EPIGENETICS

Inheritance of directed DNA cytosine methylation in mammals

A recent study performed directed C5-cytosine methylation of CpG islands to demonstrate that acquired methylation at critical loci could be reestablished for multiple generations in mice. This work provides a manipulatable system to examine how non-genetic information is transmitted across generations to regulate complex phenotypes.

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While most traits are transmitted via DNA[1] and follow Mendelian inheritance patterns, a growing list of traits has now been shown to be inherited from non-genetic information. What specific non-genetic cues are transmitted across generations and how this epigenetic information can escape the typical epigenetic erasure that occurs upon fertilization is still unknown. There is mounting evidence that a variety of different epigenetic cues, including DNA methylation, histone modifications, small non-coding RNAs, prions and microbiota can all be transmitted across generations to regulate complex phenotypes in a variety of species[2]. C5-cytosine DNA methylation (5mC) was initially demonstrated to be transmitted through DNA replication in mouse cells *ex vivo*[3]. The best examples of the maintenance of 5mC across generations are genes that are parentally imprinted, where DNA cytosine methylation from a single parental allele is maintained in a parent-of-origin specific manner[4]. The majority of non-imprinted DNA cytosine methylation is erased between mouse embryonic day 8.5 and 13.5, but intracisternal A particle (IAP) retrotransposons retain 5mC[5]. IAP insertion upstream of the agouti gene in mice produces a viable yellow mutation (A^{vy}) which causes yellow coat color, increased tumor incidence as well as adult onset obesity which is inherited in an epigenetic manner through the maternal retention of 5mC[6]. Another IAP retrotransposon insertion upstream of axin-fused ($Axin^{Fu}$) is regulated by DNA cytosine methylation and the 5mC is epigenetically heritable through both maternal and paternal lineages[7]; however examples of 5mC regulating transgenerational epigenetic inheritance in mammals are relatively rare under basal or stress free conditions[8]. What epigenetic cue is unique that allows some DNA methyl marks to persist and others to be erased? Given that DNA methylation has long been known to be transmitted intergenerationally during imprinting, the question remains as to what distinguishes which DNA methyl marks are transmitted for one generation versus multiple generations? If the signal is completely erased during gametogenesis and embryogenesis, what is the placeholder modification that allows DNA methylation to be reestablished?

The authors of a recent study[9] show that acquired de novo methylation in the promoter-associated CpG island (CGIs) can be stably maintained and transmitted across multiple generations in mice. First, the authors generated methylation-edited mouse embryonic stem cells (mESCs) in which two metabolic genes, Ankyrin repeat domain 26 (*Ankrd26*) or low-density lipoprotein receptor (*Ldlr*), were superficially silenced by inducing de novo methylation in their CGIs. The authors took advantage of a technique that they had previously optimized to induce de novo methylation in target-specific promoter-associated CGIs using CRISPR-based editing in human pluripotent stem cells. For inducing de novo methylation, the authors inserted a CpG-free DNA cassette in the promoter-associated CGIs, which induced directed DNA methylation at the endogenous CGI adjacent to the inserted cassette; then, they removed the foreign inserted cassette, and observed retained gene silencing through 20 passages in tissue culture[10]. In their most recent work[9], the authors expanded this work into mice by targeting *Ankrd26* or *Ldlr;* the inactivation of the two genes is associated with obesity or hypercholesterolemia disease phenotype in mice. The CGIs of these two genes were specifically hypermethylated (without changing global DNA methylation in mESCs), and mRNA levels of *Ankrd26* and *Ldlr* were downregulated. Similar to the previous findings, these acquired methylations were also stably maintained even after extensive cell passages (Fig. 1a).

Next, the authors asked whether these acquired epigenetic marks could be stably transmitted and maintained across generations *in vivo* despite the global epigenetic reprogramming that occurs during gametogenesis and embryogenesis. For that, they generated chimeric mice by microinjecting mESCs that had been methylation-edited (hypermethylated) at two different metabolic genes' CGIs into the 8-cell stage of a mouse embryo (Fig. 1b). Excitingly, both promoter-associated CGIs of *Ankrd26* and *Ldlr* were persistently hypermethylated, and transcription and protein production of Ankrd26 or Ldlr were downregulated in chimeric mice. The methylation-edited mice also developed associated disease phenotype due to repression of the target metabolic gene, without global methylation being altered elsewhere in the genome. This acquired methylation in the promoter-associated CGIs of both *Ankrd26* or *Ldlr* was stably maintained and transmitted across multiple generations, even after repeated DNA methylation erasure during gametogenesis and embryonic development. Importantly, the disease phenotype associated with the inactivation of these two metabolic genes was also inherited in the progeny. There are multiple correlative examples of different cancers or metabolic-associated disorders driven by epimutation in the parental generation predisposing their offspring towards the high risk of developing the same disease. This study provides a piece of direct evidence for transmission of acquired epigenetic disease predisposition across generations in mammals. With a more mechanistic understanding of the non-genetic cues that are transmitted across

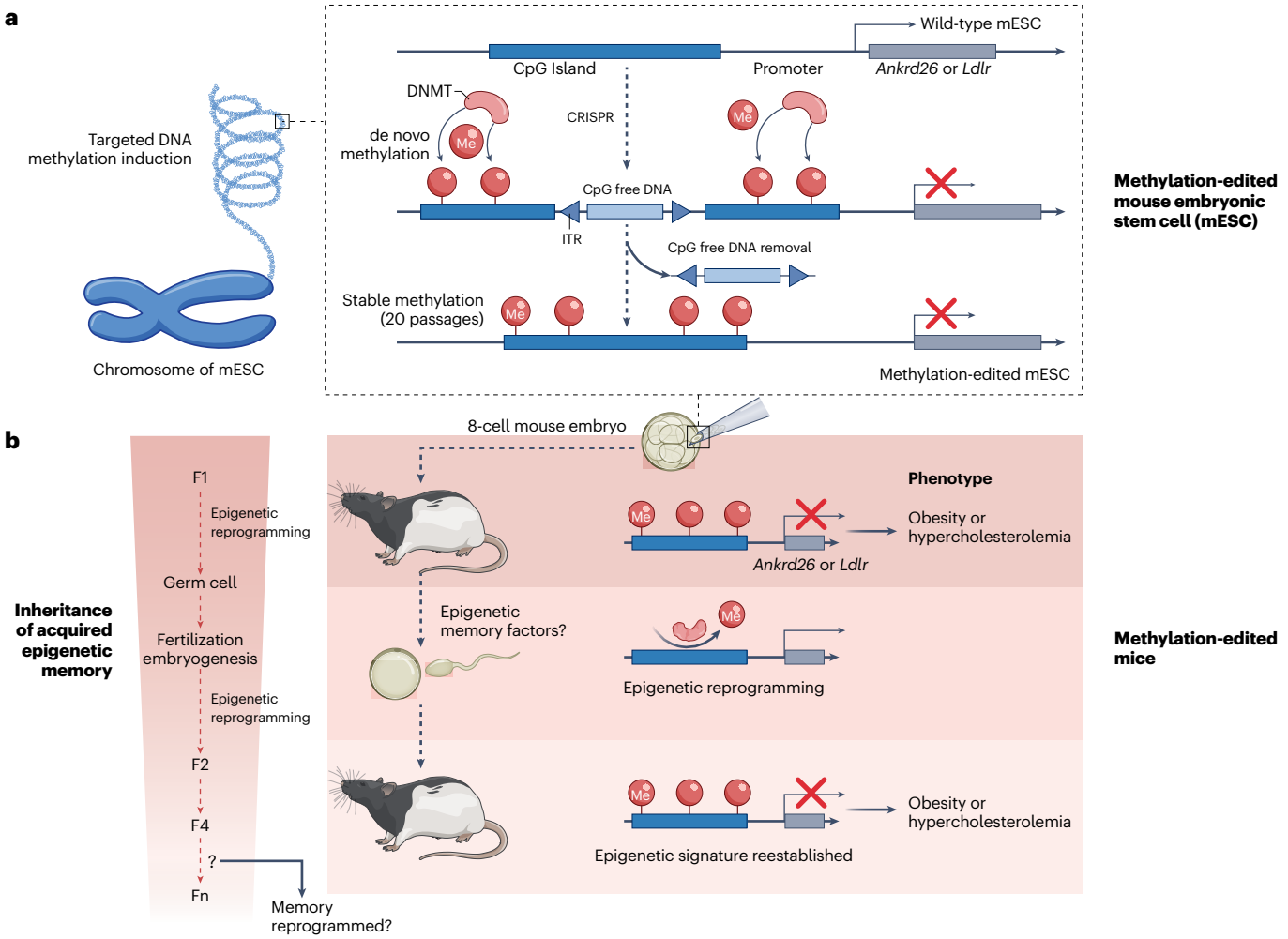

Fig. 1 | Directed DNA methylation transgenerationally inhibits Ankrd26 or Ldlr expression and drives obesity or hypercholesterolemia in mice. a, Targeted de novo methylation at the promoter-associated CpG island (CGI) in mouse embryonic stem cells (mESC) induces a stable methylation and inhibition of gene expression. Using CRISPR-Cas, a CpG-free DNA cassette was introduced into the promoter CGI of *Ankrd26* or *Ldlr* in mESC. The addition of this cassette induces recruitment of DNA methyltransferases (DNMT) by a poorly understood mechanism, to methylate C5-position of cytosines in the CpG island, which causes silencing of the gene; the silencing is stably maintained, even after the removal of the CpG-free cassette, for several passages (20 passages). The CpG-free cassette contains PiggyBac inverted terminal repeats (ITRs) to facilitate removal of the exogenous DNA cassette. **b**, Mice generated from directed DNA methylated mESC cells show DNA methylation at *Ankrd26* or *Ldlr* for up to four generations, despite the removal of 5mC during epigenetic reprogramming. Chimeric mice were generated by injecting methylation-edited mESC (panel a) into the 8-cell stage of the mouse embryo. Similar to the cell line, in chimeric mice the CGI of *Ankrd26* or *Ldlr* was hypermethylated as compared to the wild-type mice and the associated gene was also transcriptionally silenced. The F1 chimeric mice were phenotypically obese when *Ankrd26* was silenced or displayed hypercholesterolemia when *Ldlr* was silenced; the mice inherited the metabolic abnormality as well as DNA hypermethylation for four generations before it was diluted. Interestingly, the acquired 5mC are erased during gametogenesis and embryogenesis when there is a global epigenetic reprogramming and are reestablished post-implantation by some unknown mechanism. What epigenetic cues mark these specific loci for re-establishment of the epigenetic memory are still unknown.

generations and can regulate complex traits, a potential next step could be the treatment of some inherited diseases associated with epimutation or dysregulation of gene expressions.

The mechanism by which this directed DNA methylation could be transmitted to the next generation is still a mystery. While the authors did detect reacquired 5mC at the promoters of both *Ankrd26* and *Ldlr* CGIs in subsequent generations, there was a distinct loss of 5mC at the promoter of *Ldlr* CGI in both male and female primordial germ cells (PGCs) at E13.5 when there is a wave of DNA demethylation. This finding suggests that 5mC directs some alternative epigenetic cue that is responsible for marking the loci and then facilitates the reacquisition of 5mC at these precise loci. While it is interesting to speculate on what is the true carrier of epigenetic information in this instance and others[2], these mice provide a useful tool for attempting to address this question experimentally. This remains the big unanswered question for the field of epigenetics, potentially because there are so many different epigenetic molecules that can communicate with each other to establish and amplify non-genetic cues[2].

It is also interesting to note that the inheritance of the mouse phenotypes persisted for four generations before

beginning to dissipate, like many other transgenerational epigenetic inheritance traits in mice and other organisms[2]. What sets this epigenetic clock and prevents transgenerational phenotypes, for the most part, from persisting beyond those four generations? From an evolutionary prospective, if we anthropomorphize epigenetics, it could be advantageous to transmit non-genetic information, such as the knowledge of a drought or lack of available food, for only a few generations before returning back to a basal state. How this internal epigenetic clock can be achieved on a molecular level is an interesting mystery for the field. ❐

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Competing interests

The authors declare no competing interests.