

Functions and mechanisms of epigenetic inheritance in animals

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Abstract | The idea that epigenetic determinants such as DNA methylation, histone modifications or RNA can be passed to the next generation through meiotic products (gametes) is long standing. Such meiotic epigenetic inheritance (MEI) is fairly common in yeast, plants and nematodes, but its extent in mammals has been much debated. Advances in genomics techniques are now driving the profiling of germline and zygotic epigenomes, thereby improving our understanding of MEI in diverse species. Whereas the role of DNA methylation in MEI remains unclear, insights from genome-wide studies suggest that a previously underappreciated fraction of mammalian genomes bypass epigenetic reprogramming during development. Notably, intergenerational inheritance of histone modifications, tRNA fragments and microRNAs can affect gene regulation in the offspring. It is important to note that MEI in mammals rarely constitutes transgenerational epigenetic inheritance (TEI), which spans multiple generations. In this Review, we discuss the examples of MEI in mammals, including mammalian epigenome reprogramming, and the molecular mechanisms of MEI in vertebrates in general. We also discuss the implications of the inheritance of histone modifications and small RNA for embryogenesis in metazoans, with a particular focus on insights gained from genome-wide studies.

Transgenerational

Spanning more than two generations, from F0 to F2 and beyond.

Intergenerational epigenetic inheritance

Inheritance of an epigenetic trait across two generations, from F0 to F1.

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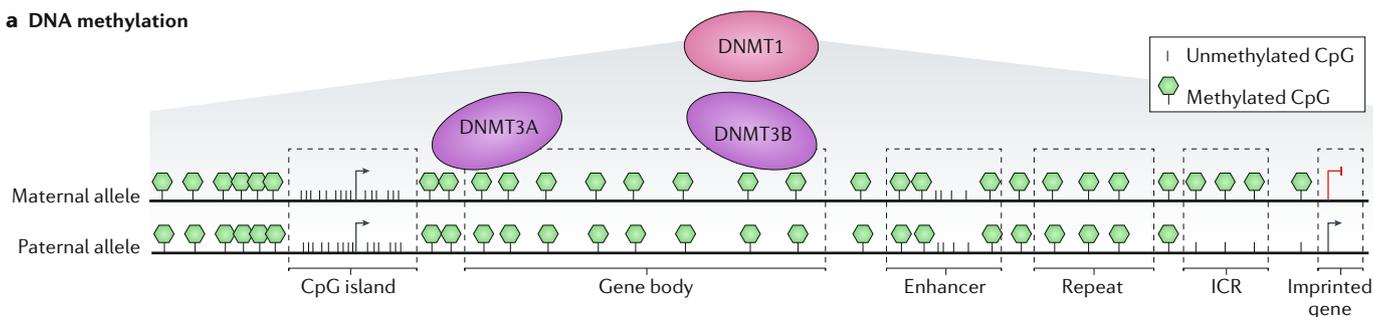
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Genetic information transmitted from parents to their offspring underpins the inheritance of traits across generations. Nevertheless, a number of examples of heritable phenotypic variation in different organisms as well as clinical cases in humans cannot be fully explained by Mendelian genetics, which encompasses DNA-sequence-based inheritance^{1–3}. Apart from DNA sequence, gene regulation determinants that can be transmitted through mitosis and meiosis, such as covalent chemical modifications to the DNA, histone post-translational modifications (PTMs) and diverse RNA species, can also be transmitted from parental gametes to the zygote (FIG. 1). Such factors, which comprise the layer of regulatory information that is superimposed on the DNA sequence and imparts cell-type-specific function, are often referred to as ‘epigenetic’ factors⁴. These epigenetic factors are frequently invoked when discussing potentially heritable cellular responses to environmental signals in the absence of detectable DNA sequence alterations.

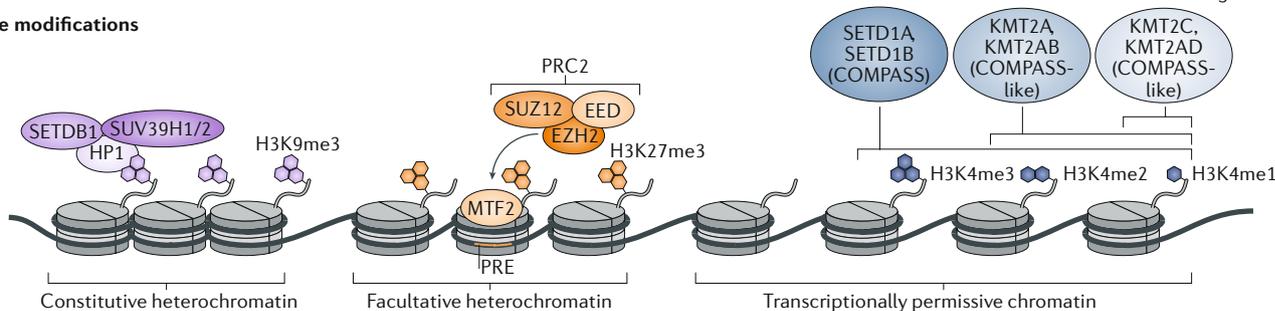
The idea that particular characteristics acquired in response to environmental exposure can be transferred from parents to progeny has its roots in the doctrines of Hippocrates⁵, which were later propagated by Jean-Baptiste Lamarck⁶ and others. Work from Conrad Waddington^{7,8} demonstrated that the exposure of *Drosophila melanogaster* pupae to ether vapour or heat shock resulted in phenotypes that through selection had

become genetically assimilated; the induced phenotypic traits had eventually manifested at high frequency in the selected population even without the exposure to the initial environmental agent. Such genetic assimilation of environmentally induced phenotypes could therefore act as a driver of evolutionary change⁹ and possibly contribute to the origin of new species. Although now we know that the phenomena observed by Waddington are underpinned by a genetic basis rather than by transgenerational propagation of an acquired trait, the precise mechanisms of such genetic assimilation remain unknown. Such experiments, however, raise the question of which molecular components are implicated in the complex interactions between the genome and the environment and in their potential heritability. A number of studies published in the past couple of decades have postulated that traumatic experiences, exposure to chemicals and deficiency in nutrients can alter cellular epigenetic states^{10–15} and that, in some cases, such altered states might be transmitted to the offspring^{2,16–21}. In this Review, we explore the principles of meiotic epigenetic inheritance (MEI) in animals, which we define as epigenetic inheritance through meiotic products that spans at least two generations (that is, from F0 to F1 and beyond). MEI includes both intergenerational epigenetic inheritance and transgenerational epigenetic inheritance (TEI). Comprehensive reviews of TEI in microorganisms,

a DNA methylation



b Histone modifications



c Small non-coding RNAs

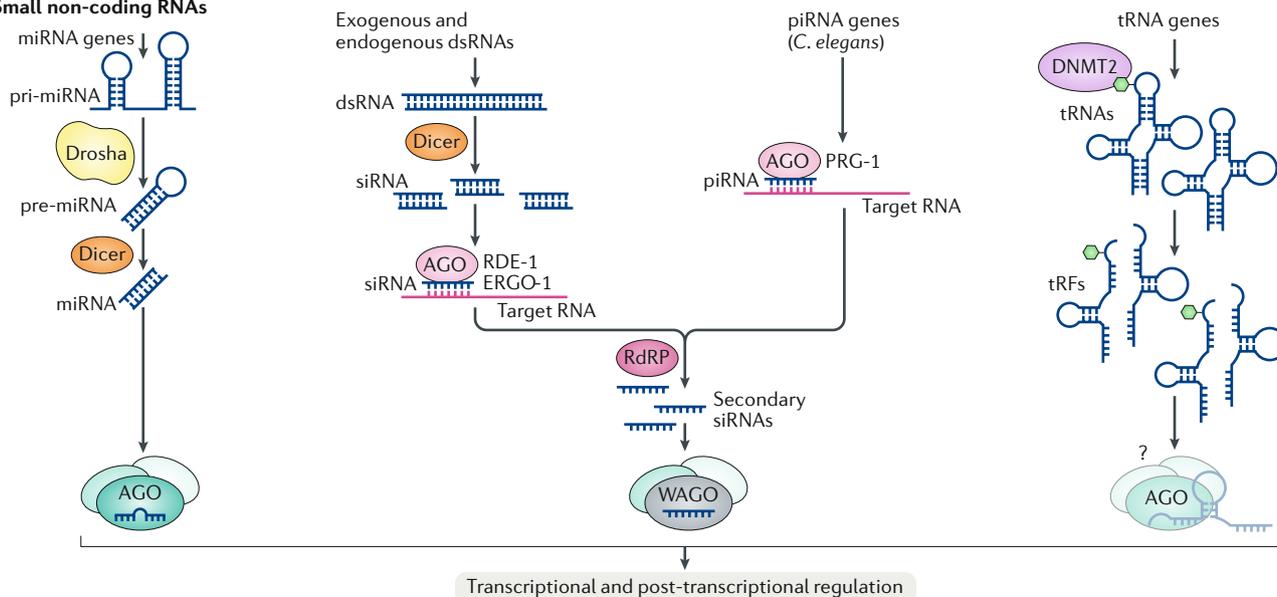


Fig. 1 | DNA methylation, histone post-translational modifications and small non-coding RNAs.

a | DNA methylation in vertebrates occurs primarily at CpG dinucleotides and is catalysed by DNA methyltransferases (DNMTs)^{89,90}. DNMT1 is involved in the maintenance of DNA methylation whereas DNMT3A and DNMT3B catalyse de novo DNA methylation¹⁸⁶. Although the majority of CpG sites in vertebrates are methylated, promoter-associated CpG-rich regions (CpG islands) are generally unmethylated¹⁸⁷. Active enhancers display reduced DNA methylation¹³². Imprinted genes — genes expressed from only one of the parental alleles — display allele-specific methylation at imprinting control regions (ICRs)⁵⁸. **b** | Trimethylation of histone H3 lysine 9 (H3K9me3) characterizes constitutive heterochromatin¹⁸⁸. H3K9me3 is catalysed by histone methyltransferases SETDB1, SU(VAR)3-9 homologue 1 (SUV39H1) and SUV39H2 (REFS^{189,190}) and is bound by heterochromatin protein 1 (HP1) proteins^{191,192}. H3K27me3 characterizes facultative heterochromatin¹⁹³ and is catalysed by EZH2 (enhancer of zeste homologue 2)^{194,195}, part of the Polycomb repressive complex 2 (PRC2), which also includes SUZ12 (REFS^{196,197}) and EED¹⁹⁸. PRC2 is recruited by Polycomb response elements (PREs) in flies^{199,200} or by MTF2 (metal-response element-binding transcription factor 2) in vertebrates²⁰¹. H3K4 mono-methylation

(H3K4me1), H3K4me2 and H3K4me3 characterize transcriptionally permissive chromatin^{202–204} and are catalysed by SET domain-containing protein 1A (SET1A)–COMPASS (complex proteins associated with Set1) or SET1B–COMPASS, by histone-lysine *N*-methyltransferase 2A (KMT2A)–COMPASS-like or KMT2B–COMPASS-like and by KMT2C–COMPASS-like or KMT2D–COMPASS-like complexes²⁰⁵. **c** | Primary microRNAs (pri-miRNAs) are cleaved by the endoribonuclease Drosha to yield precursor miRNAs (pre-miRNAs)²⁰⁶. Pre-miRNAs and double-stranded RNAs (dsRNAs) are cleaved by the endoribonuclease Dicer to produce mature miRNAs and small interfering RNAs (siRNAs)²⁰⁶. In *Caenorhabditis elegans*, Argonaute (AGO) proteins such as RNAi-defective 1 (RDE-1) and ERGO-1 (REF³³) bind to siRNAs, which leads to the recruitment of RNA-dependent RNA polymerases (RdRPs) and their subsequent amplification to secondary siRNA^{34,35}. Similarly, in the germ line, PIWI-interacting RNAs (piRNAs) are bound by Piwi proteins such as PRG-1 to recruit RdRPs, which results in the production of secondary siRNAs^{42,43}. DNMT2 methylates tRNAs and alters their stability and the formation of tRNA fragments (tRFs)^{161,207}. Processed small RNAs are loaded onto AGO and worm-specific AGO (WAGO) proteins to exert gene silencing²⁰⁸. It is not yet clear how tRFs exercise their function.

yeast, plants and in the nematode *Caenorhabditis elegans* have been published previously^{22–29}. Here, we discuss examples of MEI and epigenome reprogramming in vertebrates and elaborate on the mechanisms of MEI, namely through DNA methylation, histone modifications, tRNA fragments (tRFs) and other small RNAs. Finally, we discuss the implications of parental transmission of histone modifications and small RNAs for embryonic development in metazoans.

MEI in *Caenorhabditis elegans*

One of the best-studied mechanisms of epigenetic inheritance in animals is RNAi, which is thoroughly described in the nematode *C. elegans*. Apart from RNAi responses that are heritable through many generations, *C. elegans* also displays robust transgenerational inheritance of both active and repressive histone PTMs.

RNAi pathways. In *C. elegans*, double-stranded RNA (dsRNA) triggers RNAi, which promotes systemic mRNA degradation and is heritable through the germ line³⁰. The RNAi response in *C. elegans* is dependent on dsRNA cleavage by the RNase III family nuclease Dicer^{31,32}, which results in the production of small interfering RNAs (siRNAs) (FIG. 1c). siRNAs are subsequently bound by the Argonaute (AGO) proteins, such as RNAi-defective 1 (RDE-1)³³. This results in the recruitment of RNA-dependent RNA polymerase (RdRP) and the production of secondary siRNAs^{34,35}. Secondary siRNAs are loaded onto worm-specific AGO (WAGO) proteins³⁶ that localize to the nucleus and initiate silencing^{33,37}. The transgenerational effect of RNAi in *C. elegans* is dependent on the inheritance of small RNA molecules and their amplification, as demonstrated by virus-derived small RNAs³⁸. The heritable maintenance of silencing is dependent on nuclear RNAi pathways³⁹. Additionally, very recent work has identified a highly conserved RNA helicase, ZNFX-1, that together with WAGO proteins forms phase-separated nuage granules in the cytoplasm of germ cells and that has a role in the propagation of heritable RNAi^{40,41}. Endogenously derived PIWI-interacting RNAs (piRNAs), which suppress the expression and activity of transposons in the germ line, can also trigger heritable RNAi^{42,43}. Together with PIWI-like protein PRG-1, piRNAs exert their function through the generation of secondary siRNAs^{42,43} (FIG. 1c). Such piRNA-initiated silencing is heritable across generations and is dependent on nuclear RNAi pathways^{44–46}. Chromatin remodelling activity and repressive-chromatin pathways, including the histone H3 lysine 9 (H3K9) methyltransferase SET-25, also participate in the maintenance of long-term silencing^{44,46–48}. Interestingly, transgenerational inheritance of RNAi can continue in the absence of H3K9 trimethylation^{49,50} and the H3K9 methyltransferase MET-2 was recently shown to inhibit the biogenesis of heritable siRNAs, thereby constraining transgenerational transmission of RNAi⁵⁰.

Histone PTM inheritance in *Caenorhabditis elegans*.

TEI of both active and repressive histone PTMs occurs in the germ line of the nematode *C. elegans*. Mutations in proteins of the Trithorax methyltransferase complex (myeloid/lymphoid or mixed-lineage leukaemia, in

mammals) WD repeat-containing protein 5 (WDR-5), ASH-2 and SET-2 decreased levels of H3K4 trimethylation (H3K4me3; a positive regulator of gene expression) and resulted in approximately 20% extension of the *C. elegans* lifespan, which was inherited across several generations⁵¹. Depletion of the H3K4me3 demethylase RBR-2 abolished the transmission of extended lifespan in WDR-5-deficient worms, suggesting that TEI of prolonged lifespan due to deficiency in H3K4 trimethylation depends on histone demethylation. Similarly, knockouts of *spr-5*, which codes for an orthologue of the H3K4 dimethylation (H3K4me2) lysine-specific histone demethylase 1A LSD1 (also known as KDM1A), result in a phenotype characterized by an increased incidence of sterility across generations (germline mortality)⁵². This phenotype is driven by the accumulation of H3K4me2, causing the misregulation of genes expressed during spermatogenesis, and is indicative of TEI of H3K4me2 patterns. Repressive histone PTMs such as H3K27me3 and H3K9me3 can also be inherited in *C. elegans*. X chromosome inactivation through Polycomb repressive complex 2 (PRC2)-mediated H3K27 trimethylation can be intergenerationally transmitted from both oocytes and sperm to the embryos⁵³. Also, a recent study demonstrated TEI of exposure to high temperature, which directly inhibited the H3K9 methyltransferase SET-25; this resulted in derepression of SET-25 target loci in the germ line and was inherited for multiple generations of worms raised at normal temperature⁵⁴ (TABLE 1). This effect persisted for up to 14 generations on an integrated transgene array, whereas for endogenous repetitive elements, considerable changes in expression levels were observed for up to 6 generations. How small RNAs cooperate with repressive histone PTMs in epigenetic inheritance is currently unclear^{44,46,48,50}. Although the inactivation of RNAi components such as HRDE-1 or NRDE-2 did not affect the transmission of this H3K9me3-mediated epigenetic memory⁵⁴, very recent data argue that H3K9me3 might be required for siRNA-dependent TEI that specifically targets newly evolved genes and is particularly sensitive to transgenes⁵⁵.

MEI in mammals

Although MEI is well-documented in plants (Supplementary Box 1) and in *C. elegans*, in mammals such phenomena remain more of an exception than the rule. This is partly because in mammals, the epigenome inherited from the gametes is heavily reprogrammed upon fertilization and during the formation of primordial germ cells (PGCs)^{56,57} (see below). However, although these epigenome remodelling processes reduce DNA methylation to its lowest levels during the mammalian life cycle, methylation is not erased and re-established with the same efficiency at all sequences, thereby constituting a potential route for MEI. For clarity, we use the term ‘intergenerational’ epigenetic inheritance if the potentially heritable effect experienced by the pregnant female (F0) was observed in F1 and F2, and we use the term ‘transgenerational’ epigenetic inheritance if the effect persisted to F3 or beyond. Accordingly, we use ‘intergenerational’ if the effect experienced by the male (F0) or a non-pregnant female (F0)

Facultative heterochromatin
Condensed, transcriptionally silent chromatin that retains the ability to decondense and license transcription within temporal and spatial contexts.

PIWI-interacting RNAs (piRNAs). A class of endogenous small non-coding RNAs that interact with Piwi-domain-containing proteins and have a role in retrotransposon silencing in the germ line.

Primordial germ cells (PGCs). Primary germ cells that give rise to gametes.

Table 1 | Inheritance of histone post-translational modifications and small RNAs

Histone post-translational modification or small non-coding RNA	Species	Maternal (M) or paternal (P) inheritance	Function	Loss-of-function mechanism	Offspring phenotype	Refs
H3K27me3	<i>Drosophila melanogaster</i>	M	Regulation of embryonic development and establishment of proper H3K27ac patterns at ZGA	<ul style="list-style-type: none"> E(z) knockdown (maternal) E(z) mutation 	Aberrant accumulation of H3K27ac in the embryo; homeotic phenotype	137
	<i>Drosophila melanogaster</i>	M, P (preference for M)	Modulation of stably inherited 3D chromatin interactions	E(z) mutation	Gradual establishment and inheritance of derepressed but not repressed epialleles	136
	<i>Caenorhabditis elegans</i>	M, P	Memory of gamete-of-origin-specific repression	Mutation of Polycomb protein MES-3	Gradual dilution of H3K27me3 from gamete-of-origin chromosomes	53
	<i>Mus musculus</i>	M	Gene-distal chromatin structure?	NA	NA	122
	<i>Mus musculus</i>	M	DNA-methylation-independent maternal imprinting in the embryo	KDM6B overexpression	Biallelic expression at 18% of imprinted regions in the zygote	66
H2A.Z and H3K4me1	<i>Danio rerio</i>	P	Deterring DNA methylation in early embryos and establishment of poised, pre-ZGA chromatin	<ul style="list-style-type: none"> srcap morpholino Recombinant Anp32e protein 	Reduction of nuclear H2A.Z(FV) levels, coinciding with acquisition of DNA methylation	128
H3K4me2	<i>Caenorhabditis elegans</i>	M, P	Epigenetic memory?	SPR-5 (KDM1) mutation	Progressive derepression of genes that regulate spermatogenesis, defects in oogenesis and spermatogenesis and sterility	52
	<i>Mus musculus</i>	P	Epigenetic memory?	KDM1A overexpression in developing sperm	H3K4me2 reduction coinciding with deregulation of genes in the early embryo and developmental defects	150
H3K4me3	<i>Caenorhabditis elegans</i>	M, P	Lifespan regulation	<i>wdr-5</i> , <i>ash-2</i> and <i>set-2</i> mutations	Reduction of H3K4me3, coinciding with lifespan extension inherited for several generations	51
	<i>Mus musculus</i>	M	Broad H3K4me3 domains associated with transcriptional silencing	KDM5A and KDM5B overexpression	Aberrant reactivation of H3K4me3-marked loci	120,121
H3K9me3	<i>Drosophila melanogaster</i>	M, P	Heterochromatin organization	High-temperature-induced phosphorylation of Atf-2	Reduction of H3K9me2, disruption of heterochromatin formation and gene silencing	175
	<i>Caenorhabditis elegans</i>	M, P	Temperature-sensitive transcriptional repression	<i>set-25</i> mutation	Reactivation of SET-25-silenced transposons	54
	<i>Homo sapiens</i>	P	Heterochromatin build-up in early embryos	NA	NA	145
Small non-coding RNAs	<i>Mus musculus</i>	P	Intergenerational transmission of diet-induced metabolic phenotypes?	Low-protein diet	Increase in the abundance of sperm tRNA-derived small RNAs and decrease in let-7 miRNAs, downregulation of genes regulated by the retroelement MERVL in the early embryo and altered metabolism in the offspring	156
	<i>Mus musculus</i>	P	Intergenerational transmission of diet-induced metabolic phenotypes?	High-fat diet	Changes in the abundance of sperm tRNA-derived small RNAs and increase in 5mC and m ² G tRNA modifications coinciding with the deregulation of metabolic pathway genes in the F1 offspring, glucose intolerance and insulin resistance	154
	<i>Mus musculus</i>	P	Intergenerational and transgenerational transmission of diet-induced metabolic phenotypes	High-fat diet	Changes in F1 sperm RNA content (tRNA fragments and miRNA) and inheritance of latent metabolic phenotypes in F1 and F2	155

Table 1 (cont.) | Inheritance of histone post-translational modifications and small RNAs

Histone post-translational modification or small non-coding RNA	Species	Maternal (M) or paternal (P) inheritance	Function	Loss-of-function mechanism	Offspring phenotype	Refs
Small noncoding RNAs (cont.)	<i>Mus musculus</i>	M, P	Execution of the first zygotic division	Chemical inhibition of sperm-derived miR-34c and deletion of maternal Dicer	Reduced DNA synthesis and suppression of the first zygotic division	157,163
	<i>Mus musculus</i>	M, P	NA	Mutation of KIT tyrosine kinase receptor	Increased levels of aberrant Kit RNA species coinciding with white tail tip phenotype in the offspring	153

m²G, N²-methylguanosine; 5mC, 5-methylcytosine; Anp32e, acidic (leucine-rich) nuclear phosphoprotein 32 family member E; Atf-2, activating transcription factor 2; E(z), enhancer of zeste; H3K27ac, histone 3 lysine 27 acetylation; H3K4me1, H3K4 mono-methylation; H3K4me2, H3K4 dimethylation; H3K4me3, H3K4 trimethylation; KDM, lysine-specific histone demethylase; MERVL, mouse endogenous retrovirus with leucine tRNA primer; miRNA, microRNA; ZGA, zygotic genome activation.

was observed in F1 and ‘transgenerational’ if the effect persisted to F2 and beyond (BOX 1).

Genomic imprinting. In mammals, a small number of genes (~125 in mice and ~100 in humans; **Geneimprint**) are expressed in a parent-of-origin-specific manner, a phenomenon termed genomic imprinting⁵⁸. This differential allelic expression is the result of epigenetic silencing of the inactive allele, a process that involves DNA methylation^{59–62}. Such imprinted genes are regulated through imprinting control regions (FIG. 1a), which are resistant to post-fertilization DNA methylation reprogramming, thereby forming a platform for intergenerational epigenetic inheritance. Many imprinted genes are expressed during prenatal development and are required for proper placental function^{63,64}. Errors occurring during the establishment of imprinting in the parental germ line were shown to account for a number of behavioural and neurodevelopmental disorders, such as Prader–Willi syndrome and Angelman syndrome⁶⁵. Interestingly, recent work in mouse embryos provided evidence for imprinting that occurs independently of DNA methylation and is associated with maternal inheritance of the repressive modification H3K27me3 (see below)⁶⁶. The number of genes ($n = 76$) regulated by such non-canonical imprinting mechanisms appears to be similar to the number of genes regulated by canonical, DNA-methylation-dependent imprinting.

Epigenetic reprogramming of intracisternal A particles. Intracisternal A particles (IAPs) are retrotransposons present in many copies (~1,000) in the mouse genome⁶⁷. To suppress their capacity for retrotransposition in the germ line, IAPs are predominantly methylated and silenced in germline cells. Similarly to genomic imprints, IAPs can largely resist embryonic DNA methylation reprogramming, which makes them potentially attractive targets for MEI studies⁶⁷. One of the best examples of MEI in mammals is the agouti viable yellow (*A^y*) locus in mice, which harbours an insertion of the IAP retrotransposon upstream of the agouti gene that determines coat colour⁶⁸. *A^y* expression is highly variable among littermates and is dependent on the

DNA methylation state of the IAP inserted in the agouti locus^{68,69}. While the phenotype of the silent, methylated allele (pseudoagouti) is wild-type dark-brown coat colour, the hypomethylated, active allele gives rise to a yellow coat. The spectrum of agouti phenotypes in the offspring is dependent upon the phenotype of the dam. For example, a dam with the yellow coat phenotype is more likely to have yellow coat offspring⁶⁸. Interestingly, dietary supplementation of methyl donors during mid-gestation results in the shift of agouti phenotypes towards pseudoagouti in both F1 and F2, suggestive of increased IAP methylation in the germ line and the intergenerational retention of this altered epigenetic state¹⁶. Nevertheless, bisulfite sequencing data suggest that both the maternally contributed and paternally contributed *A^y*-associated IAPs are reprogrammed during early embryogenesis, albeit with different dynamics⁷⁰. This suggestion implies that DNA methylation might not be the primary inherited mark controlling the spectrum of agouti phenotypes in F1. It is currently not clear whether histone modifications or RNA-based mechanisms have a role in the inheritance of the *A^y* epialleles; however, recent studies have demonstrated robust intergenerational inheritance of H3K4me3 and H3K27me3 histone modifications through the maternal germ line⁷¹. Methyl-donor supplementation during mid-gestation affects the percentage of agouti phenotypes only when the *A^y* allele is contributed through the paternal germ line. This finding suggests that the paternal allele might undergo more extensive epigenetic reprogramming and is thus affected by environmental cues during the period of PGC methylome reprogramming (embryonic day 8.5 (E8.5)–E.15.5)¹⁶. Therefore, it is possible that the inheritance of the maternal *A^y* locus is more tightly regulated, perhaps through the coordinated action of diverse gene-regulatory mechanisms, including histone modifications, and is not limited only to DNA methylation.

Contribution of MEI to human diseases. Considerable efforts have been made in identifying the contribution of epigenetic modifications to the heritability of complex diseases. In particular, the possibility that epimutations confer predisposition to cancer has garnered much

Imprinting control regions
cis-regulatory regions for which the allele-specific epigenetic state mediates differential expression of the parental alleles.

Prader–Willi syndrome
A complex disorder characterized by developmental and neural phenotypes that can be caused by a paternal imprinting defect.

Angelman syndrome
A neurological disorder characterized by intellectual disability that can be caused by a maternal imprinting defect.

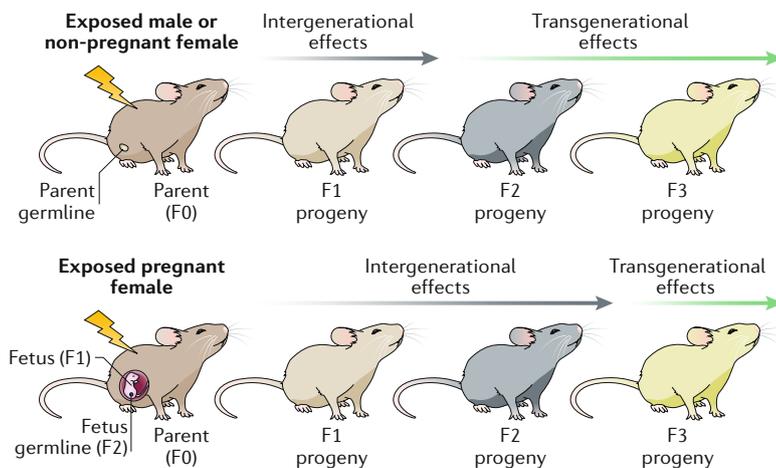
Bisulfite sequencing
Treatment of DNA with sodium bisulfite followed by sequencing; determines cytosine methylation status, as unmethylated cytosine is converted into uracil upon this treatment.

Epialleles
Genetically identical alleles displaying distinct epigenetic modifications.

Epimutations
Heritable changes in gene expression that are caused by changes in the epigenetic state.

Box 1 | Interpreting meiotic epigenetic inheritance studies in animals

Although many studies of meiotic epigenetic inheritance (MEI) in animals have been published to date, the field is still facing substantial challenges. Whereas MEI in plants and nematodes encompasses a series of well-studied phenomena^{24–28}, MEI studies in mammals have proved especially challenging to set up and to interpret owing to many external confounding factors, which can easily result in the misinterpretation of acquired data⁸⁸. First, studies dealing with MEI should, where possible, incorporate robust DNA sequencing controls to exclude potential genetic contributions to the studied phenomena⁸⁴. Another important consideration in interpreting MEI studies is the nature and persistence of the phenotypic effect under investigation. For example, if stress is applied to the paternal lineage (F0), the germ cells (sperm) that would participate in the formation of the F1 generation likely would have also been affected by the stress. This likelihood would make the F0–F1 transmission of phenotypic effects intergenerational, whereas for transgenerational epigenetic inheritance the phenotypic effect would have to be preserved to F2 and beyond (see the figure). In mammals, if the stress is applied to a pregnant female (F0), then the fetus (F1) as well as its primordial germ cells, which have the potential to form the F2 generation, would be affected. In such cases, only effects that would be visible in and beyond F3 would be perceived as truly transgenerational (see the figure). Parental care and shared environment can also considerably affect offspring epigenomes¹⁸⁵, making it difficult to distinguish between MEI and de novo acquisition of epigenetic modifications in response to particular environmental cues. Additionally, the tissue of origin can be a major confounding factor in epigenetic inheritance studies. Most assays used for quantification of epigenetic modifications do not discriminate between different cell types in the interrogated sample; therefore, more subtle epigenetic changes or changes present in just a small subpopulation of cells could easily be missed. Altogether, MEI studies in animals, and particularly in mammals, frequently suffer from these methodological shortcomings, and it is important to be aware of these confounding factors when designing studies aimed at demonstrating intergenerational or transgenerational inheritance of epigenetic traits.



attention over the past few decades⁷². One of the best examples of such an epimutation is the silencing of the mutL homologue 1 (*MLH1*) gene, which encodes a DNA mismatch repair factor, through hypermethylation of its promoter. The *MLH1* epimutation is widespread in somatic tissues derived from all three major embryonic lineages (ectoderm, mesoderm and endoderm), suggesting that it has an embryonic or germline origin^{73,74}. Such *MLH1* epimutations contribute to Lynch syndrome, which is characterized by early onset of multiple cancers, including colorectal cancer and endometrial cancer, and in general cancers displaying microsatellite instability⁷⁵. In the majority of cases, *MLH1* epimutations arise de novo in affected individuals and are reprogrammed to the unmethylated state in their offspring⁷². Nevertheless, to date at least one case was

reported in which the methylated state was inherited from the affected mother to one of her three sons; however, it was then erased in his germ line⁷⁴. This observation demonstrates the possibility that epimutations might be passed on from parents to their offspring, albeit infrequently, likely owing to the DNA methylation reprogramming that occurs post-fertilization and in PGCs. Although additional data support the (rare) occurrence of intergenerational epigenetic inheritance at the *MLH1* locus^{76,77}, evidence for this type of heritability in humans remains very limited. Finally, it should also be stressed that, on the basis of currently available data, it is very difficult to unequivocally rule out the involvement of other *cis*-acting or even *trans*-acting factors in the intergenerational heritability of disease-causing epimutations in humans.

MEI of environmental cues in humans. Understanding how the environment affects the human epigenome and how potentially acquired traits can be propagated through generations remains an area of intense investigation^{78–81}. One of the most noted studies on this topic is the Dutch Hunger Winter study^{79,82,83}. DNA methylation profiling of blood samples extracted from individuals who were conceived during the Dutch Hunger Winter (1944–1945) revealed small DNA methylation differences in the differentially methylated region (DMR) associated with the insulin-like growth factor 2 (*IGF2*) gene⁸². Five CpG dinucleotides located within the *IGF2* DMR were assessed by quantitative mass spectrometry to reveal on average 5.2% lower DNA methylation in the affected siblings. These results were successfully validated (5.6% lower in the affected individuals) by locus-specific bisulfite sequencing approaches. Notably, only the cohort exposed to maternal malnutrition during early gestation but not during later stages of pregnancy displayed these changes in DNA methylation at the *IGF2* locus⁸². Although this understandably invites speculation about epigenetic mechanisms of adaptation to the environment, one has to keep in mind that MEI studies in humans frequently suffer from serious methodological flaws. A major limitation, even in the presence of a testable hypothesis, is the fact that such analyses are often performed on a complex tissue such as blood, which consists of different cell types. Therefore, it is difficult to exclude the possibility that the observed differences in DNA methylation merely reflect the differences in cell-type composition between samples. Furthermore, even if those differences were truly caused by the proposed environmental stress, it remains challenging to prove that such modest changes could result in an adaptive phenotype. Finally, a major confounding factor that is often disregarded in MEI studies is the difference in DNA sequence composition between individuals, which is well known to be a major contributor to the inheritance of epigenetic states^{84–87}.

Whereas the Dutch Hunger Winter study aimed to assess the potential impacts of early intrauterine exposure to famine on DNA methylation during adult life, a more recent study tried to address whether intergenerational inheritance of stress effects, such as those caused

Microsatellite instability
Genetic instability in short tandem repeats due to impaired DNA mismatch repair.

Differentially methylated region
(DMR). A genomic region displaying statistically significant change in DNA methylation between at least two samples.

to survivors of the Holocaust, can be demonstrated in humans⁸¹. The offspring of Holocaust survivors displayed diminished DNA methylation of the *FKBP5* gene, which was previously linked to post-traumatic stress disorder, whereas this gene was marginally more methylated in the Holocaust survivors themselves than in the non-affected controls. The concept of non-genetic trauma inheritance has previously been postulated in mice²⁰; however, drawing any definite conclusions regarding the potential intergenerational epigenetic inheritance of trauma in humans would be very challenging (BOX 1). Apart from the methodological issues discussed above, many of which apply also to this study, it is very difficult to distinguish between the effects of parental upbringing on a growing child and epigenetic inheritance per se. Owing to the genetically heterogeneous nature of the human population and long human lifespan, longitudinal studies that would span multiple generations and that would be able to properly distinguish genotype from phenotype remain very difficult to set up⁸⁸. In the absence of those, the published research on the topic of MEI in humans requires highly circumspect treatment.

MEI through DNA methylation

DNA methylation has often been proposed as a MEI carrier in animals owing to its relative stability and well-defined mechanisms of de novo deposition and maintenance^{89,90} (FIG. 1a). Although DNA methylation is present in fungi, plant and animal kingdoms, its genomic abundance and distribution vary considerably between species^{91,92}. For example, species with easily demonstrable MEI, such as *D. melanogaster* and *C. elegans*, are virtually devoid of DNA methylation^{93,94}. Thus, it is unclear to what extent DNA methylation contributes to MEI in animals and in what biological contexts. However, recent insights from genome-wide methylome studies suggest that a considerable fraction of mammalian genomes might potentially bypass the removal of DNA methylation that occurs during preimplantation and PGC reprogramming (TABLE 2).

Reprogramming of the preimplantation methylome.

DNA methylation is mostly static in adult somatic tissues; however, global DNA methylation patterns are reprogrammed twice during the mammalian life cycle⁵⁶. The first reprogramming event takes place

Table 2 | Reprogramming of embryonic DNA methylation in vertebrates

Species	Occurrence of post-fertilization global 5mC reprogramming	Genomic regions possibly escaping post-fertilization global mC reprogramming	Occurrence of global mC reprogramming in primordial germ cells	Genomic regions possibly escaping reprogramming in primordial germ cells	Refs
<i>Homo sapiens</i>	+	ICRs, gene bodies and LINE-1	+	NA	104
	+	ERVK, ICRs and LINEs	+	NA	105
	+	NA	+	Repeat-free regions including promoters, enhancers, gene bodies, CGIs ($n = 1,426$) and repeats ($n > 100,000$)	115
	+	Exons, 3' UTRs, promoters, splice sites, L1HS and HERVK transposons ($n = 1,471$) ^a	+	Exons, 3' UTRs, promoters, splice sites, L1HS and HERVK transposons ($n = 1,471$) ^a	113
	+	NA	+	LINE-1, SINEs (Alu) and ERVK	114
	<i>Mus musculus</i>	+	IAPs	+	NA
+		Single-copy germline-expressed genes and somatically expressed genes ($n < 20$)	+	NA	103
+		Oocyte-specific CGIs, LINEs, LTRs and ICRs	+	NA	98
+		ICRs	+	NA	100
+		NA	+	IAPs, LTR-ERV1 and single-copy genes ($n = 23$)	109
+		NA	+	IAPs and non-IAP-related CGIs ($n = 265$)	112
+		NA	+	Promoter CGIs ($n = 11$) and IAPs	110
<i>Xenopus laevis</i> and <i>Xenopus tropicalis</i>	-	Global methylome inheritance	NA	NA	124,125,143
	-	Global methylome inheritance (paternal methylome configuration inherited)	NA	NA	126,127

5mC, 5-methylcytosine; AGCs, advanced germ cells; CGIs, CpG islands; ERVK, endogenous retrovirus K; HERVK, human endogenous retrovirus K; IAP, intracisternal A particle; ICM, inner cell mass; ICRs, imprinting control regions; L1HS, L1 *Homo sapiens*-specific; LINE, long interspersed nuclear element; LTR, long terminal repeat; LTR-ERV1, LTR-endogenous retrovirus sequence 1; NA, not applicable; PGCs, primordial germ cells; SINEs, short interspersed nuclear elements; UTRs, untranslated regions; +, occurrence; -, no occurrence. ^aGenomic regions found to commonly escape 5mC reprogramming in the blastocyst (ICM) and germ cells (PGCs and AGCs).

post-fertilization and involves widespread DNA demethylation of the paternal pronucleus, followed by a progressive drop in global DNA methylation in the zygote, which reaches its lowest point in the blastocyst^{95–98}. The precise mechanisms of embryonic DNA methylome remodelling remain a topic of debate^{99–101}; however, it is likely that both active (enzymatic) and passive (replication-coupled dilution) demethylation mechanisms have a role in this process. Active DNA demethylation is mediated by ten-eleven translocation (TET) enzymes, which oxidize 5-methylcytosine, whereas passive reduction in DNA methylation levels is dependent on the inhibition of DNA (cytosine-5)-methyltransferase 1 (DNMT1) activity in the nucleus⁵⁶. Whereas rapid post-fertilization demethylation of the paternal pronucleus was observed in mouse, rat, pig, bovine and human embryos, sheep and rabbit preimplantation development involves less demethylation of the paternal genome, indicative of species-specific differences in mammalian embryonic epigenome remodelling¹⁰². The embryonic genome is remethylated following implantation, coinciding with the loss of cellular pluripotency^{97,98,103}. Nevertheless, not all genomic loci are reprogrammed with the same efficiency. Apart from imprinted loci and IAPs, single-base-resolution studies in mouse embryos revealed the persistence of DNA methylation on oocyte-hypermethylated DMRs and a number of long interspersed nuclear element transposons during the initial wave of global hypomethylation⁹⁸. Similarly, a number of DMRs identified as hypermethylated in the oocytes were found to be intermediately methylated during human preimplantation development, suggestive of maternal DNA methylome inheritance¹⁰⁴. Another important feature of human preimplantation development is the persistence of DNA methylation within gene bodies^{104,105}. As gene bodies are often enriched in enhancer elements^{106,107}, such persistence of DNA methylation might allow for the intergenerational inheritance of *cis*-regulatory states. In summary, these observations provide evidence for locus-specific retention of DNA methylation (TABLE 2) during a regulatory phase in which DNA methylation maintenance is largely inactive. This evidence suggests that a previously underappreciated fraction of mammalian genomes, including promoters, gene bodies and repeats, confer resistance to early DNA methylation reprogramming and thus constitute a potential platform for MEI.

DNA methylation reprogramming in PGCs. The second wave of mammalian DNA methylation reprogramming takes place in PGCs and also involves a combination of active and passive mechanisms^{108–111}. The hallmark of this process is erasure of imprinting followed by gender-specific re-establishment of imprinting in the gonads. As in the post-fertilization demethylation process, not all genomic sequences are reprogrammed with the same efficiency^{109,112}. Studies in mice revealed that the feature most resilient to reprogramming are IAPs, which remain predominantly hypermethylated throughout PGC demethylation^{109,112} (TABLE 2). Human PGCs exhibit similar genomic DNA methylation erasure

patterns to those observed in mice^{113–115}. However, more than 116,000 genomic regions were identified that remained significantly hypermethylated following methylome reprogramming in human PGCs¹¹⁵. Of those, more than 7,000 regions were repeat-poor and mostly coincided with CpG-rich regions known as CpG islands, promoters, enhancers and gene bodies. Many of these regions were associated with obesity, multiple sclerosis and schizophrenia. Comparisons with mouse bisulfite sequencing data revealed 794 such escapees that were shared between mice and humans (TABLE 2). Of note, many of these loci were linked to neural and metabolic functions and could thus be of potential interest for further exploration within the context of disease-related risk inheritance.

In mice, metabolic deficiencies coinciding with perturbed DNA methylation in the germ line of adults can be inherited intergenerationally¹¹⁶. Undernourishment of pregnant F0 dams during a critical phase of embryonic PGC reprogramming resulted in metabolic phenotypes and modest DNA hypomethylation at discrete loci in adult F1 sperm (F1 DMRs). Whereas these altered methylation patterns were not detectable in brain and liver tissues obtained from E16.5 F2 embryos, genes found in the vicinity of a small number of F1 DMRs had significantly altered transcriptional profiles in those tissues in F2. Indeed, several of these genes were attributed with metabolic functions, and pancreatic islets isolated from 4-month-old F2 mice displayed impaired insulin secretion. The altered transcriptional state of those genes in E16.5 embryos, in the absence of corresponding DNA methylation changes, suggests that F1 sperm DMRs might exercise an effect during early F2 embryogenesis. This effect would not be detectable in terms of altered DNA methylation; however, it would be conveyed into an altered transcriptional output detectable at E16.5. Alternatively, DNA methylation might not be the primary regulatory mechanism responsible for the formation of F1 sperm DMRs as well as for the observed transcriptional changes in F2. In mammalian sperm, the majority of nucleosomes are replaced with protamines. However, certain loci of developmental importance, such as *Hox* genes, promoters of developmental genes, imprinted genes and microRNA (miRNA) clusters, can retain nucleosomes and associated histone PTMs, such as H3K27me3, H3K4me2 and H3K4me3 (REFS^{117,118}). Interestingly, 21% of the identified F1 DMRs ($n = 23$) were found within such nucleosomal regions¹¹⁶. This finding raises the possibility that the observed differences in F1 sperm DNA methylation might merely be secondary effects of altered chromatin structure, potentially caused by defects in regulatory mechanisms other than DNA methylation, such as nucleosome positioning or histone PTMs, both of which have potential for intergenerational heritability^{119–122}. In light of these findings, it is worthwhile mentioning that in many cases epigenetic inheritance might be limited only to certain genomic contexts. For example, targeting trimethylation of both H3K27 and H3K9 requires input from specific DNA sequences^{85–87}. Thus, it is plausible that sequences that escape various rounds of epigenetic reprogramming might be genetically defined.

Ten-eleven translocation (TET) enzymes

Enzymes required for active DNA demethylation, which catalyse a series of iterative oxidations of 5-methylcytosine to 5-hydroxymethylcytosine and further to 5-formylcytosine and 5-carboxylcytosine.

CpG islands

Genomic regions of high GC content and high frequency of CpG sites relative to the genome average; often associated with gene promoters and maintained in an unmethylated state.

Methylome reprogramming in anamniotes. Unlike mammals, anamniote vertebrates, such as fish and frogs, do not undergo global DNA methylation remodelling during embryogenesis^{123–127}. Instead, zebrafish remodel their methylomes before zygotic genome activation (ZGA) in a fascinating fashion: the maternal methylome is reconfigured to match the paternal methylome pattern, thus making the early embryonic methylome adopt a paternal-like configuration at ZGA^{126,127}. This harmonization of paternally and maternally inherited epigenetic states before ZGA is achieved through the usage of ‘placeholder’ nucleosomes that repel DNA methylation but, on the other hand, are incompatible with transcriptional activation¹²⁸ (see below). Other notable differences in early embryonic reprogramming also exist between zebrafish and mammals, including the absence of early TET expression and of active demethylation in zebrafish^{127,129,130}. The lack of global embryonic demethylation and remethylation makes zebrafish a potentially useful model organism for MEI studies in vertebrates¹³¹. However, whether such principles of embryonic reprogramming are common to teleosts and other anamniotes is currently unclear. Similarly, it is currently unknown whether the erasure and re-establishment of DNA methylation observed in mammalian PGCs is conserved more broadly.

MEI through histone PTMs

Despite the increasingly precise classification of regions that might escape DNA methylome reprogramming in mammals (TABLE 2), direct evidence for the role of DNA methylation in meiotic inheritance in animals is largely lacking^{70,116}. This is perhaps not surprising considering that in many cases DNA methylation merely anti-correlates with the activity of gene-regulatory regions, suggestive of its role as a secondary, reinforcing regulatory mark^{132,133}. Instead, an increasing number of recent studies have demonstrated intergenerational epigenetic inheritance and TEI of histone PTMs (FIG. 1b) in diverse animal species. We have already discussed the inheritance of histone PTMs in *C. elegans* above.

Histone PTM inheritance in *Drosophila melanogaster*.

D. melanogaster has a long history of exploration of MEI phenomena^{134,135}. The role of histone PTMs in TEI in this organism was recently demonstrated by the formation of long-range chromatin interactions accompanied by PRC2-mediated H3K27me3 deposition, which delineated the inheritance of repressed or derepressed epialleles through multiple generations¹³⁶. Interestingly, the establishment of these epigenetic states was shown to be sensitive to environmental stimuli such as temperature, thereby demonstrating the interplay between the environment and the epigenome. In line with these observations of stable Polycomb TEI, the maternal inheritance of H3K27me3 and enhancer of zeste (E(z)) protein in *D. melanogaster* was recently shown to have instructive roles for gene expression at ZGA¹³⁷ (see below). Taken together, these examples demonstrate the requirement of meiotic histone PTM inheritance for responses to environmental stimuli and the supervision of early embryogenesis in insects.

Histone PTM inheritance in anamniotes. The degree of nucleosome retention in vertebrate sperm varies greatly. Whereas zebrafish retain their entire nucleosomal content¹³⁸, in *Xenopus laevis*, only ~10% of the nucleosomes are retained in the mature sperm¹³⁹. This level is similar to that in humans, whereas in mice the degree of nucleosome retention is close to 1%¹¹⁸. The sperm chromatin of both zebrafish and mammals is characterized by the presence of active (H3K4me2 and H3K4me3) and repressive (H3K27me3) histone PTMs, thereby allowing for the possibility of intergenerational transmission of these marks^{117,118,138}. In *X. laevis*, sperm maturation entails a global loss of H3K4me2 and H3K4me3 and the retention of H3K27me3, and this process appears to be important for the execution of correct gene expression patterns during embryogenesis¹³⁹. In zebrafish, the presence of both H3K27me3 and H3K4me3 at a number of developmental genes pre-marked in sperm by the same histone PTMs was observed before the ZGA¹⁴⁰. Those observations notwithstanding, the major chromatin signatures of embryonic development are established at or after the ZGA^{141,142}. Moreover, the deposition of histone marks at the ZGA seems to be largely maternally defined, as recently described in *Xenopus tropicalis*¹⁴³.

Currently, very little is known about the fate of germline-inherited chromatin modifications during embryogenesis. A plausible explanation of how parental chromatin patterns might be maintained in early zebrafish embryos was recently proposed to involve placeholder nucleosomes¹²⁸. These nucleosomes are specific to the paternal germ line, are enriched in H3K4 mono-methylation (H3K4me1) and the histone variant H2A.Z(FV) and occupy hypomethylated regions (FIG. 2). Interestingly, the genomic locations of placeholder nucleosomes in sperm and early embryos correlate well, suggestive of a role for placeholder nucleosomes in establishing the pre-ZGA chromatin structure. The maternal genomic contribution, which initially lacks the placeholder conformation, accumulates placeholder nucleosomes during pre-ZGA development and is progressively remodelled to match its paternal counterpart, in line with previous notions of maternal–paternal DNA methylome reprogramming^{126,127}. Coexistence of H3K4me1 and H2A.Z on placeholder nucleosomes deters DNMT activity while keeping the chromatin in a transcriptionally inactive state. This provides a plausible mechanism for DNA methylation remodelling in the absence of TET protein activity in the early zebrafish embryo¹³⁰. At the onset of ZGA, placeholder-marked loci become either active (H3K4me3-marked) or poised and silent (H3K4me3–H3K27me3-marked) (FIG. 2). However, it is important to note that placeholder nucleosomes are established even in parthenogenetic embryos (maternal haploids that lack sperm DNA)¹²⁸, suggestive of a considerable maternal contribution in the establishment of placeholder chromatin. It remains to be determined to what extent placeholder nucleosomes are conserved beyond zebrafish.

Histone PTM inheritance in mammals. In mammalian sperm, the majority of nucleosomes are replaced with protamines to facilitate the compaction of the paternal genome¹⁴⁴. Nevertheless, a small percentage

Zygotic genome activation (ZGA). Activation of zygotic genome transcription for the first time after fertilization.

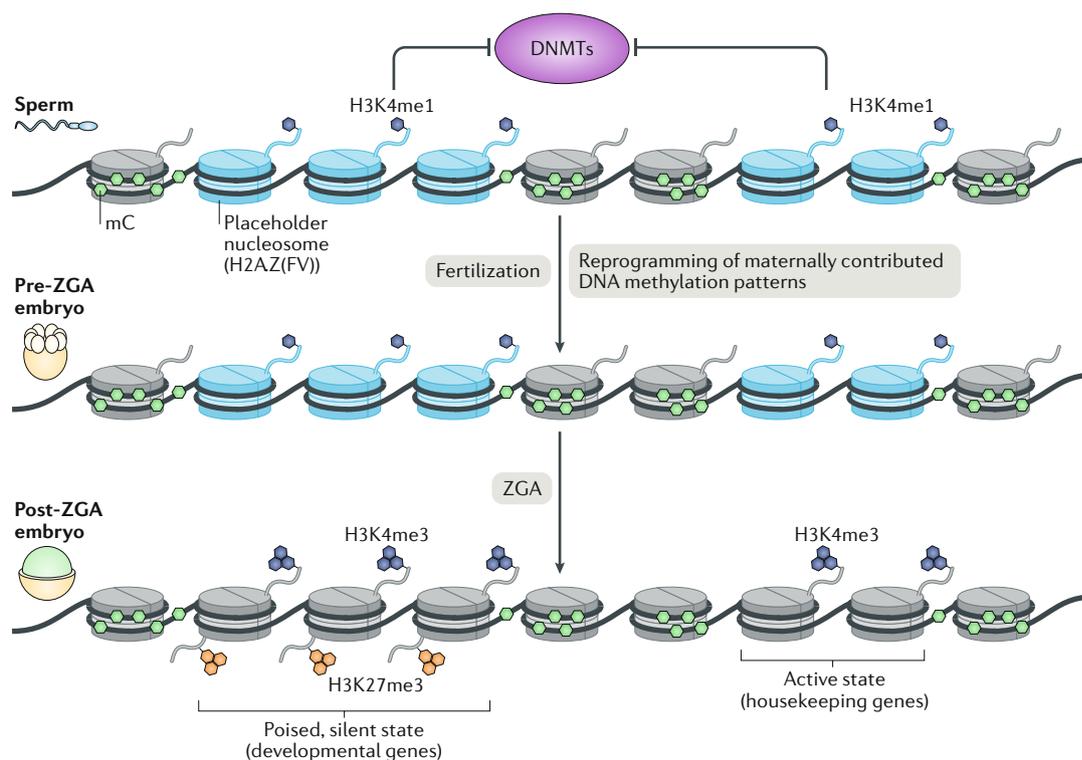


Fig. 2 | The role of placeholder nucleosomes in germline-to-embryo epigenetic reprogramming in zebrafish.

In zebrafish sperm and embryos before zygotic genome activation (ZGA), DNA hypomethylated regions are occupied by ‘placeholder’ nucleosomes bearing the histone variant H2A.Z(FV)¹²⁸ and mono-methylated histone H3 lysine 4 (H3K4me1). The presence of placeholder nucleosomes mediates the maintenance of paternal hypomethylated DNA patterns through the transcriptionally quiescent cleavage stages by deterring de novo DNA methylation without activating transcription. The maternal contribution to the embryonic epigenome, which initially lacks the placeholder conformation, is remodelled through the accumulation of placeholder nucleosomes to match the sperm epigenome in pre-ZGA embryos. Upon ZGA, placeholder nucleosomes at DNA hypomethylated regions resolve into either active (H3K4 trimethylated (H3K4me3)-marked) or poised and inactive (H3K27me3-marked and H3K4me3-marked) nucleosomes¹²⁸. DNMTs, DNA (cytosine-5)-methyltransferases; mC, methylcytosine.

of nucleosomes and their associated histone PTMs are retained, thereby forming a potential platform for the intergenerational transmission of regulatory states^{117,118}. Both active (H3K4me2 and H3K4me3) and repressive (H3K27me3, H3K9me3 and H4K20me3) histone PTMs have been detected in mammalian sperm; however, their distribution and dynamics vary considerably between species. For example, in humans, H3K9me3-marked and H4K20me3-marked nucleosomes are transmitted through the sperm into the zygote, where they participate in the build-up of constitutive heterochromatin¹⁴⁵. In mice, where only ~1% of the nucleosomes are retained in sperm¹¹⁸, the build-up of paternal heterochromatin in the zygote is initiated through maternally contributed PRC1 (REF.¹⁴⁶). Until recently, genome-wide studies of MEI in mammals were very challenging to orchestrate owing to the limited amounts of embryonic material that can be obtained for investigation. This limitation is now being overcome by the advent of novel genomics technologies, which allow for low-input transcriptome and epigenome profiling, often at single-cell resolution^{147–149}. Recent genome-wide studies revealed the existence of robust inheritance of H3K4me3 and H3K27me3 patterns through oocytes in mice and their role in the regulation of embryonic development^{66,71,120–122}. In line with these

findings, overexpression of human LSD1 in the developing mouse sperm resulted in the reduction of H3K4me2 at promoters of genes regulating developmental and metabolic processes and was accompanied by deregulation of gene expression in early F1 embryos¹⁵⁰. Notably, these changes promoted developmental defects in the offspring and were transmitted across three generations, indicative of TEI. Thus, both active and repressive histone PTMs can be transferred from the gametes to embryos in mammals (TABLE 1), with potential implications for the regulation of embryonic development (see below).

MEI through small RNAs

The meiotic inheritance of silencing through small non-coding RNAs has been widely studied in nematodes^{30,38,39,44–48} and flies^{151,152} but much less so in vertebrates^{153–156} (TABLE 1). A wealth of data suggest that distinct RNA species present in sperm and oocytes can be carried into the zygote upon fertilization^{20,153–158}. Nonetheless, the molecular mechanisms by which these RNA species might act in the embryo to potentially regulate early development remain largely unknown. Different external cues, such as viral exposure³⁸, nutrition^{154–156,159}, psychological stress^{20,21} and high temperature¹⁶⁰, can alter the abundance and composition of RNAs in the germ line, affect embryogenesis

Constitutive heterochromatin
Highly condensed, permanently transcriptionally silent late-replicating chromatin.

and induce phenotypic changes in diverse species (see below). Recent work also suggests that RNA modifications might have an important role in the heritability of metabolic phenotypes. DNMT2, which is a tRNA methyltransferase, might have a major role in the transmission of paternally acquired metabolic disorders in mice¹⁶¹. Specifically, injection of total RNA from sperm of obese (high-fat diet (HFD)-fed) males into zygotes resulted in a metabolic phenotype that was not observed upon injection of RNA extracted from *Dnmt2*^{-/-} HFD-fed males. It is currently unknown whether the loss of tRNA methylation can have a broader, indirect effect on other RNA species present in the sperm¹⁶¹.

piRNAs (FIG. 1c) are found in a broad range of organisms, such as *C. elegans*^{44–46}, *D. melanogaster*^{151,152}, zebrafish¹⁶² and mammals, in which they have major roles in maintaining the stability of germline genomes. However, unlike in *C. elegans* and *D. melanogaster*^{45–47,151}, where piRNAs also have a role in germline-inherited epigenetic silencing (FIG. 1c), in mammals such a function for piRNAs remains unproved. Examples of intergenerational transmission of small RNAs in mammals with clear implications to offspring phenotypes involve miRNAs^{153,157,163} and tRFs^{154–156,161}. Intergenerationally inherited miRNAs have a role in embryonic development^{157,163}, transmission of stress effects^{20,21} and paramutation of the *KIT* gene¹⁵³, whereas tRFs have mostly been linked to the transmission of metabolic phenotypes^{154–156}. Altogether, these findings highlight a role for small RNAs in heritable silencing of genomic elements in different species, regulation of embryonic development and transmission of acquired phenotypes. Although many of these examples await independent experimental validation, in the future, it will be of great interest to unravel the contribution of RNA chemical modifications¹⁶⁴ to their MEI potential¹⁶¹ and to explore the evolutionary conservation of such mechanisms and their potential relevance to humans.

MEI and embryogenesis

A number of studies published over the past few years have suggested that intergenerational transmission of histone PTMs and small RNAs might have important implications for the regulation of early embryogenesis of the F1 offspring. These observations have been made in both vertebrate and invertebrate organisms and have revealed novel modes of embryonic gene regulation.

Regulation of embryogenesis by inherited histone PTMs. In *D. melanogaster*, H3K27me3 can be transmitted from oocytes to the embryo together with maternally supplied E(z), which is the methyltransferase component of the PRC2 complex¹³⁷. Ablation of maternally inherited E(z) in *D. melanogaster* as well as its homologue enhancer of zeste homologue 2 in mice compromises embryonic development by causing homeotic transformation^{137,165}. Therefore, by establishing appropriate chromatin states in the maternal germ line, Polycomb proteins and H3K27me3 ensure intergenerational control of early embryonic development before ZGA^{137,165,166}. Specifically, oocyte-specific depletion of E(z) results in the aberrant accumulation of the active-genes histone

PTM H3K27 acetylation (H3K27ac) at enhancers during ZGA, thereby linking maternally inherited, Polycomb-mediated gene silencing with proper enhancer usage in the early embryo¹³⁷ (FIG. 3a).

In mammalian embryos, H3K27me3 appears to have an important role in DNA-methylation-independent maternal imprinting⁶⁶. Mapping open chromatin regions using the DNase I-hypersensitivity assay in mouse zygotes revealed several hundred parental-allele-specific (imprinted) DNase I-hypersensitive sites (DHSs), which are indicative of gene expression, including novel imprinted regions. Of note, only ~20% of paternal-allele-specific DHSs showed DNA methylation at the maternal allele, implying the existence of an alternative mechanism for reducing chromatin accessibility at the maternal allele (FIG. 3b). One-fifth of these maternal-allele imprints were lost upon overexpression of lysine-specific demethylase 6B (KDM6B), which in the zygote is H3K27me3-specific and forms open chromatin at the maternal alleles. H3K27me3-regulated maternal alleles were hypomethylated in the oocyte, suggesting that it is the repressive histone PTM H3K27me3 rather than DNA methylation that constrains maternal-allele accessibility at these imprinted loci⁶⁶. Moreover, highly sensitive chromatin immunoprecipitation followed by sequencing (ChIP-seq) profiling of 14 stages of mouse embryogenesis and gametogenesis revealed the existence of inheritance of maternally provided H3K27me3, which persists on thousands of distal regulatory regions until the blastocyst stage¹²². This finding is in line with the proposed role of maternally contributed H3K27me3 in enhancer regulation at ZGA in *D. melanogaster*¹³⁷. However, unlike in *D. melanogaster*, in mice, H3K27me3 is erased from *Hox* genes upon fertilization and the repressive state of the *Hox* cluster is maintained through alternative mechanisms; H3K27me3 is re-established in post-implantation embryos¹²² (FIG. 3b). Other Polycomb targets in mouse embryos displayed similar H3K27me3 dynamics.

In mouse oocytes, non-canonically broad H3K4me3 domains occupy almost one-quarter of the genome and coincide with DNA hypomethylation^{120,121}. The great majority of genes activated in the major ZGA — the major burst of zygotic transcription by RNA polymerase II — as well as distal regulatory elements involved in ZGA and in the establishment of totipotency, reside within these broad H3K4me3 domains^{120,121}. However, ZGA is associated with the decomposition of these broad H3K4me3 domains into sharper, transcription start site-associated H3K4me3 ‘peaks’ in late two-cell embryos, which is accompanied by the deposition of H3K27ac¹²⁰ (FIG. 3c). Establishment of broad H3K4me3 domains in oocytes, which is mediated by the histone-lysine N-methyltransferase 2B (KMT2B), and their resolution upon ZGA, which is mediated by the histone demethylases KDM5A and KDM5B, are crucial for H3K4me3-associated genome silencing during oogenesis and reactivation in late two-cell embryos, respectively^{120,121}. Depletion of KMT2B, KDM5A or KDM5B compromised early embryonic development^{120,121,167,168}. Together with the recent observations of intergenerational H3K27me3 inheritance^{66,137}, these findings provide intriguing clues

Paramutation

Heritable change in gene expression of a (paramutable) allele, which is mediated by *trans*-interaction with the homologous (paramutagenic) allele.

Homeotic transformation

The formation of a body structure or an organ in place of another in an abnormal location.

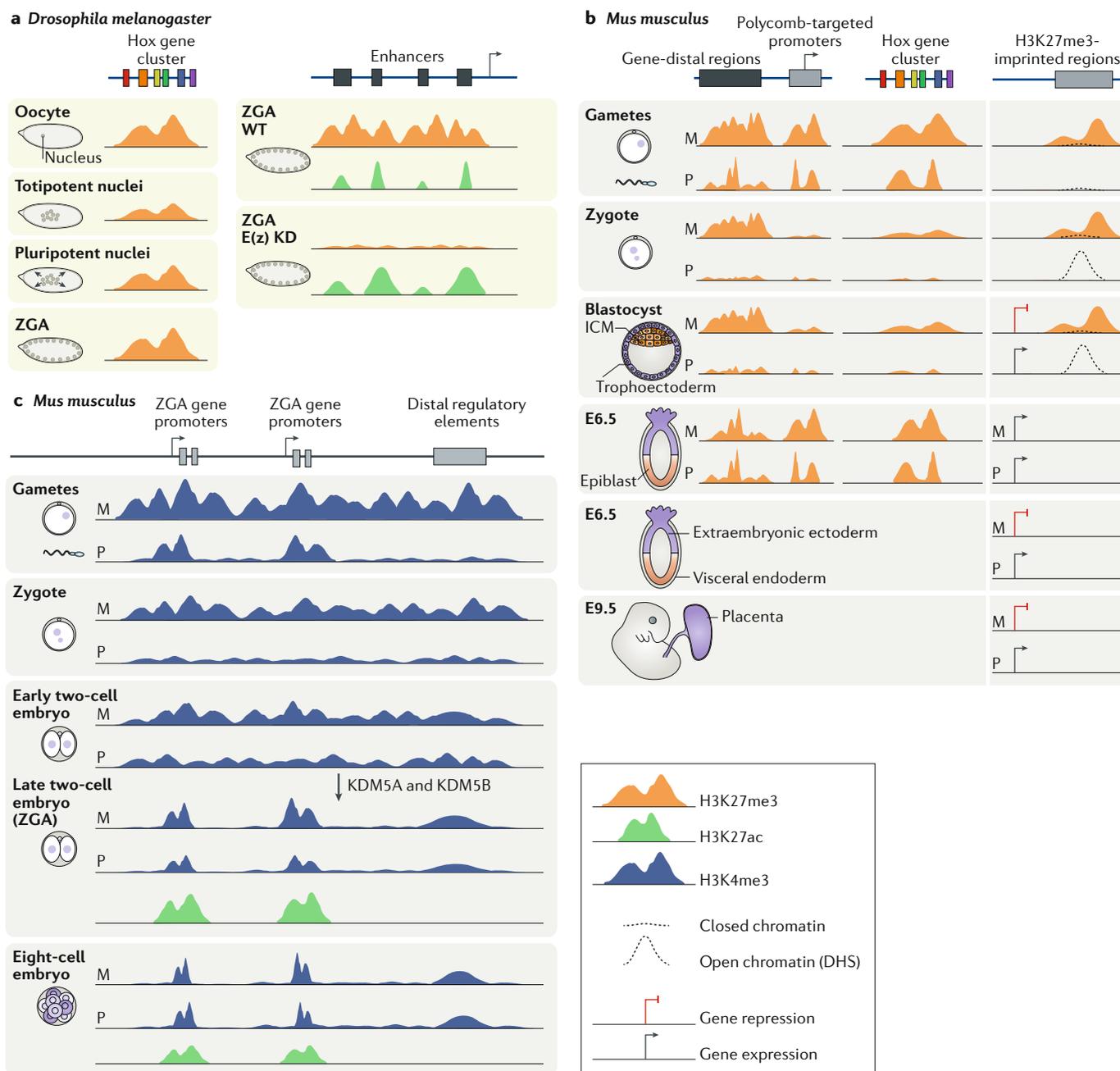


Fig. 3 | Intergenerational inheritance of histone post-translational modifications contributes to gene regulation during embryogenesis. **a** | In *Drosophila melanogaster*, histone H3 lysine 27 trimethylation (H3K27me3), which is a repressive post-translational modification, is maternally transmitted to the embryo and maintained until zygotic gene activation (ZGA), including at *Hox* loci¹³⁷. Depletion of the maternally inherited Polycomb protein enhancer of zeste (*E(z)*) results in loss of H3K27me3 and aberrant H3K27 acetylation (H3K27ac) at enhancers, leading to erroneous gene activation during ZGA. **b** | In mouse gametes, *Hox* loci and the majority of promoters targeted by Polycomb proteins are marked by H3K27me3; however, in the zygote and two-cell embryos, H3K27me3 is reduced at the maternal allele and is completely lost at the paternal allele. H3K27me3 at these regions emerges in the inner cell mass (ICM) of the blastocyst and is re-established in the epiblast at embryonic day 6.5 (E6.5). H3K27me3 at gene-distal regions is inherited from oocytes and propagated on the maternal allele in the zygote, two-cell embryos and ICM¹²². DNA methylation-independent imprinted loci, characterized by the presence of DNase I-hypersensitive sites (DHSs) at the paternal allele

and H3K27me3 at the maternal allele, are inherited by the zygote and propagated to the blastocyst stage⁶⁶. Their nearest genes ($n = 76$) display paternal-allele-specific gene expression that is largely maintained in the blastocyst (more so in the trophectoderm than in the ICM). In post-implantation embryos, H3K27me3-dependent imprinting is erased in the epiblast but is partially maintained in extra-embryonic ectoderm and visceral endoderm at E6.5 and in the placenta until E9.5. **c** | Non-canonically broad H3K4me3 domains in mouse oocytes are transmitted to the zygote upon fertilization and are transformed into canonical H3K4me3 peaks in late two-cell embryos, where they characterize genes and regulatory elements activated during ZGA. Decomposition of non-canonical H3K4me3 domains coincides with the appearance of H3K27ac at ZGA genes in late two-cell and eight-cell stages. Compared with the sperm, the paternal pronucleus has considerably less H3K4me3, which is re-established by lysine-specific histone demethylase 5A (KDM5A) and KDM5B in late two-cell embryos and onwards^{120,121}. KD, knockdown; M, maternal; P, paternal; WT, wild type.

as to how parentally contributed histone PTMs (TABLE 1) provide crucial signals required for the proper establishment of transcriptional programmes at ZGA.

Regulation of embryogenesis by inherited small RNAs.

Small non-coding RNAs inherited from gametes also participate in the regulation of early embryonic development. In mice, inhibition of a sperm-derived miRNA, miR-34c, reduced DNA synthesis and suppressed the first zygotic division¹⁵⁷. Similarly, maternal loss of the endonuclease Dicer, which is essential for miRNA formation, was not compatible with the first cell division in mouse embryos¹⁶³, while paternal loss of Dicer resulted in severely impaired preimplantation development¹⁶⁹. However, in zebrafish, this is not the case, and embryos derived from maternal Dicer mutants only start displaying developmental defects during gastrulation, somitogenesis, brain and heart development¹⁷⁰. These studies are further supported by recent observations that small RNAs found in mature sperm are essential for embryogenesis. Specifically, zygotes generated via intracytoplasmic sperm injection (ICSI) of immature sperm (derived from caput epididymis) largely failed to support embryogenesis and exhibited multiple defects during and after implantation¹⁷¹. However, these defects were rescued by the injection of small RNAs (18–40 nucleotides) derived from extracellular vesicles (epididymosomes) that deliver their small RNA repertoire to mature sperm. This finding suggests that small RNAs in mature sperm^{171,172}, which consist mostly of miRNAs but also of tRFs, likely have major roles during early mouse embryogenesis.

Inheritance of metabolic phenotypes

A major area of MEI research is related to the study of potential adaptive advantages of intergenerational inheritance of histone PTMs, small RNAs and DNA methylation. Of particular interest is the possibility that environmental influences might affect the epigenome of parental gametes, which can then be transferred to the zygote to alter gene regulation. The importance of meiotic inheritance of the histone PTMs H3K9me3 and H3K27me3 in response to environmental stimuli was recently demonstrated in *D. melanogaster*. Paternal high sugar consumption was shown to alter the chromatin state of embryonic, larval and metabolic genes in F1 offspring, accompanied by increased triglyceride levels and body weight¹⁷³. This alteration was due to the transcriptional activation of genes residing in ‘black’ (lamin/histone-H1-associated) and ‘blue’ (Polycomb-associated) heterochromatin¹⁷⁴, in line with previous findings of heterochromatin MEI in flies¹⁷⁵. Notably, the H3K9me3 mark was reduced in the fat body cells of F1 offspring males whose fathers were fed a high-sugar diet¹⁷³.

Small RNAs have also been recently described as key determinants of intergenerational transmission of metabolic phenotypes in mammals. Recent work shed light on how small (28–34 nucleotide) tRFs derived from tRNA 5' ends and inherited through sperm can potentially regulate embryogenesis in mice^{154–156,171} (TABLE 1). tRFs derived from tRNA-Gly-GCC of fathers fed a low-protein diet were shown to mediate repression of

genes regulated by the long terminal repeats of MERVL (mouse endogenous retrovirus with leucine tRNA primer) in mouse embryos and embryonic stem cells (FIG. 4a). These tRFs were trafficked from the epididymis, thereby providing remarkable evidence of soma-to-germline transmission of paternal dietary effects^{156,172}. Similarly, the injection into zygotes of tRFs from fathers that were kept on a HFD resulted in downregulation of metabolic pathways in both eight-cell embryos and blastocysts¹⁵⁴. In another study, the transmission of a latent metabolic phenotype from obese, prediabetic F0 males to F1 and to F2 was linked to the inheritance of several RNA species. Those included tRFs and miRNAs such as miR-10, which constituted ~25% of all miRNAs in the paternally obese F1 mice¹⁵⁵ and the targets of which were enriched in functions related to transcriptional regulation (FIG. 4b). Notably, the transmission of metabolic phenotypes discussed above appears to be highly linked to the methylation status of small RNA species by DNMT2, which is an area that merits further investigation¹⁶¹. DNA methylation was also recently implicated in the transmission of metabolic phenotypes associated with in utero malnutrition in mammals^{116,176}. However, the precise function of DNA methylation in these processes and its underlying causality remain unclear. In summary, these results provide evidence for widespread MEI through diverse molecular mechanisms in insects and mammals and demonstrate how environmental cues might be sensed and transmitted to the offspring.

Conclusions and future perspective

Numerous aspects of MEI in plants and in short-lived organisms, such as *C. elegans* and *D. melanogaster*, have been thoroughly described and excellently reviewed^{24–28,134,135}. By contrast, the functions, mechanisms and dimensions of MEI in most animal phyla still remain poorly understood. The recent advent of genomics technologies has enabled the thorough exploration of MEI phenomena across a broad range of species and in different biological contexts. For historical reasons¹⁷⁷, and owing to its precise, replication-coupled enzymatic machinery^{89,90}, DNA methylation has garnered much attention as a potential mechanism of MEI in animals. Nevertheless, the links between DNA methylation and MEI remain a topic of debate^{70,72,116,178}. Recent insights from genomics studies suggest that other molecular mechanisms, such as histone PTMs^{66,120,121,136,137} and small RNAs, including tRFs^{154–156,161}, likely have important roles in the intergenerational transmission of gene-regulatory information in the animal kingdom.

A major criticism related to the study of MEI in mammals is the lack of associated causality⁸⁸. In other words, it is very challenging to unambiguously demonstrate that the regulatory changes acquired in the parental germ line can be efficiently relayed to the offspring to alter gene expression. An even more challenging aspect of MEI studies is the lack of proof that such epigenetic changes, acquired in one generation and passed on to the next, have any adaptive roles³. The recent developments in CRISPR-Cas9-driven targeted epigenome remodeling applied to easily tractable model organisms such as zebrafish could tackle these challenges. For example,

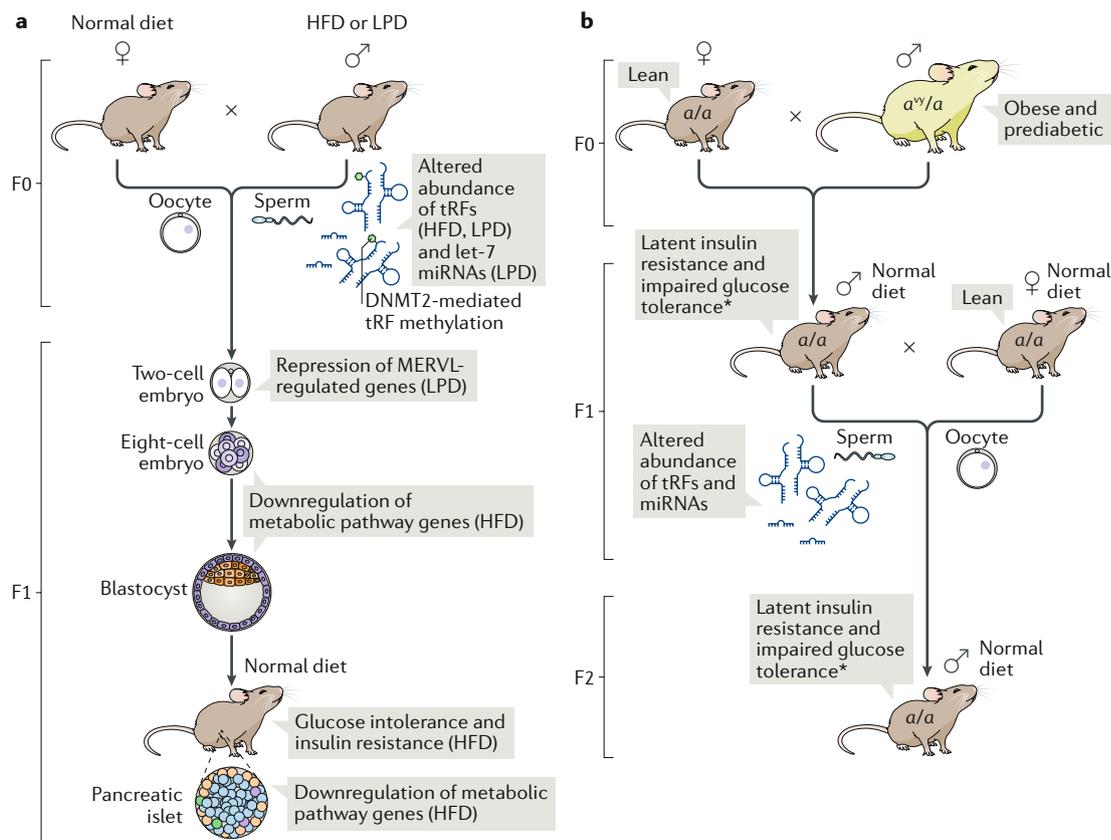


Fig. 4 | Inheritance of metabolic phenotypes through small RNAs in mammals. a | The abundance of tRNA fragments (tRFs) and *let-7* family microRNAs (miRNAs) is altered in the sperm of mice exposed to a high-fat diet (HFD) or low-protein diet (LPD). Specifically, increased abundance of tRFs (such as tRF-Gly-GCC) and decreased abundance of the *let-7* miRNA was observed in the sperm of male mice fed a LPD¹⁵⁶. The sperm of mice fed a HFD showed upregulation of tRFs and downregulation of miRNAs¹⁵⁴. Downregulation of genes controlled by the long terminal repeats of MERV1 (mouse endogenous retrovirus with leucine tRNA primer) was observed in two-cell embryos fathered by LPD mice, and antisense-mediated inhibition of tRF-Gly-GCC resulted in derepression of the MERV1-regulated genes¹⁵⁶. Downregulation of metabolic pathway genes was observed in eight-cell embryos and blastocyst-stage embryos of HFD fathers¹⁵⁴. Adult F1 offspring of HFD fathers exhibit impaired glucose tolerance, insulin resistance and downregulation of metabolic genes in pancreatic islets¹⁵⁴. F1 offspring of LPD fathers exhibit upregulation of a cholesterol biosynthesis enzyme in the liver¹⁵⁶. DNA (cytosine-5)-methyltransferase 2 (DNMT2) is a tRNA methyltransferase that participates in the transmission of metabolic phenotypes by altering the secondary structure and stability of tRNAs¹⁶¹. **b** | F1 progeny (a/a) of obese agouti viable yellow (a^y) heterozygous males exhibit impaired glucose tolerance and insulin resistance following HFD challenge (asterisk)¹⁵⁵. The transmission of these phenotypes might be caused by altered levels of tRFs and miRNAs such as miR-10, which were present in the F1 sperm of mice fed a normal diet but sired by obese fathers. The impaired glucose tolerance and insulin resistance phenotype was also observed in F2 mice during HFD challenge (asterisk). No metabolic phenotypes were detected in F3 (REF. 155).

the fusion of the Cas9 protein to catalytic domains of DNMTs^{179–181} or of histone-modifying enzymes¹⁸² could enable targeted epigenetic modification of genomes that could then be monitored through generations. This targeted modification will facilitate precise functional studies that do not depend on often-lethal gene knockouts, which can hamper the interrogation of heritable effects, and will also allow the study of the potential dependence of MEI phenomena on the DNA sequence. It is worth noting that, in many cases, *cis*-acting factors are required for the initiation of epigenetic inheritance^{85–87}, but in some cases the deletion of the initiating sequence after a chromatin state has been established does not preclude epigenetic inheritance¹⁸³. Thus CRISPR-Cas9 epigenome and genome editing systems will have the ability to assess such factors in a high-throughput manner and in

diverse species. The ever-decreasing cost of sequencing technologies is also expected to result in more whole-genome sequences to complement MEI studies, which will help in differentiating whether the observed inheritance phenomena are epigenetic, genetic or both. Until then, the evidence for MEI that spans multiple generations in mammals remains limited to sporadic examples. To date, the strongest example of such a phenomenon remains the inheritance of the DNA methylation state of the agouti locus⁶⁸.

In this Review, we discussed various molecular mechanisms of parental reach into the embryonic development of the offspring^{66,120,121,136,137,154–156}. Whether such phenomena are conserved in humans and to what extent awaits further clarification. It is plausible that some of these mechanisms would function in humans owing

to the high conservation of the underlying enzymatic machinery¹⁸⁴. On the other hand, it is worth noting that considerable differences in sperm nucleosome retention and early embryonic chromatin establishment have been observed between humans and mice^{118,145}. Studying epigenetic inheritance in humans will remain a problem owing to long generation times, which restrict the collection of biological specimens, and owing to the genetic diversity of the human population. Nevertheless, with the reduced amounts of input material required for

genome-wide assays and the increased frequency of studies carried out on donated human embryonic material, it is reasonable to expect such proof-of-principle studies sooner rather than later. Overall, the recent revolution in genomics technologies, including targeted genome and epigenome manipulation, is expected to provide long-sought answers to fundamental questions about the functions and extent of MEI in animals.

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