotide), which is also widespread in plants. As it is asymmetrical, maintenance of CHH methylation cannot rely on the same mechanisms as CG or CHG methylation, and must be re-established at each replication cycle. This re-establishment is mediated by paternally inherited siRNAs, thus providing evidence that RNAs can act as the heritable epigenetic signal in plants[49]. Recent studies in *A. thaliana* have shown that 24-nucleotide siRNAs produced in nurse cells are transported into meiocytes, the male gamete precursor cells, in order to tame transposons and silence hundreds of genes, driving their DNA methylation[50]. This result underscores how ncRNAs and DNA methylation-mediated epigenetic inheritance play an essential part in genome maintenance and physiology.

In animals, the most common RNA-based mechanisms involved in TEI are the small RNA silencing pathways. The piRNA pathway was first identified in *D. melanogaster* as a defence against transposable element proliferation in the germline[51,52]. This is achieved by both post-transcriptional degradation of the transposable element transcript and transcriptional regulation by targeting of H3K9me3 heterochromatin[53–55]. This H3K9me3 is targeted not only to transposable elements but also to so-called 'piRNA clusters', from which piRNA precursors are transcribed, feeding into the pathway to ensure that silencing is maintained. Although counterintuitive to the general view of H3K9me3 as a silencing mark, the targeting of heterochromatin to piRNA clusters actually promotes transcription in this case by the direct recruitment of the core transcriptional machinery, in the form of the transcription factor IIA paralogue Moonshiner, by the HP1 variant Rhino[56,57].

Box 3 | **Non-coding RNAs in TEI**

Non-coding RNAs (ncRNAs) are varied and have wide-ranging roles across eukaryotes. Among the most relevant to transgenerational epigenetic inheritance (TEI) are the silencing of transcription by small RNAs, a process broadly referred to as RNA interference (RNAi) and that includes small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs). Other major ncRNA categories with less established but emerging roles in TEI include microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and the less well understood tRNA-derived small RNAs (tsRNAs).

siRNAs

Among model organisms, siRNA silencing is present in plants, *Schizosaccharomyces pombe*, *Caenorhabditis elegans* and *Drosophila melanogaster*. Despite the large evolutionary gap between these species, similarities are evident at the core of these pathways[42]. siRNAs are 21–24-nucleotide double-stranded RNA (dsRNA) molecules. They are generated from a longer single-stranded RNA (ssRNA) transcript by processing involving homologues of the Dicer ribonuclease and, in certain species, RNA-dependent RNA polymerases (RdRPs), which cleave them to the appropriate size (see the figure, part **a**). Once they are fully processed, mature siRNAs associate with an Argonaute complex and target it by complementary base pairing to the nascent RNA being transcribed at the target locus. From there, Argonaute mediates gene silencing by association with chromatin modifiers. Known siRNA-directed effectors include H3K9 methyltransferases (Clr4 in yeast, SET-25 in *C. elegans*), H3K27 methyltransferases (MES-2 in *C. elegans*[61]) and DNA methyltransferases (DRM2 in plants) among others[62].

piRNAs

Most animals do not possess RNA-dependent RNA polymerases and so must depend on an alternative means of amplifying small RNAs for RNAi. This is achieved by the use of PIWI-interacting RNAs (piRNAs) for RNA-directed silencing (see the figure, part **b**). piRNAs are most traditionally known for their role in silencing transposable elements in *D. melanogaster*. In this context primary piRNA biogenesis is initiated by bidirectional transcription from a piRNA cluster. This precursor transcript is cleaved to 21–30 nucleotides by Zucchini (Zuc) and associates with one of two Argonaute complexes: Piwi or Aubergine (Aub). Piwi translocates to the nucleus to direct silencing of a transposable element by a similar process to siRNA silencing. Meanwhile Aub cleaves the transposable element transcript to generate complementary ssRNA fragments, which in turn associate with Ago3 to cleave additional precursor transcripts. This method of secondary piRNA biogenesis is known as the 'ping-pong cycle' and is one of the defining characteristics of the piRNA pathway[43,44].

miRNAs

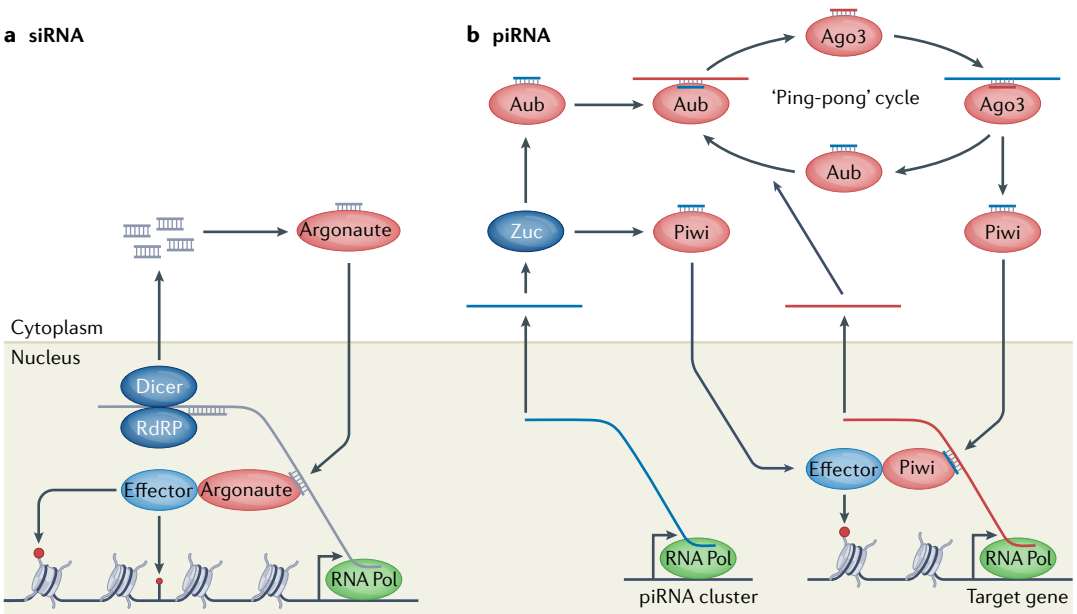
miRNAs are ssRNAs of around 22 nucleotides. They are co-transcriptionally processed by a microprocessor complex including the ribonuclease Drosha, forming a stem-loop structure, and then in the cytoplasm by Dicer to form mature miRNA[45]. miRNAs are best known for their post-transcriptional regulation of gene expression by degrading complementary mRNA in association with an Argonaute complex[161]. However, recent results suggest that both positive and negative regulation of transcription by miRNA also occurs[162–165], although the mechanism remains unclear.

lncRNAs

lncRNAs are RNA molecules longer than 200 nucleotides that do not code for proteins. Unlike the more specific categories above, this somewhat arbitrary size limit encompasses a wide range of ncRNA molecules with diverse functions in the cell[166]. Several studies have implicated lncRNAs in cases of TEI[17,81]; however, the mechanism of their involvement at the molecular level remains unclear.

tsRNAs

tsRNAs are 29–34-nucleotide RNA fragments derived from the 5′ end of tRNAs. The mechanism of their biogenesis is still unclear but they are the dominant small ncRNA in mammalian sperm[167]. Work on tsRNAs is still in its infancy, but early results suggest they may be carriers of epigenetic information in sperm, as well as harbouring numerous RNA modifications[76].



Long non-coding RNAs
(lncRNAs). RNA molecules longer than 200 nucleotides that are not translated into proteins.

Paramutation
Horizontal transmission of a heritable epigenetic state from one allele of a locus to the other.

Comparison of fly strains reveals differences in piRNA biogenesis correlating with differences of H3K9me3 over piRNA clusters[58,59]. These differences are epigenetically inherited and depend on maternally deposited piRNAs in the oocyte for transmission[60]. piRNAs and H3K9me3 thus form a positive feedback loop that ensures transposable element silencing across generations by TEI.

Small RNA silencing is particularly prevalent in *C. elegans*, with 27 distinct Argonaute proteins encoded in its genome[42], and implicates silencing marks including H3K9me3[42], H3K27me3[61] and H3K23me3[62]. It is perhaps not surprising then that some of the most striking RNA-based examples of TEI are found in *C. elegans*. The ability of exogenous small RNAs to induce silencing over generations in *C. elegans* has been known for decades[63,64]. However, for many years most naturally occurring examples of such TEI were limited to the silencing of foreign DNA such as viral genes or transposons[65–68]. Later work has made it clear that certain environmental conditions can trigger transgenerational gene regulation by endogenous siRNAs lasting several generations. Starvation conditions, for instance, were shown to induce expression of a pool of endogenous siRNAs targeting several nutritional genes[69]. These siRNAs were inherited for at least three generations after returning the nematodes to nutritionally rich conditions, potentially transmitting an epigenetic memory for coping with food shortage. Consistent with this idea, these descendants had increased lifespan compared to control nematodes. Heat stress has similarly been found to alter gene expression transgenerationally through small RNAs, lasting two to three generations after a return to normal temperature conditions[70]. More recently, and as an interesting bridge between endogenous and exogenous siRNA, an exogenous source of small RNAs from a pathogenic bacterium was found to induce TEI at an endogenous gene[71]. This study showed that exposure to a pathogenic strain of *Pseudomonas aeruginosa* induced avoidance of the same pathogen for four generations. This behaviour is due to siRNA silencing of the neuronal *maco-1* gene by a single *P. aeruginosa* RNA molecule to which it is complementary. This process is in line with similar results showing TEI of neuronal siRNAs governing chemotaxis[72]. The finding of these spectacular transgenerational effects raises the question of what advantages these mechanisms may have that might be selected for in natural conditions. The fact that small RNAs keep transposable elements at bay[73], combined with recent findings that they regulate the expression of histone genes[74], and that germline-inherited small RNAs facilitate the clearance of untranslated maternal mRNAs[75], suggests that these molecules are evolutionarily selected for their ability to regulate essential physiological and developmental processes rather than for their functions in TEI, which may thus be derived characteristics.

In mammals, much recent work has focused on sperm ncRNAs as a vector for epigenetic inheritance (reviewed in REFS[14,76]). Several studies in mice have shown alteration of sperm RNAs, notably microRNAs (miRNAs) and tRNA-derived small RNAs (tsRNAs), upon paternal exposure to insults or dietary changes[77–79]. Others have intriguingly shown that zygotic injection of total RNA[80], long non-coding RNAs (lncRNAs)[81], or in some cases specific miRNAs[82,83], from other mice can reproduce behavioural and metabolic phenotypes of the father, suggesting that ncRNAs can act as carriers of epigenetic information across generations. However, few have conclusively tracked these changes over several generations, and it remains more plausible that these non-coding RNAs are paternally supplied mediators of intergenerational inheritance, rather than true transgenerational inheritance. Studies in rats, the other major mammalian model organism, are hindered by the lack of inbred stocks, making it virtually impossible to conclusively discount DNA sequence-based effects on phenotype. However, one intriguing phenomenon involves the transgenerational pathologies induced by exposure to the pesticides vinclozolin and DDT[17]. Although the observations are based on correlation alone, with no clear mechanism as of yet, these pathologies, which are heritable for at least three generations, are accompanied by changes in short and long ncRNA expression, DNA methylation and histone retention in the sperm, with frequent overlap of the loci for each of these signals, once again demonstrating that in most physiological conditions these signals are difficult to separate from each other. On the other hand, the nature of the relationship between these signals may have important implications for the mechanism by which the epigenetic signal is passed from one generation to the next, which will be discussed in the next section.

Mechanisms of epigenetic signal transmission

Now that we have discussed the primary carriers of epigenetic information and the mechanisms by which they affect gene expression, we turn our attention to the processes by which these elements are passed from one generation to another, forming a transgenerational signal. These processes will depend on the nature of the signal but will also form complex relationships, just as the signals themselves interact in the regulation of transcription. As has been done elsewhere[84] we will distinguish between more direct 'replicative' means of transmission, by which the signal is transmitted through meiosis in a similar manner to its mitotic maintenance, and indirect 'reconstructive' transmission, in which the primary epigenetic signals are erased but faithfully reconstructed in the progeny based on a secondary signal. Distinguishing between these processes is not always straightforward, not least because a 'true' demonstration of replicative inheritance requires proof that the epigenetic signal is maintained constantly. Providing evidence that a mark is not erased at any point between generations is a difficult task. However, in several instances it has been shown that no significant change in an epigenetic signal is detected across key stages, such as gametogenesis and fertilization, which we take to be good evidence of replicative transmission. To these mechanisms of transgenerational inheritance we will also add a discussion of the special case of paramutation, a process by which epigenetic information is transmitted horizontally between alleles in trans. After this initial step, paramutated 'epialleles' are then transmitted vertically between generations

by other TEI mechanisms. When combined, these processes thus become an important factor in the spreading of an epiallele through a population.

Replicative transmission

DNA methylation and histone modifications are the features most often referred to as 'epigenetic' because they are major regulatory signals that can be faithfully transmitted across mitosis. This mitotic transmission occurs by a replicative process in parallel to DNA replication: the unmethylated daughter strand of hemi-methylated DNA is recognized and methylated post-replication[85] (BOX 1) while nucleosomes are reassembled after the replication fork, with old modified histones distributed between the strands and combining with new naive ones, allowing their epigenetic states to be re-established by self-reinforcing loops[86–89] (BOX 2). Transgenerational replicative inheritance requires the extension of these mitotic processes to meiosis. Conceptually, this remains the most intuitive means of inheritance. In single-celled organisms such as fission yeast, mitotically inherited epigenetic signals have been found to be maintained through meiosis[31,36], although whether this is by a similar mechanism remains to be seen[90]. However, in higher eukaryotes, maintenance through gametogenesis, fertilization and embryonic development is also necessary (FIG. 2a). These steps present a number of obstacles to TEI, and the extent to which replicative means of transmission occur remains a topic of debate[84].

Many organisms, including animals, undergo one or more genome-wide reprogramming steps in

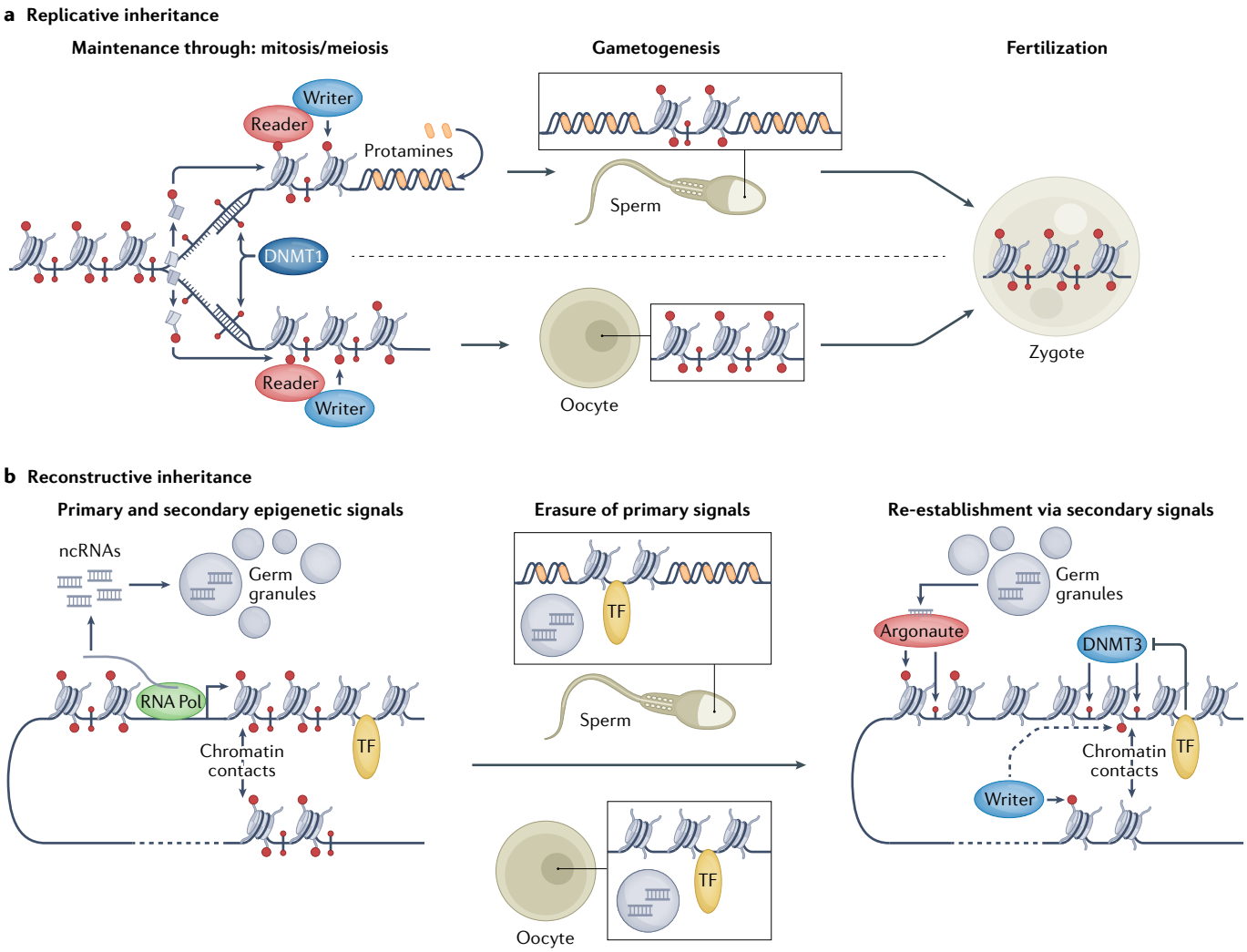


Fig. 2 | **Replicative and reconstructive inheritance.** The two primary models for inheritance of epigenetic information across generations are the replicative and reconstructive inheritance models. **a** | In replicative inheritance models, the processes by which epigenetic signals are transmitted across mitosis are extended to meiosis. Hemi-methylated DNA is recognized by DNMT1 and the unmethylated cytosine is methylated (small red lollipops). Modified histones (large red lollipops) are evenly distributed to the daughter strands after the replication fork and the mark is re-established by reader–writer coupling. These signals are then maintained through gametogenesis and fertilization, surviving both any potential reprogramming steps and replacement of histones by protamines in sperm nuclei. In the zygote, the epigenetic marks are thus carried over directly from the previous generation. **b** | In reconstructive inheritance models, primary epigenetic signals are inherited despite their erasure by recapitulating them from secondary signals. These secondary signals may include non-coding RNAs (ncRNAs), 3D chromatin contacts and transcription factor (TF) binding. Epigenetic marks such as DNA methylation or histone modifications are erased during gametogenesis or early embryonic development, but can be faithfully reconstructed from the information carried by these secondary signals, which are inherited directly.

development during which DNA methylation is erased before being established anew for the next generation. Mammals undergo two such reprogramming events: in primordial germ cells and in the early embryo[91]. Any claim of replicative transmission in mammalian TEI must therefore explain how the epigenetic signal escapes both these reprogramming events. Mechanisms for protection from reprogramming do exist. Imprinting control regions, for instance, resist embryonic (but not germline) reprogramming by the binding of specific factors that inhibit demethylase activity[92–94], but evidence for such mechanisms at non-imprinted regions is scarce. Some studies show that IAPs, which are known to be important for mammalian metastable epialleles, are resistant to both reprogramming events[95,96]. However, more recent work suggests that they may be remethylated post-fertilization by a specific group of KRAB zinc finger proteins[20], and analysis of the metastable epialleles A^{vy} and $Axin^{Fu}$ themselves shows that they are subject to methylation erasure[97,98], arguing against replicative transmission in mammalian TEI. On the other hand, some organisms undergo very little developmental reprogramming, if any. This is the case for plants, where evidence suggests that loss of DNA methylation occurs either by passive dilution or targeted reprogramming, not genome-wide erasure[48]. This would suggest that DNA methylation can be readily maintained across generations in plants, unless actively de-methylated by some targeted event. It is perhaps not surprising then that DNA methylation-based epialleles appear to be relatively common in plants[27].

Histone modifications also undergo large-scale changes during gametogenesis and fertilization in animal species. These changes are particularly extensive in mammals, but they do not constitute a complete erasure, as with DNA methylation reprogramming, and many regions appear to retain their identity, particularly regions of silenced chromatin[99–103]. The largest obstacle to the inheritance of histone marks is the replacement of histones by protamines in sperm nuclei. However, recent work has shown that sperm retain far more histones than previously thought, with estimates around 7.5% of somatic levels for mouse[104] and 4% for human[105]. These histones bear post-translational modifications that are both influenced by paternal chromatin landscapes and prefigure the expression patterns in the early embryo[104,106,107], raising the possibility of replicative inheritance at certain loci at least. Interestingly, in one of the most studied examples of environment-triggered TEI, the transgenerational phenotypes induced upon vinclozolin exposure in rats, initial responses involving ncRNA expression and sperm DNA methylation in the F1 and F2 generations were accompanied in the F3 generation by an increase in sperm histone retention at the same sites[17]. These results suggest that histone retention can be locally altered at certain regions to promote the inheritance of epialleles in subsequent generations.

Reconstructive transmission

The obstacles presented by large-scale reprogramming in animals mean that direct transmission as in mitosis is unlikely to be the prevailing means of inheritance. Nevertheless, signals that are erased can still be inherited by the following generation if they are reconstructed from a secondary epigenetic signal. In mammals, for instance, whole-genome bisulfite sequencing reveals that the majority of CpGs faithfully retain their methylated or unmethylated state after reprogramming in both the germline and embryo[96,108,109]. Despite this, analysis of the dynamics clearly shows that they are de-methylated during reprogramming before being subsequently re-methylated, or not, as the case may be. This clearly indicates that DNA methylation is not the signal being inherited but is instead recapitulated based on some other inherited signal. While this signal may be genetic (for example, recognition of sequence by transcription factors), it may also be epigenetic. Here we will discuss the types of secondary signal that may contribute to the reconstruction of primary epigenetic signals, such as DNA methylation and histone modifications, and thus either reinforce TEI or ensure it by bypassing reprogramming (FIG. 2b).

Non-coding RNAs as templates for reconstruction. As described above, ncRNAs are an integral part of many epigenetic pathways and have been implicated in TEI in several organisms. Within these pathways they act via their effect on chromatin, initiating DNA methylation and histone modifications to regulate gene expression. Several studies have demonstrated that substantial quantities of ncRNAs are transmitted to the following generation both maternally via egg[110–114] and paternally via sperm[115–117]. ncRNAs are thus emerging as major candidates for secondary signals from which these primary chromatin epigenetic signals might be reconstructed.

Ongoing work in *C. elegans*, where RNA-based TEI is most studied, is revealing the role of self-assembling structures known as germ granules in RNA-directed epigenetic regulation and inheritance. Germ granules are germline liquid-like condensates that concentrate various components of the RNAi pathway involved in small RNA processing, amplification and specification, including several Argonaute proteins and RNA-dependent RNA polymerases[118]. In addition, germ granules have been found to be crucial regulators of small RNA inheritance. Disruption of germ granule formation leads to aberrant siRNA expression and runaway silencing of key germline genes in later generations[119,120]. Germ granules thus appear to be the main organizing centres of both function and inheritance of small RNA silencing pathways in the *C. elegans* germline. Germ granules, as well as similar structures such as the piRNA-associated 'nuage', are present in many other organisms including *D. melanogaster, Xenopus laevis* and mice[121]. Whether this role in ncRNA inheritance is also conserved remains to be determined.

Biophysical properties. Many investigations into cases of TEI both in the laboratory and in the wild seek to link the heritable phenotype to an environmental trigger such as a drug or a change in temperature or food source (see TABLE 1 for examples). How these environmental signals might be translated into epigenetic changes remains unclear, but a leading hypothesis is that these

Germ granules
Membraneless cytoplasmic organelles found in metazoan germ cells.

signals are registered by changes in the biophysical properties of biomolecules within epigenetic pathways. Whether these responses are unintentional consequences of the environmental stimulus or adaptations selected to respond quickly to a changing environment is an interesting question, and the answer probably varies from case to case.

Germ granules are a perfect example of how this translation may occur. Mounting evidence suggests that germ granules form by phase separation[122], a self-organizing physical process that is highly sensitive to environmental conditions and can be altered or disrupted by temperature, pH and the concentration of various molecules. Small changes in these factors can produce rapid switch-like effects in organization which can be taken advantage of by the cell to make decisions, such as cell fate decisions in development[123]. Given the emerging role of germ granules in TEI, notably in *C. elegans*, it has been suggested that this environmental sensitivity could be the mechanism by which abiotic factors are translated to heritable epigenetic changes via the intermediate of RNAi[118], as has been observed in many instances of TEI[38,69,70].

Temperature is also known to be a factor affecting TEI in other organisms. For instance, Polycomb-mediated silencing is affected by growth temperature in *D. melanogaster*, and certain temperatures have been found to be more conducive to TEI establishment in Polycomb-dependent TEI[32]. These examples highlight the biophysical properties of the molecules involved in TEI as an added layer within which epigenetic information can be carried, whether it be for the triggering of heritable epigenetic changes or for their transmission and reconstruction in subsequent generations.

3D genome organization. In recent years the degree to which the genome is organized within the nucleus, and the role of this organization in gene regulation, cell identity and development, has become increasingly clear[124]. This organization combines both genetic and epigenetic elements to form a complex landscape of chromatin contacts, which has added another layer to the factors that determine the chromatin state of a locus, potentially contributing to TEI as well. Given that chromosome architecture and nuclear organization are completely altered during mitosis and meiosis and re-established subsequently, such a 'memory' would necessarily fall under the category of 'reconstructive' inheritance.

The relationship between the position of a locus within the nucleus and its expression and chromatin state is well established. Broadly, genes located towards the nuclear periphery tend to be heterochromatic and silenced whereas those near the nuclear interior are more euchromatic and active[125]. The causality of this relationship is not entirely clear, but tethering of a locus to the periphery can induce silencing[126], suggesting that nuclear localization informs the chromatin state. In *S. pombe* nuclear positioning has been shown to be a crucial element in the inheritance of heterochromatin across generations. Maintenance of H3K9me at both a naturally occurring heterochromatic locus and an artificially induced epiallele required the nuclear pore protein Amo1, which sequestered the loci near the periphery[127]. Although nuclear pore proteins certainly play a part in gene silencing in higher eukaryotes[128], their involvement in TEI has not yet been shown. An intriguing open question is whether loci could maintain a memory of their nuclear position across generations, for instance by the binding of some lamina or nuclear pore associated protein that could act as the signal for re-establishment of heterochromatin in the following generation.

Although this has not been shown, something akin to it has been observed in *D. melanogaster*, involving not the position of a locus within the nucleus but its spatial relationship to another locus. As described above, a Polycomb-dependent epiallele could be selected over several generations to either express or repress an eye colour gene by altering H3K27me3 levels over a transgenic locus called Fab2L[32]. Intriguingly, both the establishment and maintenance of H3K27me3 TEI at Fab2L was dependent on its physical association within the nucleus with another Polycomb-targeted locus containing a homologous regulatory sequence called *Fab7*. Deletion of *Fab7* resulted in the loss of TEI at Fab2L, resetting the chromatin to a naive state. In *D. melanogaster* and other species, Polycomb target genes associate in the nucleus to form clusters, which aid in maintaining their chromatin states and repression[129]. These results suggest that in this example of TEI the Fab2L locus does not transmit its chromatin state directly but rather retains a memory of its association with *Fab7*, and is thus able to reconstruct its H3K27me3 levels via this association. This indicates that nuclear chromatin organization may serve as a secondary signal from which inherited epigenetic signals are reconstructed. Elucidating the exact mechanism for this process remains an interesting topic for future work.

Transcription factor binding. The binding of transcription factors (TFs) to their target loci is a complex process influenced by many factors, both genetic and epigenetic, including the levels of cytosine methylation[130]. Integration of several large datasets from germ cells and early embryos has revealed that hypomethylated regions of DNA correlate with the binding of TFs during the epigenetic reprogramming stages of germline and embryonic development[109]. This has led to the suggestion that TFs could hinder methylation of their bound loci during global re-methylation, thus becoming a carrier of epigenetic information. In this way a transient, and potentially environmentally induced, change in chromatin accessibility of a particular region or in the biophysical properties of a TF could lead to the binding of a TF to a new locus and thus hypomethylation in the following generation. This transient change would thus translate to a persistent epigenetic change if the newly hypomethylated region remained accessible to the binding of the TF and would therefore continue to be protected from methylation in subsequent generations.

An example of this process may be found in the obesity phenotype following bisphenol A (BPA) exposure in mice, one of many TEI phenotypes induced by BPA[131].

Exposure of pregnant mice to BPA results in large-scale changes in chromatin accessibility, leading to increased TF binding. Most notably, and related to the previous section, binding of the chromatin organizing protein CTCF results in changes in genome organization and the activation of an enhancer in the *Fto* gene[132], which has previously been implicated in obesity in humans[133]. This enhancer activation, as well as the obesity phenotype, persist for 5 to 6 generations, at which point both disappear. This provides an intriguing example of how many of the signals discussed here (biophysical factors, 3D genome organization and TFs) may be at play in a single case of TEI, combining to contribute to the reconstruction of an epigenetic phenotype induced by environmental perturbations.

Paramutation

All of the mechanisms described above involve the vertical transmission of an epigenetic state between generations, similar to the Mendelian inheritance of a genetic mutation. However, as epigenetic information can be added or removed independently of changes in the underlying DNA sequence, and because these epigenetic changes might then be transmitted to subsequent generations, the possibility of non-Mendelian inheritance mechanisms is open to epigenetic marks. In some cases of TEI, such non-Mendelian transmission is observed through a process known as paramutation. Paramutation is the process of transmission of epigenetic information in *trans* from one allele of a gene or locus to its pair on the sister chromosome[134] (FIG. 3). Paramutation was first described in plants, where it is relatively common and most studied[135], but has since been documented in *C. elegans*[67,136], *D. melanogaster*[32,137,138] and more controversially in mice[139–141], with transfer of both DNA methylation and histone modification states between alleles having been observed.

The term 'paramutation' itself simply describes the transfer of information from one allele to another, and thus the mechanisms by which this transmission occurs are likely to be quite different between these diverse organisms and are not well understood. However, certain common aspects are beginning to emerge that may speak to either a common origin of paramutation in these species or of the evolution in parallel of paramutation from similar epigenetic pathways that are conducive to it. Notably, small ncRNA silencing pathways have been implicated in paramutation in all of the species mentioned above[134,142]. Another factor that may be involved is physical contact within the nucleus between the two allelic loci. This may be particularly relevant in species such as *D. melanoaster*, in which pairing of homologous loci is commonplace[143] and in which the similar process of transvection occurs, involving contacts between trans-homologues[144]. Recent data indicate that chromatin contacts between homologues occur in a substantial number of species across evolution[145], suggesting that a contribution by chromosome pairing might be more frequent than previously thought. This possibility is further supported by the observation that paramutation in many organisms frequently occurs at transgenes, repeat elements or in polyploid organisms, in which several copies of similar loci are present elsewhere in the genome[32,67,136–138,146]. Indeed, one case of paramutation at a transgene in *D. melanoaster* is known to involve 3D chromatin contacts of the transgenic locus both with its homologous allele and with a homologous endogenous locus[32].

Paramutation is thus likely to encompass several mechanisms across different organisms by which epigenetic information is transmitted in trans. Combined with the previously described methods of inheritance across generations, it can allow for the rapid spread of a heritable epiallele within a population by propagating epigenetic information within an individual as well as from one individual to its offspring. As opposed to the slower spread of genetic mutations, which often confer a more permanent advantage to an organism but necessarily propagate by Mendelian inheritance, this could be a crucial property for an epiallele that may confer a more urgent but transient advantage in response to a rapid but temporary change in conditions.

Conclusions and perspectives

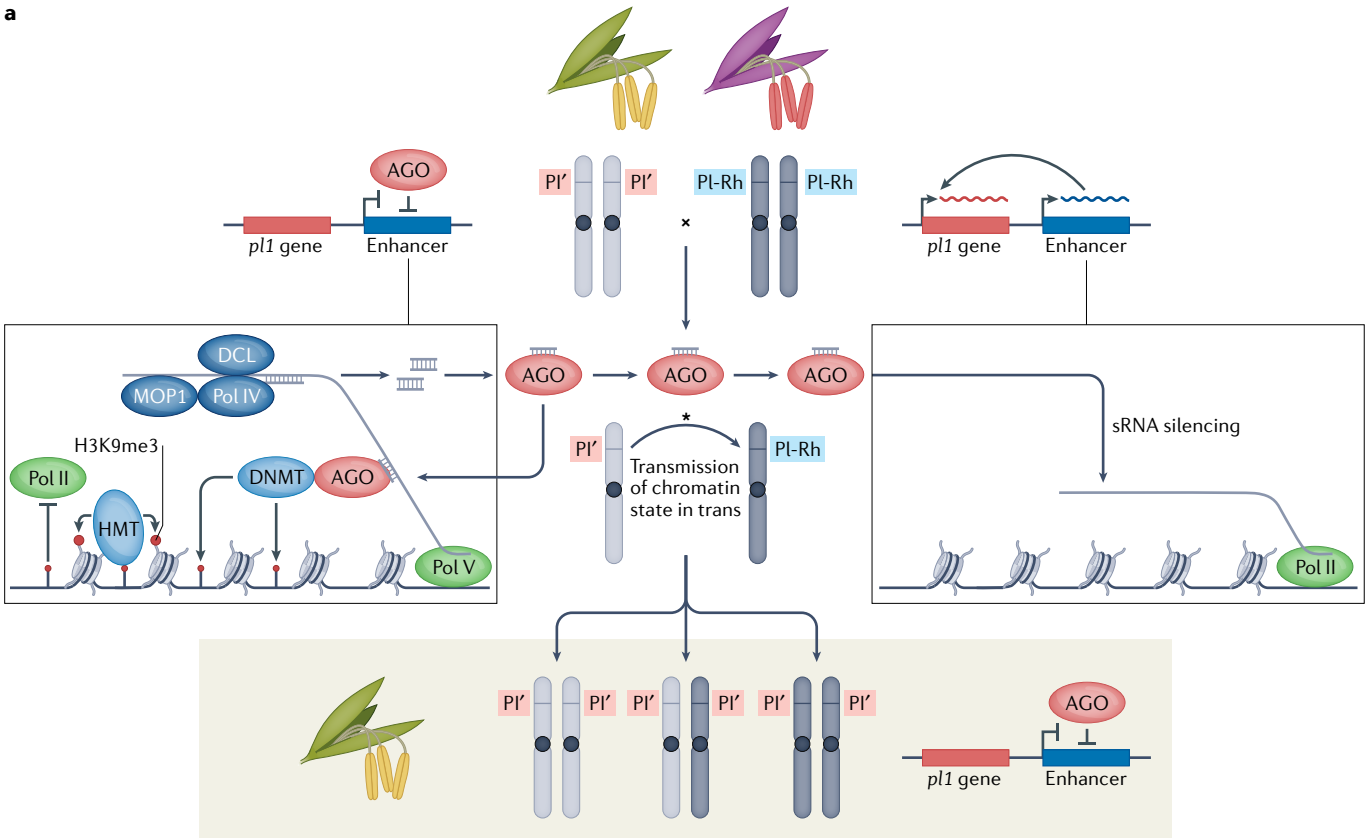
The contribution of epigenetics to phenotypes inherited across generations has remained controversial for many years. Now, as examples of TEI are accumulating, not only is its importance becoming clearer but the mechanisms by which it occurs are being increasingly described. This work has revealed common features, including the involvement of similar epigenetic signals across organisms and their transmission by either replicative or reconstructive means. However, it has also highlighted the extent to which these mechanisms can differ in their details both between and within organisms. The involvement of many different types of epigenetic signal, both primary and secondary, in any number of combinations reveals a complex landscape of interactions that can regulate TEI in many different ways.

One major question is the place of TEI mechanisms in evolution. Are these processes adaptations to survive better in a changing environment, or are they unintentional consequences of environmental pressure being placed on cellular machinery with other roles? If TEI is indeed adaptive, what, if any, has been its role in evolutionary history?

In terms of mechanisms, we might also ask to what extent replicative means of inheritance are important compared to reconstructive inheritance. Although the existence of true TEI in mammals remains controversial, in part because of the extensive epigenetic reprogramming that occurs during their development, reconstructive inheritance provides a mechanism by which TEI could take place, whereas replicative inheritance appears to be more prominent in organisms that do not undergo such reprogramming. Nonetheless, both types of mechanism are likely to be at play in many organisms. In the case of reconstruction, a more thorough investigation of the secondary signals that allow for the re-establishment of epigenetic signals is called for to truly understand how they are faithfully recapitulated.

The question of the prominence of TEI in human health also raises itself. Epigenetic contributions to diseases are already a major and fruitful topic of research.

Transvection
A process that is common in *Drosophila* species by which one allele of a gene or its regulatory sequence on one chromosome can regulate the transcription of its homologue on the other chromosome in trans, mediated by pairing of the two loci.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AGO
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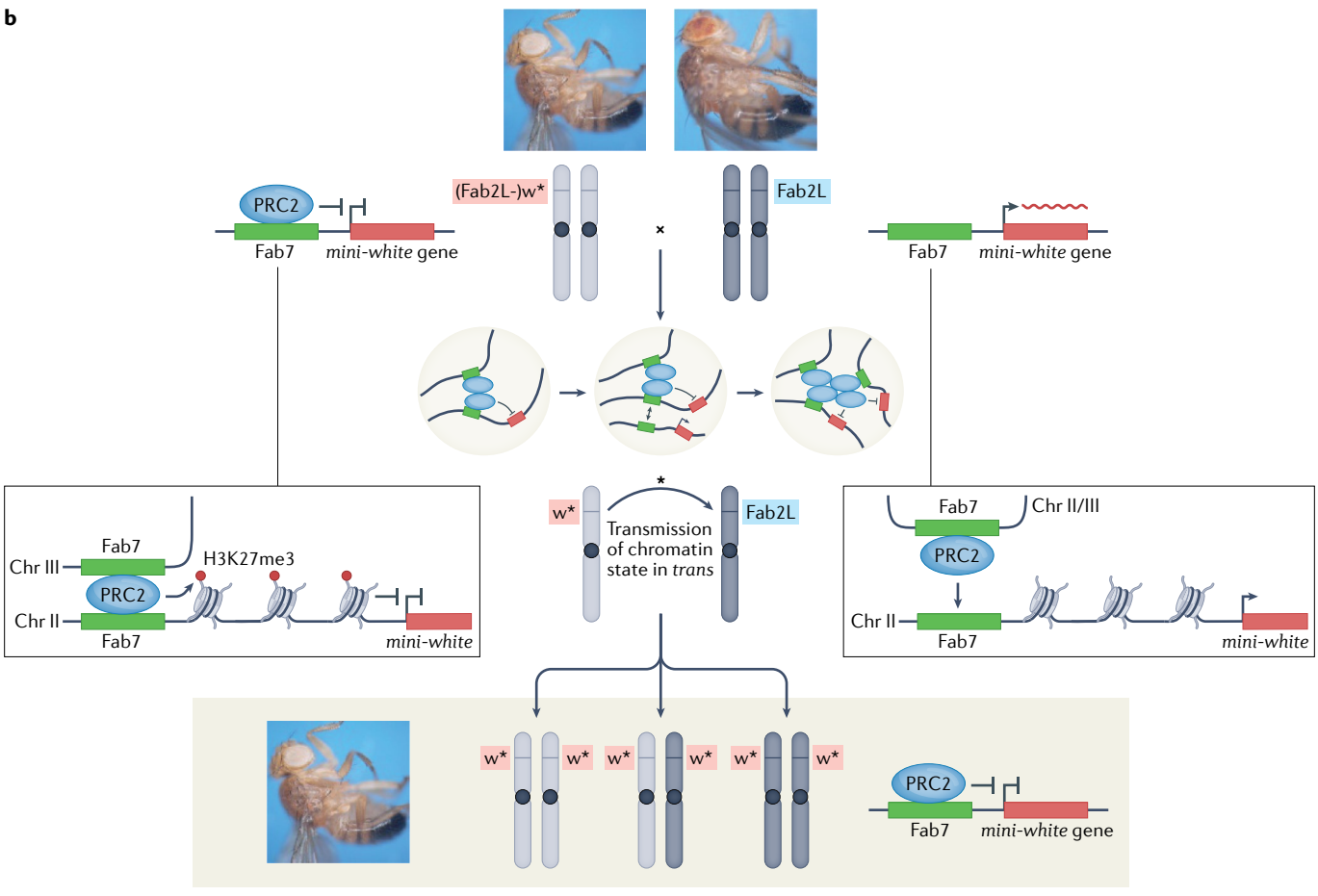
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PRC2
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Fab7
mini-white
w*
Transmission of chromatin state in trans
Fab2L
Fab7
Chr II/III
PRC2
Chr II
Fab7
mini-white
w*
w*
w*
w*
w*
w*
PRC2
Fab7
mini-white gene

◀ Fig. 3 | **Paramutation: horizontal transfer of epigenetic information.** Paramutation is a process by which epigenetic information is transmitted in *trans* between alleles of a particular locus. The actual mechanism of this transmission can vary, implicating different epigenetic marks and pathways, as illustrated by the two examples shown here. **a** | A classic case of paramutation in *Zea mays*[134]: expression of the *Pl1-Rhoades* (Pl-Rh) allele at the *pl1* locus leads to visible phenotypes, including pigmentation of the plant body and flowers (depicted). An alternative allele Pl′ does not express the *pl1* gene. This is thought to be achieved by small RNA (sRNA) silencing of a *pl1* enhancer. In Pl-Rh the enhancer is transcribed by RNA polymerase II (Pol II), stimulating the expression in turn of *pl1*. In Pl′, Argonaute (AGO)-targeted silencing switches the enhancer to Pol V transcription and recruits DNA methyltransferases (DNMTs), which in turn recruits H3K9 histone methyltransferases (HMTs), inhibiting transcription by Pol II. When these plants are crossed together, small RNAs generated from the Pl′ allele by the action of the RNA-dependent RNA polymerases MOP1 and Pol IV and the Dicer protein DCL will not only target the allele from which they originated, but also the hitherto unsilenced Pl-Rh allele. Thus, the silenced Pl′ epigenetic state is transferred from one allele to the other, leading to silencing in all of the offspring. **b** | A more recently described case of paramutation, from *Drosophila melanogaster*[32]. Trimethylation of histone H3 lysine 27 (H3K27me3) by Polycomb repressive complex 2 (PRC2) over the Fab2L locus leads to silencing of the *mini-white* gene and a white eye colour (denoted w*). This silencing is partly dependent on 3D chromatin interactions between the Fab7 regulatory element of Fab2L and another Fab7 element present elsewhere in the genome. Crossing with a naive, red-eyed individual leads to transfer of the H3K27me3 mark to the naive allele. This is stimulated by interactions between the Fab7 element of the naive allele and other Fab7 elements both at the silenced Fab2L allele and on chromosome (Chr) III. In the F2 generation all offspring have H3K27me3 and white eyes, including those with two alleles from the naive parent. Part **a** is adapted from REF.[134], Springer Nature Limited. Part **b** is adapted from REF.[32], Springer Nature Limited.

Shifting some of this attention to the epigenetic inheritance of disease and disease susceptibility may reveal further insights. Investigating transgenerational effects in humans is difficult, meaning that robust evidence for the presence of TEI in humans is lacking. On the other hand, examples in model organisms suggest that many diseases could be responses to environmental insults in previous generations[12,17,132,147], raising questions for the involvement of pollutants and pesticides in the health problems of modern society. However, these studies often rely solely on the observation of a correlation between such insults and disease. It is only by careful dissection of the molecular basis of these observations that we may determine if they are truly transgenerational and epigenetic, and gain a better understanding of how the environment may affect our health for generations after exposure.

Finally, as we began this Review by presenting our definition of epigenetics, we may wish to reassess its relevance to recent work in the field, whether discussed in these pages or not. Although our definition aims to be quite broad, in practical terms it remains tied to the concept of chromatin modifications. However, the case of small RNAs, which can silence genes at the post-transcriptional as well as transcriptional level, illustrates that chromatin modifications are not an indispensable component of epigenetic inheritance. Indeed, mechanisms such as the ping-pong cycle (BOX 3) could allow for such signals to be perpetuated exclusively in the cytoplasm, silencing gene expression without recourse to any change in chromatin. It is debatable whether this regulation falls under our chosen definition of epigenetics perpetuating 'alternative gene activity states'. However, we have already seen how one such mechanism in the fungus *M. circinelloides* produces, in practical terms, an 'epimutation' highly similar to another chromatin-based example in *S. pombe*[35,47]. If it is by the outcome of these processes that we judge them, it is likely that the definition of epigenetics will need to evolve to include such instances of heritable non-chromatin-based change as well as similar phenomena such as prions, which we elected not to include in this Review. As always, our definitions of many concepts will have to be updated as our understanding of the mechanisms that guide them grows.

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