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Review

Metastable epialleles and their contribution to epigenetic inheritance in mammals

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ABSTRACT

Many epigenetic differences between individuals are driven by genetic variation. Mammalian metastable epialleles are unusual in that they show variable DNA methylation states between genetically identical individuals. The occurrence of such states across generations has resulted in their consideration by many as strong evidence for epigenetic inheritance in mammals, with the classic A^{vy} and $Axin^{Fu}$ mouse models – each products of repeat element insertions – being the most widely accepted examples. Equally, there has been interest in exploring their use as epigenetic biosensors given their susceptibility to environmental compromise. Here we review the classic murine metastable epialleles as well as more recently identified candidates, with the aim of providing a more holistic understanding of their biology. We consider the extent to which epigenetic inheritance occurs at metastable epialleles and explore the limited mechanistic insights into the establishment of their variable epigenetic states. We discuss their environmental modulation and their potential relevance in genome regulation. In light of recent whole-genome screens for novel metastable epialleles, we point out the need to reassess their biological relevance in multi-generational studies and we highlight their value as a model to study repeat element silencing as well as the mechanisms and consequences of mammalian epigenetic stochasticity.

1. Introduction

Inter-individual phenotypic and epigenetic variation is most often explained by underlying genetic polymorphism. There is evidence, however, that in certain instances genetically identical individuals can differ epigenetically. The mechanisms driving such differences are poorly understood and likely involve both external (environmental) factors as well as intrinsic stochastic processes. There is considerable interest in determining the extent to which epigenetic information can be passed on from one generation to the next, as this challenges the dogma dictating that heritable traits are strictly conferred by the sequence of DNA transmitted from parent to offspring. Epigenetic inheritance across generations has convincingly been shown to occur in a number of non-mammalian model organisms. This has been reviewed elsewhere [1–3]. In contrast, this type of inheritance is rare in mammals due to the extensive genome-wide epigenetic reprogramming that takes place during mammalian development. Where it does occur, the driving mechanisms remain poorly understood. Metastable epialleles are frequently cited as the best example of this phenomenon in mammals. This review, focused solely on mammalian biology, will explore and interpret the literature to date regarding these unusual loci.

2. Classic metastable epialleles: A^{vy} and $Axin^{Fu}$

In order for the definition of metastable epialleles to become clear, let us first consider two classic examples in the mouse: the *Agouti viable yellow* (A^{vy}) and *Axin fused* ($Axin^{Fu}$) alleles. Both exhibit ectopic gene expression due to intracisternal A-particle (IAP) insertions [4,5]. IAPs are repetitive elements of the Class II endogenous retrovirus (ERV) family, their structure characterized by protein-coding sequences flanked by 5′ and 3′ long terminal repeats (LTRs) [6]. LTRs contain regulatory sequences that can act as host gene promoters and enhancers, making retrotransposition events potential drivers of evolutionary change (reviewed in [7]).

The *Agouti viable yellow* (A^{vy}) allele arose from the spontaneous insertion of an IAP into pseudoexon1A (PS1A) of the *Agouti* coat colour gene locus [4,8,9] (Fig. 1A and B). PS1A is located approximately 100 kb upstream of the *Agouti* coding exons (Fig. 1B). Wild type *Agouti* is normally expressed transiently from a hair cycle-specific promoter, producing a paracrine signalling peptide that yields a yellow band on a black hair [10]. This tightly controlled expression pattern is responsible for brown wild-type ‘agouti’ fur. The Agouti peptide can also interfere with metabolic pathways and has been linked to obesity, glucose intolerance, and tumourigenesis [11,12]. In A^{vy} mice, a cryptic promoter

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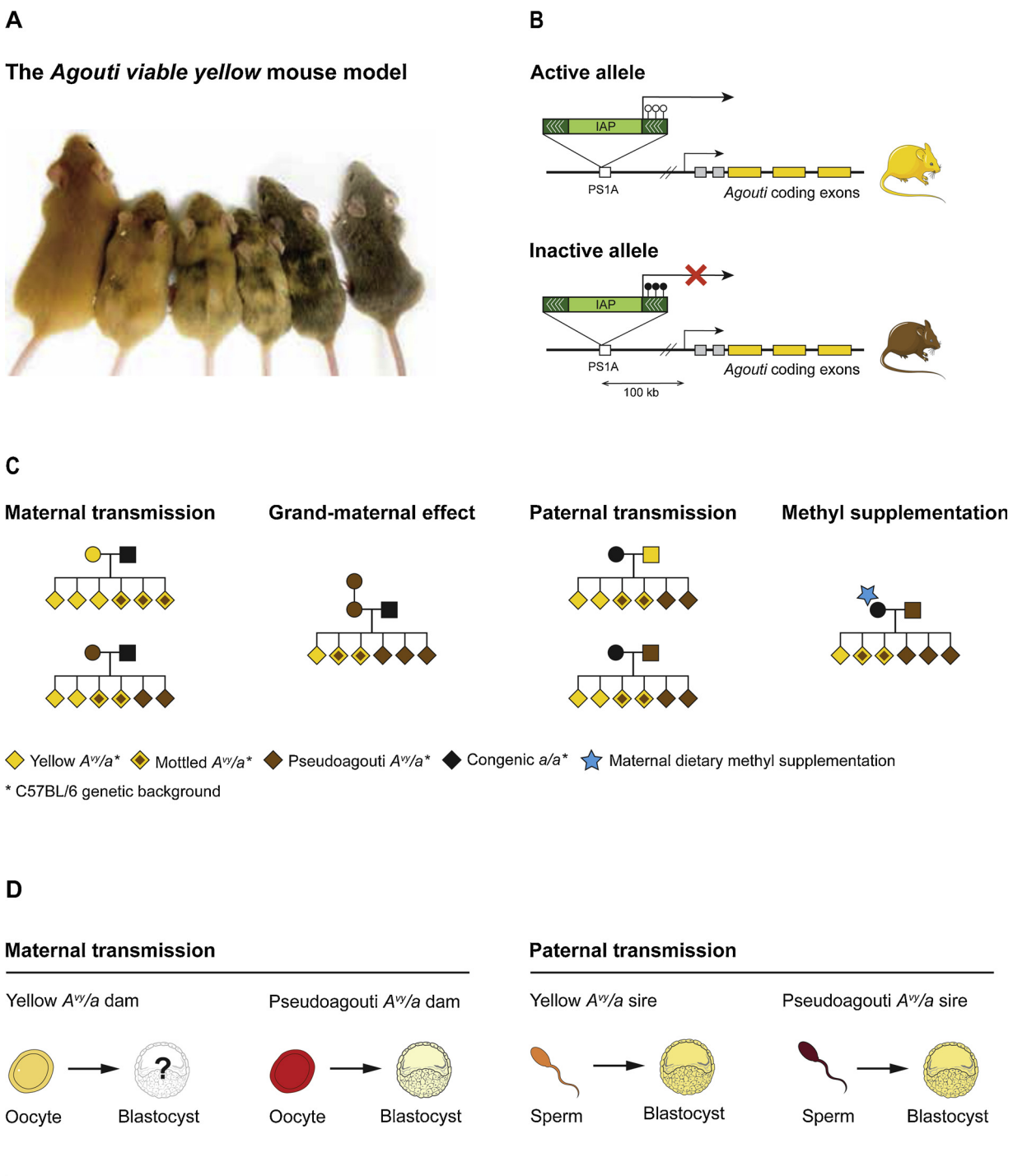

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in the IAP LTR drives constitutive ectopic *Agouti* expression [4] (Fig. 1B). This produces a mouse with a completely yellow coat as well as adult-onset obesity and diabetes (reviewed in [11]). The A^{vy} allele is part of a series of dominant *Agouti* alleles brought about by IAP insertions, suggesting the *Agouti* gene locus may be particularly prone to insertional mutagenesis. Some of these alleles, such as A^{iapy} and A^{hvy}, cause similar phenotypes to A^{vy} [13,14]. These additional dominant *Agouti* alleles have not been studied as extensively as A^{vy} and will not be discussed further.

The *Axin fused* ($Axin^{Fu}$) allele resulted from an IAP insertion in the sixth intron of the *Axin* gene [5,15]. The Axin protein is involved in the regulation of embryonic axis formation by inhibiting Wnt signaling [16]. The intragenic position of the IAP causes aberrant transcription of *Axin* downstream exons, producing an atypical protein. The resulting truncated Axin interferes with axial patterning and results in the development of a distinctive kinked tail [5,17]. Of note, while the $Axin^{Fu}$-associated IAP is intragenic, the one associated with the A^{vy} locus is 100 kb upstream of the affected exons, indicating that retrotransposon-

Fig. 1. The *Agouti viable yellow* (A^{vy}) locus.
(A) Genetically identical A^{vy} mice display a range of coat colours from yellow to pseudoagouti, including varying levels of mottling in between. The mice shown are on a C57BL/6J background and are not age-matched siblings.
(B) The A^{vy} allele is characterized by the presence of a contra-oriented IAP insertion (directionality shown with white arrows) in pseudoexon1A (PS1A) 100 kb upstream of the *Agouti* coding exons. The IAP is variably methylated between individuals and drives constitutive *Agouti* expression when unmethylated, leading to yellow fur. Full methylation results in pseudoagouti fur and partial methylation gives rise to mottling (see panel A).
(C) Maternal coat colour phenotype influences the coat colour distribution in A^{vy}/a offspring in the C57BL/6 genetic background. A grand-maternal effect is observed when the A^{vy} allele is transmitted through two generations of pseudoagouti females, resulting in a more severe phenotypic shift. No inheritance is observed upon paternal transmission on this genetic background (adapted from [18]). *In utero* dietary methyl supplementation shifts A^{vy} coat colour towards pseudoagouti [9]. In these experiments, mice carrying the A^{vy} allele were bred to congenic a/a mice homozygous for the *Agouti* null allele (a). Pedigrees: circle—female; square—male; diamond—sex unspecified; a/a offspring are not included.
(D) DNA methylation of the A^{vy} allele in gametes and blastocysts upon maternal and paternal transmission suggests reprogramming after fertilization. Methylation levels in sperm and oocytes reflect the coat colour phenotype and somatic methylation levels of the individual, while blastocysts are largely unmethylated regardless of parental phenotype. This is consistent with erasure of DNA methylation during preimplantation stages. The diagrams are constructed based on clonal bisulphite sequencing data from [52]. The methylation state of blastocysts produced by yellow A^{vy}/a dams has not been studied (depicted as a question mark).

mediated gene regulation can occur both locally and from a distance.

Unlike most mutations, A^{vy} and $Axin^{Fu}$ mice show variable penetrance and expressivity despite genetic homogeneity. The coat colour of individual inbred A^{vy} mice ranges from completely yellow to seemingly wild-type agouti (termed *pseudoagouti*), including varying intermediate degrees of mottled patterns [18] (Fig. 1A). Likewise, tail morphologies in inbred $Axin^{Fu}$ mice span from straight to severely kinked [17]. In both cases, the mechanism underlying the continuous phenotypic spectra is epigenetic in origin, with DNA methylation at the IAPs inversely correlated with expressivity. Hypomethylation at the IAP LTR thus corresponds to increased allelic expression, and vice versa [17,18]. This is highly unusual for IAPs, the vast majority of which are heavily methylated [19]. Remarkably, the full span of phenotypes can be observed within a single litter regardless of parental phenotype, illustrating the instability of their epigenetic state after passage through the germline [8,15].

The term *metastable epiallele* should make more sense now. The word *metastable* was first used in this context by plant biologists to describe alleles whose epigenetic state is capable of switching between active and repressed states from one generation to another [20–22]. Adapted for mammals by Emma Whitelaw and colleagues in 2002, the term *metastable epiallele* is intended to highlight (1) the epigenetic basis for the phenotypes associated with these alleles and (2) the apparent stochasticity of their epigenetic state [23]. In practice, this has translated to methylation variation between genetically identical individuals and, importantly, consistency in methylation levels within a single individual. While the methylation level of the IAPs associated with A^{vy} and $Axin^{Fu}$ varies across different mice, it is constant across different tissues of a single mouse, suggesting the methylation state is established early in development before tissue differentiation and maintained mitotically thereafter [9,24]. This differentiates metastable epialleles from *differentially methylated regions* (DMRs), a broader term used to designate differential but invariant methylation between biological samples, which could be cells, tissues, or individuals depending on the context.

Methylation consistency across tissues at the A^{vy} locus may seem at odds with the variegated patches of yellow and agouti fur observed on mottled mice. One might have expected pelts of intermediately methylated individuals to display a consistent intermediate pigmentation. DNA methylation, however, is dichotomous. A single CpG site is either methylated or unmethylated. Hence, the evident inter-individual range of methylation results from different proportions of methylated alleles in a cell population. Following from that, the proportion of methylated cells at A^{vy} is likely determined in a probabilistic fashion before germ layer differentiation and propagated mitotically throughout the body as it develops [9,11]. This would result in an approximately equal methylation percentage across tissues but would allow for local patches of cells to be different depending on the methylation state of their clonal origin. This is reminiscent of the black and orange mosaic fur pigmentation observed in tortoiseshell cats due to X-chromosome inactivation [25]. Given that cellular development and proliferation differ between cell types, it is perhaps more accurate to think of metastable epialleles as loci that display a substantial correlation in methylation between tissues rather than identical intra-tissue methylation.

3. Assessing the prevalence of metastable epialleles genome-wide

3.1. Murine metastable epialleles

As discussed above, retroelement insertions play a key role in the unique behaviour of the A^{vy} and $Axin^{Fu}$ loci. Considering almost half of the mouse genome is made up of repetitive elements, it is perhaps unsurprising that other metastable epialleles have been identified. While A^{vy} and $Axin^{Fu}$ were discovered decades ago due to their striking visual phenotypes [8,15], the identification of additional candidates has relied on using the genetic and epigenetic features of these classic loci to develop genome-wide screens and search algorithms. The third metastable epiallele to be identified was discovered by inspecting C57BL/6J cDNA databases in the hopes of finding transcripts containing IAP LTR sequences [26]. One such sequence contained a contra-oriented IAP element in the sixth intron of the *Cdk5rap1* gene. Much like the A^{vy} and $Axin^{Fu}$ loci, IAP LTR methylation was inversely correlated with expression of the aberrant transcript initiating from the 5′ LTR. The inter-individual methylation range of this new candidate, named $Cabp^{IAP}$, is much narrower than those observed at A^{vy} and $Axin^{Fu}$, and no identifiable phenotype is associated with its epigenetic variability [26].

The advent of high-throughput sequencing in the past decade has enabled larger-scale screens for metastable epialleles. The first attempt searched the mouse genome using genome-wide expression microarray data. Transcripts exhibiting wide-ranging inter-individual variation and low-ranging inter-tissue variation were selected as candidates in an attempt to capture the expression pattern observed for the *Agouti* gene in A^{vy} mice [27]. Only two loci (*Dnajb1* and *Glcci1*) were analysed in depth and though they showed inter-individual methylation differences, neither exhibited methylation-associated expression.

The second attempt screened for IAP insertions with promoter activity. The study identified retrotransposons near mRNA promoters associated with H3K4me3, an activating histone modification [28]. This enriched for active IAP LTR promoters and resulted in a set of 143 candidate regions, from which 13 were selected for experimental validation. Only three of these were found to exhibit significant methylation variation between individuals [28]. Also focusing on repeat elements, Faulk and colleagues recognized that the IAPs associated with A^{vy} and $Cabp^{IAP}$ both belong to the IAPLTR1_Mm subclass. They showed that IAPLTR1_Mm elements cluster into three clades, with the largest one containing the most conserved elements. A^{vy} and $Cabp^{IAP}$ segregated together in a separate smaller clade. Based on a limited selection of seven loci per clade, they provide preliminary evidence that the younger clades are more lowly methylated and display greater inter-individual methylation ranges [29]. These clades are likely enriched for

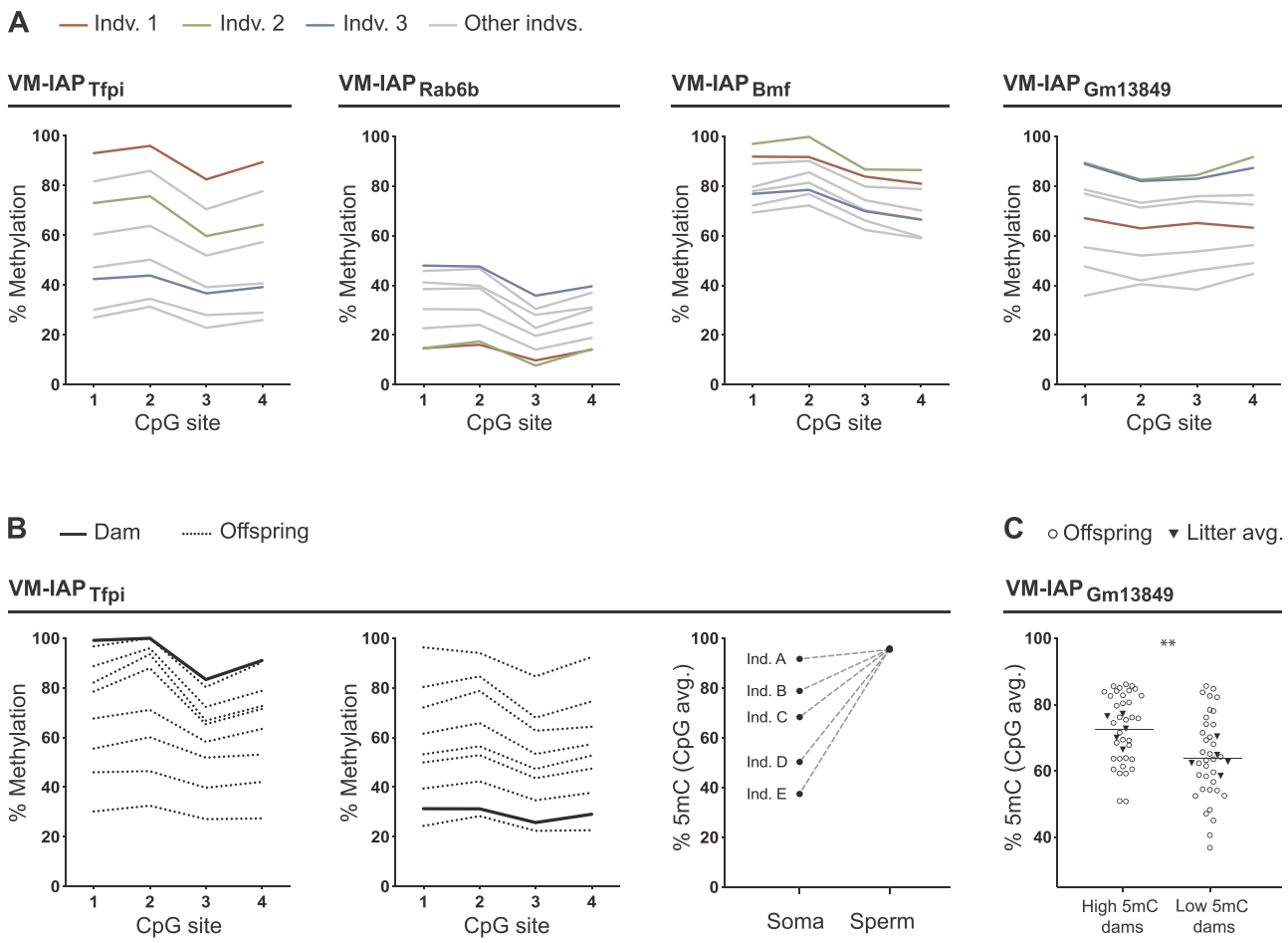

Fig. 2. Epigenetic variability and inheritance at VM-IAPs.
(A) VM-IAPs exhibit inter-individual methylation variation (four examples are shown). The methylation level at VM-IAPs is locus-specific, whereby separate loci show different methylation levels within a single mouse.
(B) The full range of VM-IAP methylation variation is predictably reconstructed from one generation to the next regardless of maternal methylation level. In contrast to the A^{vy} and $Axin^{Fu}$ loci, VM-IAPs are hypermethylated in mature sperm irrespective of somatic methylation levels (e.g. VM-IAP$_{Tfpi}$). Adapted from [31].
(C) Maternal epigenetic inheritance at VM-IAP$_{Gm13849}$. On average, offspring born to highly methylated dams show higher methylation levels than offspring born to lowly methylated dams. These results are from a replicate cohort. Statistics: one-sided *t*-test on litter averages, n = 5 dams per group. Adapted from [31].

metastable epialleles.

Taking an unbiased approach, Oey and colleagues performed whole genome bisulphite sequencing on five A^{vy} mice and identified 356 regions showing inter-individual methylation variation, including 55 ERV-overlapping regions [30]. Four of these were experimentally validated. Perhaps the most valuable aspect of this study was the whole genome sequencing analysis of two A^{vy} mice with different coat colours. Only 32 single nucleotide variants were detected in coding sequences and no mutations were found near the A^{vy} allele, contesting arguments suggesting that the phenotypic variation observed among A^{vy} littermates is due to genetic variation. However, as explained in Section 4.2, genetic background has an effect on the distribution of A^{vy} phenotypes in a parent-of-origin–specific manner.

The most recent screen for metastable epialleles was conducted by our team. We carried out a genome-wide screen identifying variably methylated IAPs (VM-IAPs) in the C57BL/6J genome [31]. Extensive characterization of the resulting candidates revealed both similarities and differences to A^{vy} and $Axin^{Fu}$. Much like the classic alleles, VM-IAP methylation levels are variable between individuals but consistent across tissues within a single individual (Fig. 2A). In addition, inter-individual methylation variation at VM-IAPs is reconstructed from generation to generation regardless of parental methylation level (Fig. 2B). However, the transcriptional effects and inheritance patterns observed at VM-IAPs, discussed in more depth in Sections 4 and 7, indicate that properties associated with A^{vy} and $Axin^{Fu}$ cannot necessarily be extrapolated to other metastable epialleles. To avoid confusion, the term VM-IAP will be used in this review when discussing findings specific to the regions identified and characterized in this screen.

Although the loci discussed here are endogenous and naturally occurring, a chimeric long interspersed nuclear element (LINE) retrotransposon of the L1 subclass was recently experimentally inserted into the *Axin* gene, inducing the kinked tail phenotype with variable penetrance much like the spontaneous $Axin^{Fu}$ insertion [32]. Unlike $Axin^{Fu}$, the $Axin^{cL1}$ mutation is not associated with variable methylation levels. Additional experimental manipulations of this sort will be of great comparative value in determining the mechanisms underlying metastability.

3.2. Metastable epialleles in humans

The identification of human metastable epialleles is a very challenging task due to the extensive genetic variation present in human populations. It is nevertheless an important endeavour in order to assess the extent to which they contribute to human phenotypic variation, to understand the roles of the human repeat genome, and to evaluate the relevance of using murine metastable epialleles as models to study epigenetic variation in humans. While analysing monozygotic (MZ)

twin cohorts can aid in overcoming some of the challenges associated with genetic variation, a recent cautionary study describes the phenomenon of "epigenetic supersimilarity" between human MZ twins and highlights their non-equivalence to isogenic mice that do not originate from the same zygote [33].

An alternative strategy to control for genetic differences in human populations has been to use a large number of genetically diverse methylomes. This approach has given rise to a growing list of putative human metastable epialleles, some of which are sensitive to environmental factors such as maternal nutrition and season of conception [34–37]. The most recent of these studies argues that the definition of metastable epialleles should be relaxed to include variably methylated regions that are susceptible to genetic influence, at least in the human context where this issue is unavoidable [37]. Under this framework, additional sequence-dependent candidates identified in the past year can be added to the list of potential human metastable epialleles [38,39].

Interestingly, although IAP elements do not exist in humans, the bordering regions of putative human metastable epialleles appear to be enriched for transposable elements of the ERV and LINE families [36,37]. While some of the murine screens were specifically restricted to transposable elements based on the presence of IAPs at the A^{vy} and $Axin^{Fu}$ loci [28,29,31], others were unbiased and still found enrichment for repeats [27,30]. Taken together, the mouse and human studies indicate that repeat elements play an important and conserved role in the establishment of inter-individual epigenetic variability. It is possible that metastable epialleles are a product of conflicting interactions between activating factors recruited to insertion sites and repeat repressive modifiers, an idea we will return to in Section 5. This does not preclude, however, the possibility of metastable epialleles at unique non-repetitive regions maintained by distinct mechanisms. The human studies suggest these exist, but a systematic screen for metastability at unique regions has not, to our knowledge, been conducted in the mouse.

4. Metastable epialleles as models of epigenetic inheritance

4.1. Partial inheritance of parental epigenetic state

Epigenetic inheritance across generations is one of the most striking properties of A^{vy} and $Axin^{Fu}$ mice. In the case of A^{vy}, maternal (but not paternal) coat colour phenotype affects the range of phenotypes observed in the offspring; the coat colour distribution of offspring born to yellow mothers is shifted towards yellow compared to that of offspring born to pseudoagouti mothers [40,41] (Fig. 1C). These experiments were conducted using inbred mouse strains, effectively eliminating the possibility of genetically mediated effects [18,40,41]. In light of the increased incidence of obesity observed in yellow mice, maternal inheritance of coat colour at A^{vy} was originally attributed to metabolic differences in the intrauterine environments of developing embryos [40]. Elegant embryo transfer experiments showed that this is not the case. Transferring fertilized oocytes from yellow dams to black foster mothers not carrying the A^{vy} allele produces offspring with the same coat colour distribution as offspring born to yellow dams without embryonic intervention [18]. This confirms that the transmission of maternal coat colour to the next generation is an epigenetic process rather than an environmental one. The same study reported a grand-maternal effect at A^{vy}: transmission of the allele through two generations of pseudoagouti dams appeared to cause a greater shift towards pseudoagouti than transmission through a single generation (Fig. 1C). It is unknown whether passage through a third or fourth generation of pseudoagouti females produces a further cumulative effect.

In contrast to A^{vy}, the $Axin^{Fu}$ allele exhibits epigenetic inheritance upon both maternal and paternal transmission. Parents with several tail kinks are more likely to produce offspring with kinked tails [17]. Interestingly, the effect is more pronounced following paternal inheritance, at least in the 129P4/RrRk mouse strain [17].

It is worth noting that all of the studies reporting A^{vy} or $Axin^{Fu}$ epigenetic inheritance use coat colour and tail morphology as phenotypic readouts of DNA methylation, respectively. No comprehensive statistical analysis has been conducted on parent and offspring DNA methylation data at the A^{vy} or $Axin^{Fu}$ loci, and very few have carried out offspring phenotyping in a manner blind to parental phenotype. While the correlation between IAP LTR methylation and phenotype severity is well documented for these classic loci, assessing the epigenetic inheritance at other metastable epialleles lacking visual phenotypes cannot rely on this form of classification. Breeding intensive experiments on six VM-IAPs quantified parental and offspring DNA methylation levels and assessed the maternal and paternal contribution to offspring VM-IAP methylation state using linear mixed-effects models. Only one region (VM-$IAP_{Gm13849}$) showed evidence of maternal inheritance and no paternal inheritance was detected (Fig. 2C). Importantly, the effect size of the maternal contribution to offspring methylation level at VM-$IAP_{Gm13849}$ was distinctly small, raising questions about its biological relevance [31].

The A^{vy} mouse model is often cited as the best described instance of mammalian epigenetic inheritance and the assumption has been that other regions in the mouse genome likely behave in the same way. The lack of heritability observed at VM-IAPs calls into question the prevalence of epigenetic inheritance across generations at metastable epialleles and warns against extrapolating from isolated examples.

4.2. Genetic background effects

The genetic background used to study the heritability of metastable epialleles is an important consideration, as both A^{vy} and $Axin^{Fu}$ inheritance patterns are influenced by the mouse strain the alleles are maintained on. The magnitude of maternal A^{vy} inheritance was shown to be dependent on whether the strain of the dam was C57BL/6J, YS/ChWf, or VY-Wf [40,42]. Similar genetic background effects have been reported for the penetrance of tail kink phenotypes associated with the $Axin^{Fu}$ allele [43]. Interestingly, when $Axin^{Fu}/+$ 129P4/RrRk male mice are crossed with A^{vy}/a C57BL/6J female mice, there is no paternal inheritance of tail phenotype, mimicking the inheritance pattern observed for A^{vy} coat colour on a C57BL/6J background [17,18].

These dependencies on genetic background suggest that the A^{vy} and $Axin^{Fu}$ parent-of-origin effects are mediated by *trans* acting genetic factors. It is possible that genetic or cytoplasmic modifiers carried in C57BL/6J oocytes, but not 129P4/RrRk oocytes, promote complete epigenetic reprogramming of metastable epialleles, therefore preventing the transmission of paternal phenotype. Such strain-specific modification has been studied in other contexts but has not been tested at metastable epialleles [44]. It is nonetheless evident that genetic background has vital implications for the design and resulting generalizability of future experiments. We are once again reminded of the interdependence of genetic and epigenetic processes, and the problematic nature of investigating epigenetic variation in genetically heterogeneous contexts.

Transposable element content and distribution differ substantially across mouse strains [45]. The C3H/HeJ strain, for example, is significantly more susceptible to new IAP insertions compared to other strains [46,47], potentially predisposing it to an increased number of metastable epialleles. As genome assemblies and the ability to map repeats improve, quality high-throughput analyses across multiple strains will be essential to understand the causes of these genetic background effects as well as the evolutionary and functional relevance of metastable epialleles.

4.3. Developmental dynamics

The mammalian genome undergoes two rounds of global epigenetic reprogramming, once during pre-implantation embryogenesis and

again during early germ cell lineage specification. After fertilization, genome-wide DNA methylation erasure occurs via active and passive demethylation of the paternal and maternal genomes, respectively. The second genome-wide demethylation event occurs only in primordial germ cells, after which sperm- and oocyte-specific methylation patterns are established (reviewed in [48,49]). These processes are important for the reacquisition of cellular totipotency in the next generation and the proper differentiation of soma and germ line.

It is difficult to reconcile genome-wide reprogramming with the idea of perpetuation of epigenetic states across generations. This has led to considerable debate in the ever-growing field of epigenetic inheritance. It has been shown that some IAP elements are resistant to global demethylation in both the germline [50] and during pre-implantation development [51], providing an attractive mechanism by which the IAP-driven A^{vy} and $Axin^{Fu}$ phenotypes could be inherited. Indeed, in sperm and oocytes, the methylation levels of these alleles have been reported to reflect those observed in somatic tissues [17,52,53] (Fig. 1D). In contrast, VM-IAPs are fully methylated in mature sperm regardless of somatic methylation level [31] (Fig. 2B). Importantly, both the A^{vy} and $Axin^{Fu}$ loci are demethylated at the blastocyst stage, indicating that their associated IAPs are not resistant to the post-fertilisation wave of epigenetic reprogramming and demonstrating that DNA methylation is not perpetuated as the direct mediator of phenotypic heritability [52,53] (Fig. 1D). Little is known about the methylation dynamics at putative human metastable epialleles during early development although an analysis of human embryo methylomes suggests that the variable epigenetic states at these regions may be established during the gastrulation transition [37].

4.4. Predictable reconstruction of epigenetic stochasticity

So far our discussion on metastable epiallele epigenetic inheritance has mainly focused on a partial memory of maternal or paternal methylation levels, reflected as a phenotypic bias in the F1 generation towards parental phenotype. Notably, only three such examples have been described (A^{vy}, $Axin^{Fu}$, VM-IAP$_{Gm13849}$), and even in these cases, persistence of methylation across generations does not occur. Instead, each of these epialleles reacquires a variable methylation state in the next generation. We suggest that more careful consideration should be given to the remarkable reconstruction of this epigenetic state from one generation to the next (Fig. 3A).

While analyses of the A^{vy} and $Axin^{Fu}$ loci have largely been based on phenotypic data, VM-IAPs were identified and characterized based on their methylation profiles [31]. This created an opportunity for a more comprehensive and quantitative cross-locus assessment of methylation variability. Interestingly, VM-IAP methylation levels are highly locus-specific and are not correlated within an individual. For example, a single mouse can be highly methylated at one VM-IAP and lowly methylated at another (Fig. 2A). This suggests VM-IAPs are not uniformly targeted by the same *trans*-acting mechanism. In addition, the methylation range within a population is different from locus to locus but is remarkably constant for a given VM-IAP, even after passage through the germline and regardless of parental methylation level [31].

We suggest that it is this predictable reconstruction of epigenetic stochasticity from generation to generation that raises the most compelling mechanistic questions, rather than the subtle memory of parental methylation level observed at a minority of metastable epialleles. What is the role of genomic context in delimiting the consistent methylation ranges observed at each locus? What factors are at play during the probabilistic acquisition of methylation states within the limits of each range? At what developmental time point is parental methylation level forgotten, and when is the methylation state of offspring established? How long is the period of stochastic establishment and is there a later somatically heritable state? Which of these mechanistic aspects vary between VM-IAPs? The answers to these questions, as yet unknown, will provide considerable insight into the mechanisms underlying the resetting of epigenetic variability across generations. Once this is understood, then the basis for partial memory of parental methylation level observed for a subset of loci can be addressed.

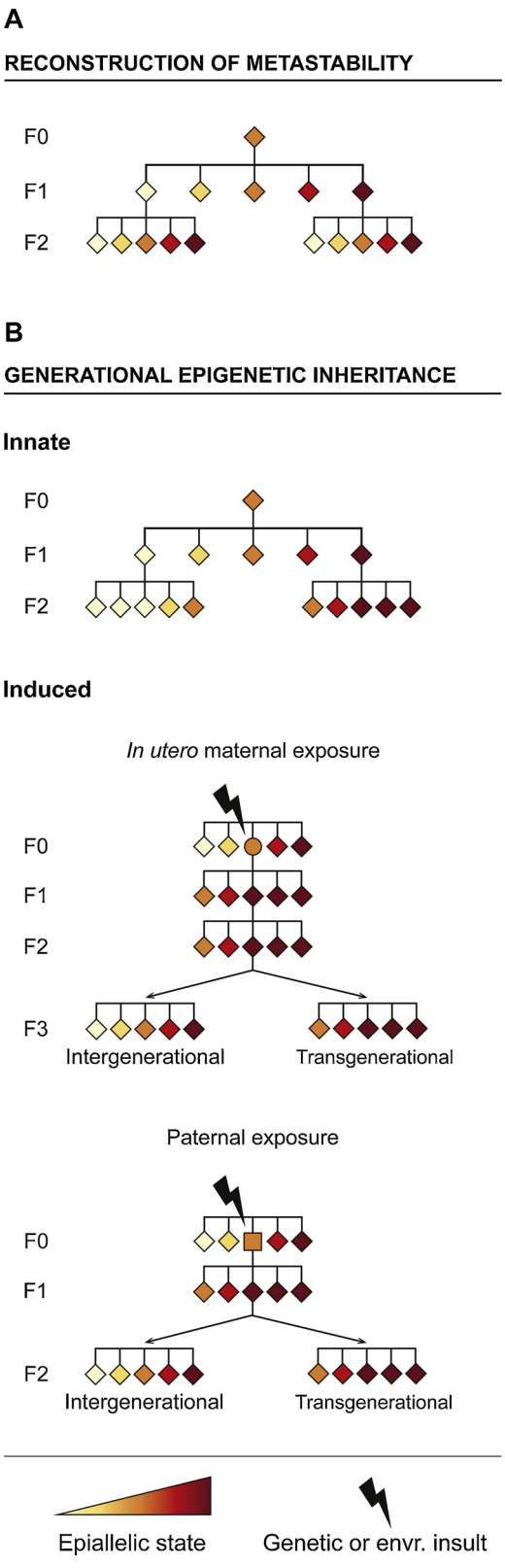

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Fig. 3. Reconstruction and heritability of epiallelic states.
(A) Reconstruction of epigenetic metastability. The full range of epiallelic states is reconstructed after passage through the germline, regardless of parental state. **(B)** Generational epigenetic inheritance occurs when the parental epiallelic state influences that of the offspring. It can either be *innate*, occurring in the absence of an external trigger, or *induced*, defined by the cross-generational persistence of an epigenetic change brought about by a genetic or environmental insult inflicted in a previous generation. In the case of induced epigenetic inheritance, if the phenotypic or epigenetic perturbation persists at least to the F3 generation following *in utero* maternal exposure (or to the F2 generation following paternal exposure), the effect is *transgenerational*. It is *intergenerational* if it only persists to F2 (or F1 upon paternal transmission) [102]. Since the distinction between trans- and intergenerational inheritance is most often associated with induced instances, it is only shown for this context above. Pedigrees: circle—female; square—male; diamond—sex unspecified.

5. Mechanistic insights into the establishment and maintenance of epigenetic metastability

5.1. Histone modifications

The inter-individual methylation variation observed at metastable epialleles is likely associated with other variable epigenetic factors. Two studies have described the presence of variable histone marks at metastable epialleles: one conducted in A^{vy} liver tissue, the other in $Axin^{Fu}$ blastocysts. Mild enrichment of H3 and H4 di-acetylation was observed at the A^{vy} IAP LTR in yellow mice while H4K20me3 enrichment was detected in pseudoagouti mice. H4K20me3 is thought to be the most prominent histone modification at IAP LTRs, targeting them specifically over other types of repeats such as L1 elements [54,55]. No difference in H3K4me3 was found [56]. The study that assessed the histone modification landscape at the $Axin^{Fu}$ locus at the blastocyst stage found significant differences in H3K4me2 and H3K9ac between blastocysts generated from penetrant and silent sires, suggesting histone marks may be involved in the transmission of tail phenotypes across generations [53]. Histone modifications have also been explored at VM-IAPs, but no consistent patterns have emerged other than an enrichment for the active marks H3K27ac, H3K9ac and H3K4me3 at the bordering regions of transcript-overlapping VM-IAPs [31]. A more in-depth characterization of metastable epiallele histone profiles, both in terms of the number of loci examined and the range of histone marks considered, will prove useful in assessing their role in establishing variable epigenetic states.

5.2. Transgene modifiers

5.2.1. Drawing parallels between metastable epialleles and transgenes

The use of transgenic mice has been vital for the study of genome function and for the modelling of human disease. One of the challenges associated with producing mice carrying exogenous DNA constructs is the often-unpredictable cell-to-cell variability in transgene expression levels within a cell population or between individuals. The cause of such variegation remains poorly understood and has been attributed to a range of factors, including the repressive effects of multi-copy transgene arrays, the proximity of the integration site to heterochromatin, and the presence of viral or plasmid-derived sequences within transgene constructs [57–61].

Reminiscent of the properties of metastable epialleles, variegated transgenes are (1) linked to variable DNA methylation levels, (2) modulated by strain background, and (3) influenced by parental origin [62–65]. For some transgenes, variable expressivity is recapitulated from one generation to the next in a predictable manner [66,67]. Others exhibit memory of parental methylation level, their silenced state stably inherited to subsequent generations after passage through the germline [65,68–70]. This heritable silencing is sometimes irreversible, and at other times reactivated upon transmission through the other parent or by crossing to a different strain.

Although fully heritable silencing is not a property of metastable epialleles, the overlapping characteristics with transgenes are worth considering while investigating the mechanisms underlying epigenetic stochasticity. Both are associated with foreign DNA sequences with regulatory potential, likely triggering similar host genome recognition and response pathways. Parallels have been drawn between transgenesis and retrotransposition before [71,72]; some have even classified variegated murine transgenes as metastable epialleles [23]. In fact, a successful screen for modifiers of variegated transgenes, described in the next section, confirms that transgenes and metastable epialleles share epigenetic modifiers.

5.2.2. MommeD mutagenesis screen

Having made key contributions to our understanding of the unique molecular behaviour of the A^{vy}, $Axin^{Fu}$ and $Cabp^{IAP}$ loci, Emma Whitelaw and her team embarked on a large-scale *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screen for modifiers of epigenetic variability [73,74]. The study used a mouse line carrying a GFP reporter transgene expressed in a variegated fashion in red blood cells. Importantly, the variegated expression of this transgene is predictable: 55% of red blood cells express GFP in multi-generational isogenic mice. Offspring born to ENU-treated males were assessed for enhancement or suppression of variegation by screening for shifts in the percentage of GFP-expressing red blood cells. The resulting mutations were designated *Modifiers of Murine Metastable Epialleles* (*Mommes*); dominant mutations were referred to as *MommeDs*.

Mapping the mutations associated with *MommeDs,* as well as characterizing the role of the affected genes in epigenetic regulatory processes, is still ongoing [73–83]. More than 50 *MommeD* enhancers or suppressors of variegation have been identified in this screen. *MommeDs* that increase the proportion of GFP-expressing cells have mutations in genes acting as suppressors of variegation and involved in transgene silencing. Conversely, *MommeDs* resulting in a decrease in the proportion of GFP-expressing cells have mutations in genes that enhance variegation and promote transgene expression. Unsurprisingly, the majority of mutations fell into genes with known epigenetic regulatory properties. These include genes involved in DNA methylation (e.g. *Dnmt1* and *Dnmt3b)*, histone modification (e.g. *Brd1*, *Hdac1, Setdb1, Trim28*), and chromatin remodelling (e.g. *Baz1b, Pbrm1, Smarca4, Smarca5*). The full list of genes is reviewed elsewhere [72]. Many of the identified genes were previously detected in screens for modulators of position effect variegation (PEV) in *Drosophila*, reflecting the highly conserved nature of epigenetic modifiers [84].

In line with previously reported similarities between transgene and metastable epiallele epigenetic states, *MommeDs* were also found to modulate the A^{vy} locus. In particular, crossing *MommeD* heterozygotes with A^{vy}/a mice resulted in offspring with shifted coat colour distributions. In general, there was concordance between positive regulation of GFP-transgene expression and a shift in coat colour towards yellow, and vice versa. Yellow-shifting *MommeDs* include the mutants $Smchd1^{MD1}$, $Dnmt1^{MD2}$, $Setdb1^{MD13}$, and $Trim28^{MD9}$; pseudoagouti-shifting ones include $Smarca5^{MD4}$, Rlf^{MD8}, and Wiz^{MD30}. These experiments notably identified paternal effect genes, whereby wild type pups born to mutant sires exhibited changes in coat colour distribution. As a result, *Smarca5* and *Dnmt1* were the first ever reported paternal effect genes in the mouse [76]. More recently, *Setdb1* was found to exhibit similar behaviour [83].

The genes underlying *MommeDs* have diverse functions at endogenous loci extending beyond the regulation of transgene variegation. For example, previously uncharacterized *Smchd1* has been shown to regulate long-range interactions on the inactive X chromosome and at Hox genes [85,86]. $Smchd1^{MD1}$ mutants shift A^{vy} coat colour towards yellow upon maternal inheritance, but only in female offspring [73]. Interestingly, sex effects have also been observed at some VM-IAPs [31].

Complex interactions between the many genes uncovered by the *MommeD* mutagenesis screen are likely involved in the maintenance of epigenetic states at metastable epialleles. However, not all *MommeDs* have been studied with regards to their effect on A^{vy} coat colour, and perhaps due to the nature of the screen, none have been shown to affect the establishment of metastability but instead regulate its maintenance.

5.3. CTCF binding at VM-IAPs

CTCF (CCCTC binding factor) has recently emerged as a potential regulator of metastable epialleles. CTCF is a multi-functional methyl-sensitive DNA binding protein, with 41% of cell-line-specific CTCF binding sites being associated with DNA methylation at unbound loci [87–89]. The methylation-dependent sites contain CpGs at specific positions in the DNA binding site [89]. While CTCF binding at the A^{vy} and $Axin^{Fu}$ loci has not been studied, CTCF is enriched at VM-IAPs compared to their methylation invariant counterparts in multiple tissues and across different developmental time points [31]. It is plausible that a molecular antagonism between repeat element silencing via DNA methylation and the maintenance of unmethylated CTCF binding sites is contributing to the stochastic establishment of metastable epiallele methylation states [31]. An inverse relationship between VM-IAP methylation level and abundance of bound CTCF would substantiate this model. Two recent studies in humans further support an association between CTCF and epigenetic variability: one finds an enrichment for CTCF binding sites at human metastable epialleles [37] and the other implicates CTCF binding affinity in the regulation of stochastic switching between epigenetic states [39].

Despite these advances, the mechanisms underlying the establishment and maintenance of variable methylation levels at metastable epialleles remain poorly understood. Other pathways that might be involved include RNA-mediated regulation and/or a role for the recruitment of KRAB zinc finger proteins (KZFPs), which represent the largest family of transcription factors in mice and target repressive epigenetic states to retrotransposons in vertebrate genomes (reviewed in [90]). The rapid evolution displayed by KZFPs may in fact explain some of the strain-specific effects observed at metastable epialleles. The development of sequencing technologies and analytical pipelines that are optimized to include repeat elements will continue to improve our ability to address the functional and regulatory impact of these elements within and across generations.

6. Environmental modulation of metastable epialleles

6.1. Methyl supplementation in the A^{vy} mouse model

The A^{vy} mouse line has become a popular model for the study of environmentally induced epigenetic change, the most documented intervention being *in utero* methyl supplementation. Maternal dietary supplementation with methyl donors and co-factors, including folic acid, vitamin B12, choline, and anhydrous betaine, has been shown to shift A^{vy}/a offspring coat colour towards pseudoagouti [9,41,91,92] (Fig. 1C). The shift in phenotype has been attributed to an increase in methylation at the A^{vy} IAP [9]. However, having shown that the silent pseudoagouti version of the A^{vy} allele is not normally fully methylated but rather averages ~65% methylation, Cropley and colleagues compared the IAP methylation levels of methyl-exposed and unexposed pseudoagouti A^{vy}/a offspring and found no difference in methylation density at the silent IAP LTR. This suggests that the observed coat colour phenotypic change following *in utero* exposure to methyl donors is driven by an increase in methylation of the more lowly methylated allele [93]. It is possible that the increased methyl donor availability is acting indirectly via substrates other than cytosine bases at the A^{vy} allele, but this has not been tested. Consistent with this, a study in wild-derived deer mice that lack a repeat element at the *Agouti* locus showed that Agouti-controlled pelt colour is susceptible to methyl donor supplementation in the absence of a variably methylated retroelement [94].

Some of the previously discussed hallmark properties of metastable epialleles re-emerge in methyl supplementation studies. These include genetic background effects, whereby the magnitude of the A^{vy} coat colour shift is dependent on the mouse strain used for the experiment [41]. Additionally, the coat colour of offspring born to methyl-supplemented dams was found to only be altered when the A^{vy} allele was inherited paternally [92]. This is reminiscent of parent-of-origin effects observed in the absence of dietary supplementation [18]. Therefore, the fully reconstructed paternal allele may be more sensitive to modulation via methyl donor supplementation at this early embryonic stage. This does not rule out environmental sensitivity of the maternally inherited allele, since a subsequent study reported methyl supplement-induced alterations in offspring coat colour phenotypes upon maternal transmission [95].

Studies investigating environmental modulation of the epigenome often consist of exposing dams for two weeks prior to breeding followed by maintenance of the experimental regimen throughout pregnancy and lactation. While this experimental design maximizes the chances of observing an effect, it limits mechanistic inferences that would otherwise be possible by narrowing the window of exposure to a specific developmental time point. For example, the confinement of methyl supplementation to a single week during mid-gestation (corresponding to primordial germ cell migration and epigenetic reprogramming) resulted in a shift in offspring coat colour [92]. Another study on A^{vy} mice showed that feeding A^{vy}/a offspring a methyl donor diet post-weaning for a period of 29 weeks neither shifts coat colour nor IAP LTR methylation levels [96]. Together, these studies reveal that early pre-implantation embryogenesis is not the only environmentally susceptible period in development yet confirms that coat colour phenotype in A^{vy} mice and its associated epigenetic control are fixed by the age of weaning. Further experiments that fine-tune the exact period of environmental vulnerability will help identify the windows of opportunity and hence possible mechanisms contributing to changes in epigenetic state.

6.2. Innate versus induced epigenetic inheritance – a sense of semantics

Most studies on epigenetic inheritance across generations in mammals follow phenotypes or epigenetic changes triggered by ancestral exposures to environmental insults (e.g. [97–99]). Others track phenotypes in wild-type offspring caused by a mutation in a previous generation (e.g [100,101]). The volume of these studies is ever expanding. In response, it has become useful to distinguish *transgenerational* from *intergenerational* epigenetic inheritance. For true transgenerational epigenetic inheritance to take place, the induced phenotype must arise from germ cells never exposed to the original stimulus [2,3,102]. In the case of maternal exposure during pregnancy, the primordial germ cells of the developing embryo (the future F2 generation) are also exposed, so the induced change must persist at least to the F3 generation, arising from unexposed germ cells. This is not an issue for paternal exposure, so the heritable effect can be considered transgenerational if it persists to the F2 generation, and intergenerational if it does not.

This nomenclature is confusing when applied to A^{vy} mice. Many have referred to the A^{vy} mouse model as one of the best lines of evidence for transgenerational epigenetic inheritance. This is based on the pivotal finding that maternal A^{vy} phenotype, which is epigenetically controlled, influences that of the offspring [18]. As mentioned previously, grand-maternal phenotype also affects A^{vy} coat colour [18]. The extent to which this compounding effect extends beyond the F2 generation is unclear. We stress that these effects occur naturally in the population and no environmentally or genetically triggered phenotype is being tracked across generations in these experiments. If it were to be reported that the coat colour of F0 females influences that of the F3

generation, regardless of F1 and F2 coat colours, then the term *transgenerational* could be used. To our knowledge, this has not been investigated. To control for confounding F1 and F2 effects, such a study would require a large number of crosses extending down multiple generations. Therefore, it is currently unknown whether innate epigenetic inheritance at the A^{vy} locus is trans- or intergenerational.

That said, the unique non-genetic inheritance of the A^{vy} pelt patterns combined with their reported environmental susceptibilities have sparked interest in determining the heritability of environmentally induced epigenetic changes at the A^{vy} locus. *In utero* exposure to methyl donors was found to shift coat colour toward pseudoagouti in both the F1 and F2 generations without additional supplementation of F1 dams [92]. This implies that aspects of the mechanism of epigenetic change in response to exposure can persist (either directly or indirectly) throughout gamete maturation and embryo development. Whether or not the complete demethylation of A^{vy}/a embryos at the blastocyst stage occurs in this methyl-supplemented context is unknown. Given this finding, it follows that continuous methyl supplementation of F0, F1, and F2 dams might result in a cumulative phenotypic shift in offspring toward pseudoagouti. This hypothesis was tested but not substantiated [95]. However, a subsequent study showed that multi-generational methyl supplementation leads to a progressive increase in the proportion of pseudoagouti mice if the supplementation is paired with selection for the silent pseudoagouti phenotype, whereby only pseudoagouti offspring are set up for breeding to produce the next generation [103]. This cumulative effect was completely reversed after discontinuing supplementation. Despite disagreements on the merits and shortfalls of studies on this topic [104], it is clear that the effects of a maternal methyl supplementation do not extend beyond the F2 generation in the absence of continuous exposure. Hence, in the environmental (or induced) context, epigenetic inheritance at A^{vy} is intergenerational.

There is therefore a need to discriminate between innate and induced epigenetic inheritance across generations. Equally important, however, is the distinction between generational and cellular (mitotic) epigenetic inheritance to differentiate parent-to-offspring and mitotic cell-to-cell transmission, respectively, so the generational qualifier must be kept. To avoid semantic headaches, we propose reserving the use of *transgenerational* and *intergenerational* for cases where the phenotype is traced to a specific generation, and employ the more generic term *generational* otherwise. Thus, instances of generational epigenetic inheritance are innate or induced and can be further categorised as inter- or transgenerational when appropriate (Fig. 3B). Considering the direction of studies in this field, the latter distinction will most often be reserved for induced contexts. Accordingly, A^{vy} mice display innate generational epigenetic inheritance and diet-induced intergenerational epigenetic inheritance.

6.3. Additional A^{vy}-influencing environmental exposures

Other environmental insults have been found to influence coat colour in A^{vy} mice. Maternal ethanol consumption shifts offspring coat colour towards pseudoagouti, regardless of whether ethanol is administered preconceptionally or during gestation [105]. A shift in the same direction and an increase in methylation levels at the IAP LTR were observed following maternal supplementation of genistein, an isoflavone abundant in soy [106]. Intrauterine ionizing radiation has been reported to favour the silenced version of the A^{vy} allele in a dose- and sex-dependent manner, rescued by dietary anti-oxidants [107]. Maternal dietary bisphenol-A (BPA) consumption and lead exposure were independently shown to have the opposite effect on A^{vy}/a offspring coat colour distribution, shifting it towards yellow [108–110]. More recently, maternal exposure to phthalates, commonly found in plastics and cosmetics, caused altered coat colour distributions and higher IAP LTR methylation levels in A^{vy}/a offspring [111]. Finally, *in vitro* culture of zygotes to the blastocyst stage was found to significantly shift pup coat colour towards yellow and decrease IAP LTR methylation levels [112].

Research on A^{vy} environmental modulation has been controversial. A 2008 study on maternal consumption of casein and soy protein isolate, which contain genistein, showed no alteration of A^{vy}/a offspring coat colour [113]. Similarly, an extensive analysis using generalized linear mixed models on a total of 426 mouse litters and six different dietary interventions was unable to reproduce previously reported effects of BPA and genistein on A^{vy}/a offspring coat colour [114]. The same study revealed a strong parity effect, whereby changes in coat colour distribution were observed in offspring born from different parities within a single treatment group, highlighting the extreme care that must be taken in designing these experiments.

6.4. Environmental modulation of other loci

While most research programs on the environmental modulation of metastable epialleles have focused on A^{vy}, there is evidence that other loci are also susceptible. Maternal methyl supplementation causes a decrease in the incidence of kinked tails in $Axin^{Fu}/+$ offspring and an increase in DNA methylation at the $Axin^{Fu}$ locus [24]. Methylation levels at $Cabp^{IAP}$ are decreased in offspring born to BPA-exposed dams [108], and mildly increased following lead exposure [115]. As observed for A^{vy}-associated phenotypes, *in vitro* culture of $Axin^{Fu}/+$ embryos from the zygote to the blastocyst stage leads to a more severe tail kink phenotype [53].

Since the A^{vy} and $Axin^{Fu}$ loci arose from insertional mutations, commonly used laboratory mouse strains do not carry these loci. The recent identification of novel metastable epialleles in the C57BL/6J genome allows for the assessment of a repertoire of regions in the same set of environmentally perturbed mice to determine whether they respond synchronously, and to the same extent, to intrauterine environmental influences. One such study detected small tissue-specific DNA methylation differences at three variably methylated IAPs following perinatal lead exposure [110,116]. Table 1 summarises the studies conducted to date concerning the environmental modulation of metastable epialleles.

6.5. The A^{vy} mouse model: an epigenetic biosensor of environmental compromise?

The A^{vy} mouse model has been documented as a sensitive epigenetic biosensor of environmental compromise [117–119]. An ideal epigenetic biosensor is (1) particularly susceptible to a given environmental change and (2) exhibits an epigenetic response that is both predictable and easily detectable. Coat colour in A^{vy} mice appears to be acutely sensitive to slight changes in embryonic environment, likely via epigenetic influences at the A^{vy} locus. However, its innate epigenetic and phenotypic variability, established in large part by a stochastic process, is precisely what makes it a poor biological readout with little predictive value. The full range of coat colour phenotypes and associated methylation levels are observed in both control and exposed mice in these studies, requiring hundreds of mice to detect an effect and reach sufficient statistical power. Indeed, when over 2000 animals were analysed to assess the effect of *in utero* BPA and genistein exposures (both separately and together) on A^{vy} offspring coat colour, no significant shifts were observed [114]. Together, this not only makes the use of the A^{vy} mouse model a costly and inefficient biosensor of environmental perturbation, but also questions its efficacy in this context. Further studies on the more recently identified metastable epialleles in C57BL/6J mice will clarify whether some regions are better biosensors than others, or whether perhaps metastable epialleles *en masse* can potentially be used to build a multifactor epigenetic biosensor with enhanced predictive capabilities.

Table 1
Environmental modulation of metastable epialleles.

In utero exposure	Locus	Effect on offspring		Refs.
		Phenotypic shift	Methylation change	
Silencing				
Methyl donors	A^{vy}	twd. pseudoagouti	Increase	[9,41,91,92,95]
	$Axin^{Fu}$	twd. straight tail	Increase	[24]
Genistein	A^{vy}	twd. pseudoagouti	Increase	[106]
	A^{vy}	None	N/A	[114]
Casein and soy protein isolate[a]	A^{vy}	None	N/A	[113]
Ethanol	A^{vy}	twd. pseudoagouti	Increase	[105]
Ionizing radiation	A^{vy}	twd. pseudoagouti	Increase	[107]
Dibutyl phthalate	A^{vy}	twd. pseudoagouti	Increase	[111]
Activating				
BPA	A^{vy}	twd. yellow[b]	Decrease	[108]
	A^{vy}	None	N/A	[114]
	$Cabp^{IAP}$	N/A	Decrease	[108]
Lead	A^{vy}	twd. yellow	Cubic trend[c]	[110]
	$Cabp^{IAP}$	N/A	Cubic trend[c]	[110]
	IAP 110	N/A	Decrease	[116]
	IAP 236	N/A	Decrease	[116]
	IAP 506	N/A	Decrease	[116]
Embryo culture[d]	A^{vy}	twd. yellow	Decrease	[112]
	$Axin^{Fu}$	twd. kinky tail	Decrease	[53]

[a] Contains genistein.
[b] Only following high levels of exposure.
[c] Dose-dependent.
[d] Not *in utero*.

7. Functional and evolutionary relevance of metastable epialleles

The well-described relationship between IAP methylation and phenotypic outcome observed in A^{vy} and $Axin^{Fu}$ mice suggests that metastable epialleles can have a profound influence on phenotype, potentially acted upon both positively and negatively by natural selection. However, direct impact on neighbouring gene expression is not an obvious prerequisite for variable methylation at IAP elements, and in fact, inverse correlations between VM-IAP methylation and expression of nearby genes are rare [31]. This suggests that metastability *per se* is not maintained as a product of host genome hijacking of the *cis* regulatory sequences present in repeat elements. It should be noted that retroelements are capable of regulating host gene expression in a *trans* capacity in addition to their better-described *cis* regulatory potential [120]. A remarkable number of transcription factor binding sites are embedded in repeat elements; 40% of mouse CTCF binding sites are derived from transposable elements [120]. This along with the recently reported enrichment of CTCF binding at VM-IAP flanking regions suggests metastable epialleles may also play a role in orchestrating long-range regulatory networks [31]. Until these other contributions are explored, the functional relevance and evolutionary consequences of metastable epialleles remain open questions.

The metastable epialleles $Axin^{Fu}$, A^{vy} and $Cabp^{IAP}$ are associated with evolutionarily young IAPs of the IAPLTR1_Mm subclass [29,121]. VM-IAPs are similarly enriched for young IAP elements and show high levels of absence/presence polymorphism across mouse strains [31]. It is possible that inter-individual methylation variation reflects a transient epigenetic state associated with recent retrotransposition before reaching full repression. Under this premise, metastability might represent a snapshot in evolutionary time and a biologically inconsequential phenomenon. In the cases where the variably methylated transposable element positively affects host genome function, selective pressures would stabilize the epigenetic state of the element accordingly, likely in a tissue-specific manner [122].

Alternatively, locus-specific epigenetic variability itself may confer an evolutionary advantage. It has been proposed through mathematical modelling that stochastic methylation at repeat elements could allow for more rapid fixation of the element and its associated genes, as well as increase the probability of fixation in the first place [123]. Other evolutionary models have also been proposed, whereby the phenotypic plasticity conferred by metastable epialleles enables rapid adaptation to sudden environmental changes [124]. Developmental epigenetic reprogramming of these loci is consistent with this theory, allowing the re-establishment of epigenetic marks according to new environmental cues and rendering the developing embryo responsive to the environment into which it will be born. The predictable reconstruction of precise inter-individual methylation ranges observed at metastable epialleles may be a product of the controlled environments experimental mice are housed in, deliberately kept free of environmental fluctuations. The jury is still out on whether metastability is symptomatic of incomplete silencing or whether selective pressures are maintaining epigenetic stochasticity at specific regions in the genome. Further research on the now-expanded repertoire of known metastable epialleles will allow questions like these to be addressed more comprehensively.

8. Conclusion

The characterization of novel metastable epialleles in mammals has provided additional insight into their biology. It has become clear that inter-individual methylation variation at a repetitive element is not always accompanied by epigenetic inheritance or transcriptional regulation of neighbouring genes. In fact, the A^{vy} and $Axin^{Fu}$ loci appear rare in this regard. The predictable reconstruction of epigenetic variability across generations is what truly sets metastable epialleles apart from other genomic loci. Therein may lie their value, as models of biological stochasticity in the absence of genetic variation. The relatively subtle effects observed so far with respect to their environmental modulation argues against their use as biosensors of environmental change, but additional studies on other loci will help resolve this.

The advent of more sophisticated mathematical modelling approaches and the optimization of high-throughput sequencing for repeat genome analyses have been and will continue to be key in unravelling the prevalence, molecular drivers, and functional consequences of epigenetic metastability. While this review has largely focused on IAP-associated murine metastable epialleles, we anticipate research in the coming years will determine the extent to which other regions of the mouse genome are associated with variable methylation, including unique non-repetitive loci and repeat elements of other subclasses. In addition, comparative and interdisciplinary research in humans and across model organisms will enrich our understanding of the functional and evolutionary implications and mechanistic conservation of inter-individual epigenetic variation.

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