Epigenetics Research in Evolutionary Biology: Perspectives on Timescales and Mechanisms

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Abstract

Epigenetics research in evolutionary biology encompasses a variety of research areas, from regulation of gene expression to inheritance of environmentally mediated phenotypes. Such divergent research foci can occasionally render the umbrella term "epigenetics" ambiguous. Here I discuss several areas of contemporary epigenetics research in the context of evolutionary biology, aiming to provide balanced views across timescales and molecular mechanisms. The importance of epigenetics in development is now being assessed in many nonmodel species. These studies not only confirm the importance of epigenetic marks in developmental processes, but also highlight the significant diversity in epigenetic regulatory mechanisms across taxa. Further, these comparative epigenomic studies have begun to show promise toward enhancing our understanding of how regulatory programs evolve. A key property of epigenetic marks is that they can be inherited along mitotic cell lineages, and epigenetic differences that occur during early development can have lasting consequences on the organismal phenotypes. Thus, epigenetic marks may play roles in short-term (within an organism's lifetime or to the next generation) adaptation and phenotypic plasticity. However, the extent to which observed epigenetic variation occurs independently of genetic influences remains uncertain, due to the widespread impact of genetics on epigenetic variation and the limited availability of comprehensive (epi)genomic resources from most species. While epigenetic marks can be inherited independently of genetic sequences in some species, there is little evidence that such "transgenerational inheritance" is a general phenomenon. Rather, molecular mechanisms of epigenetic inheritance are highly variable between species.

Key words: **epigenetics, evolution, adaptation, DNA methylation, histone modification, phenotypic plasticity.**

Perspective

This perspective is part of a series of articles celebrating 40 years since Molecular Biology and Evolution was founded. It is accompanied by virtual issues on this topic published by Genome Biology and Evolution and Molecular Biology and Evolution, which can be found at our 40th anniversary website.

Introduction

There has been much interest in exploring epigenetics in the study of evolutionary biology, as evidenced by numerous articles in the pages of *Molecular Biology and Evolution* and *Genome Biology and Evolution*. I became interested in epigenetics and its impacts on genome evolution nearly two decades ago, when researchers began discovering DNA methylation in many taxa where it was previously thought to be absent (e.g. [Wang, et al. 2006; Grbic, et al. 2011; Gao, et al. 2012]). Since then, a notable "paradigm shift" has occurred contrasting with the earlier view that DNA methylation had limited impact across taxa, bringing considerable recognition to the widespread presence of DNA methylation throughout the tree of life.

The current consensus is that DNA methylation of gene bodies ("gene body DNA methylation") was ancestrally present and that it has undergone lineage-specific changes, including losses in lineages containing the iconic laboratory model species *Drosophila melanogaster* and *Caenorhabditis elegans* (e.g. [Werren, et al. 2010; Yi 2012; Zhong 2016; de Mendoza, et al. 2020]). A notable event in animal evolution was the emergence of genome-wide DNA methylation (not just gene body DNA methylation) in the early stages of vertebrate evolution (Tweedie, et al. 1997; Suzuki and Bird 2008; Keller, et al. 2015; Angeloni, et al. 2024), although there are still some human genes (and likely other vertebrate genes) that are devoid of DNA methylation altogether (Mendizabal, et al. 2017). Remarkably, it is becoming increasingly clear

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that genomic patterns of DNA methylation can change rapidly, even between closely related species (Bewick, et al. 2016, 2017; Schmitz, et al. 2019; Sarkies 2022; Sadler 2023).

Conservation and divergence of histone modifications during evolution are less well understood than those of DNA methylation. Nevertheless, it is well established that several components of histone modifications and nucleosomes predated the emergence of eukaryotes (Sandman and Reeve 2006; Ammar, et al. 2012; Erives 2017; Talbert, et al. 2019; Grau-Bové, et al. 2022). On the other hand, histone modifications of regulatory regions are shown to evolve rapidly between species, especially those associated with intergenic regulatory elements such as enhancers (Villar, et al. 2015; Garcia-Pérez, et al. 2021).

Our understanding of the epigenome evolution has significantly improved over the past two decades since I first entered this field. We now know that the epigenomes are phylogenetically widespread and that epigenetic modifications can evolve rapidly. These recent paradigm shifts have introduced even more intriguing and unresolved questions about the associated consequences of epigenomic evolution on the genome. The importance of epigenetic changes as powerful mediators of phenotypes during evolution is becoming increasingly recognized, and comparative epigenomic studies are providing promising tools to understand genome regulation and annotation. The study of epigenetically mediated phenotypes has the potential to advance our understanding of molecular mechanisms of adaptation. Here I discuss these contemporary areas of research while pointing out important considerations that can help resolve several misunderstandings regarding the term epigenetics. Most of my experience with epigenetics comes from studies of DNA methylation, which is arguably the most phylogenetically broadly studied epigenetic mark. Consequently, many examples are from DNA methylation, although I have included some examples of other epigenetic marks.

The Focus of Epigenetic Studies is Highly Divergent in Different Contexts

The term "*epigenetics*" is used broadly, and sometimes ambiguously, across divergent realms of scientific literature. At the molecular level, epigenetics is the study of "epigenetic marks" that chemically modify biological molecules including genomic DNA, RNA, and proteins. Widely studied epigenetic marks include methylation of DNA (DNA methylation) and chemical modifications of histone subunits of nucleosomes. Small RNA molecules that influence genome integrity, such as piRNAs, are also considered epigenetic marks (Zhang, et al. 2020). Together, these marks affect how different sections of the genome are packaged, altering the three-dimensional structures and molecular accessibility of the genomic regions (e.g. [Cavalli and Misteli 2013; Allis and Jenuwein 2016]) within each and every nucleus of cells within an organism.

There is an especially prominent divide in the use of the term epigenetics between molecular genetic literature and ecological literature (Deans and Maggert 2015). Broadly speaking, the former focuses on molecular mechanisms of expression changes, while the latter tends to concern environmentally mediated phenotypes (Deans and Maggert 2015). These two areas also deal with divergent timescales, the former within an organism's lifetime, and the latter within longer timescales that can span generations. In addition, due to the limited genomic and molecular resources of nonmodel species, the latter often uses organismal phenotypes as readouts.

From my personal experience, some evolutionary biologists who are not necessarily involved in epigenetics research tend to be more familiar with the term "epigenetics" as it is commonly used in ecological literature (associated with environmentally mediated phenotypes). In reality, many evolutionary epigenetic studies focus on the molecular mechanisms of expression changes and genome regulation between species, and thus use the term "epigenetics" in the manner more typical for molecular genetic literature. The misunderstandings and complexities that arise due to the ambiguity of the term epigenetics have been discussed elsewhere (e.g. [Deans and Maggert 2015; Richards, et al. 2017]). Scientists may be thinking about entirely different phenomena if the term epigenetics is used without clarifying details. In principle, it may be possible to bridge these divergent scales through molecular mechanisms that explain how epigenetic marks influence cellular phenotypes (referred to as "epigenetic mechanisms" in this article) and how these marks are inherited across cell generations. However, it is becoming clear that epigenetic mechanisms may be highly variable between different taxa, and sometimes even within the same species depending on the genetic background and/or molecular methods used to study them, as I will demonstrate below. Consequently, extrapolating the impacts of epigenetics across different timescales and between taxa can be misleading.

Epigenetic Marks are key Regulators of Development, but the Specific Outcomes are Dependent on Genetic Background

Epigenetic marks are known for their crucial roles in developmental processes. Importantly, DNA methylation marks, and some histone modifications, are transmitted through mitotic cell divisions (Probst, et al. 2009; Alabert, et al. 2017; Brickner 2023). Consequently, these epigenetic marks are largely conserved in descendant cell populations, or "cell lineages," although spontaneous mutations of epigenetic marks, referred to as "epimutations," are also known to arise (see [Bogan and Yi 2024] for discussion on this topic). As a result, epigenetic marks laid during early development will have long-lasting functional consequences during the lifetime of an organism via impacts on long-lived cell lineages and their descendant cell populations. This concept is well displayed in the so-called "epigenetic landscape" figure by Waddington (Waddington 1957), where the "epigenetic state" of different cell populations and their descendants is depicted as a ball following through different downward paths, analogous to different cell lineage fates during organismal development (See Fig. 1 for the author's modification of the epigenetic landscape). More broadly, variation in epigenetic marks

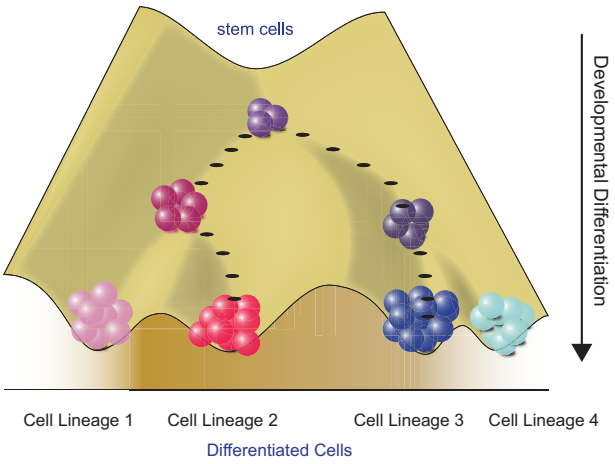

Fig. 1. A modified version of the "epigenetic landscape" in Waddington (1957). Stem cell populations (near the top of the landscape) experiencing different epigenetic modifications follow different "paths" toward more differentiated states, resulting in populations of cells that harbor distinct epigenetic marks from other cell populations. Each population of differentiated cells is specialized for distinct cellular niches. Figure is modified from Yi (2017).

during any point in development can influence many subsequent cell generations.

The critical importance of epigenetic mechanisms in development is supported by a myriad of studies, yet this research has also revealed substantial differences between taxa. I will demonstrate this point using studies that examined the consequences of modulating key DNA methylation enzymes, named "DNA methyltransferases (*dnmt*s)" (Goll and Bestor 2005; Lyko 2018). These are one of the most conserved proteins in the tree of life. Starting with mammalian studies, mutations of *dnmt*s result in lethality in mice embryos (Li, et al. 1992; Okano, et al. 1999). Mutations in human and mouse *dnmt*s in somatic cells are also generally lethal (e.g. [Trowbridge, et al. 2009]). Deletion of *dnmt1* in human embryonic stem cells results in cell death (Liao, et al. 2015). Mutations in other epigenetic regulators cause developmental disorders and cancers, and phenotypic alterations of multiple organs (Feinberg 2007).

Similar studies in nonmodel animal species are much rarer compared to those in mammals. While they generally support the importance of epigenetic marks in development, the degree and severity of the consequences of *dnmt* manipulation vary considerably across different studies, depending on the methods used and the developmental stages when the manipulations were performed. For example, knockdown of a *dnmt3* homolog in newly hatched honey bee larvae changed the course of development between queen- and worker-like phenotypes (Kucharski, et al. 2008). Knockdown of a *dnmt1* homolog using parental RNAi in a parasitic wasp *Nasonia vitripennis* resulted in developmental failure and embryonic lethality (Arsala, et al. 2022). In the milkweed bug *Oncopeltus fasciatus*, knockdown of a *dnmt1* homolog in embryos negatively affected testis development and resulted in the reduction of genome-wide DNA methylation (Bewick, et al. 2019; Washington, et al. 2021). In the clonal raider ant *Ooceraea biroi*, mutating *dnmt1* homolog using CRISPR/Cas9 in eggs resulted in sterile individuals with a significantly reduced lifespan (Ivasyk, et al. 2023). Knockout of *dnmt3a* isoform using CRISPR/Cas9 in zebrafish embryos resulted in organismal deaths in a temperature-dependent manner (Loughland, et al. 2021). These results support that epigenetic marks are important regulators of development and cell lineage differentiation in the studied animals. However, apart from changes of genome-wide DNA methylation, molecular consequences of *dnmt* changes on gene expression are not consistent across animal studies, and some authors suggest DNA methylation may play a role in reproduction via (an) unrelated mechanism(s) to gene expression (e.g. [Amukamara, et al. 2020; Washington, et al. 2021; Ivasyk, et al. 2023]). Therefore, DNMT1 orthologs may have important regulatory functions aside from DNA methylation. One confounding factor in these studies may be cell heterogeneity. As we learn that epigenetic marks in different cell types are highly distinctive (Kundaje, et al. 2015; Cusanovich, et al. 2018; Mendizabal, et al. 2019), assessing epigenetic changes and their consequences in samples consisting of heterogeneous cell types could "mask" the true causal effects due to the lack of necessary resolution.

Studies in plants reveal yet another degree of divergence. In rice and maize, loss-of-function of single DNA methyltransferase results in lethality (Hu, et al. 2014; Fu, et al. 2018). On the other hand, in *Arabidopsis thaliana*, loss-of-function mutants of one or more DNA methyltransferases, including those orthologous to animal *dnmt1*, exhibit relatively minor phenotypic impacts (Kankel, et al. 2003; Stroud, et al. 2014), although more severe effects were also observed (Mathieu, et al. 2007). Even *A. thaliana* individuals with four out of the five DNA methyltransferase deleted were viable and exhibited weak phenotypic differences such as curled leaves and reduced rosette size (Henderson and Jacobsen 2008; Stroud, et al. 2014). Two recent studies reported mutants where all five currently known DNA methyltransferases were deleted from *A. thaliana* (He, et al. 2022; Liang, et al. 2022). In one study, the quintuple mutants were still viable, although they exhibited serious developmental delays, including small size and the inability to undergo floral transition (He, et al. 2022). However, in another

study, the resulting quintuples were nonviable (Liang, et al. 2022). These two studies utilized different genetic backgrounds to generate the quintuples. These examples highlight that the consequences of experimental manipulations of key epigenetic enzymes are highly divergent in different taxa, and even in the same species depending on specific genetic backgrounds and molecular methods used, a theme that is repeatedly observed in other areas of research as I discuss below.

Promises and Challenges of Comparative Epigenetic Studies

Given its importance in development and cell lineage differentiation, and the rapid evolutionary changes of epigenetic marks across the tree of life, comparative studies of epigenetic marks may advance our understanding of the evolution of development, phenotypic divergence, and genome evolution. More broadly, epigenetic mechanisms make attractive candidate causative agents of regulatory evolution, a prime topic of interest for many molecular evolutionists.

Consequently, there is a growing interest in the comparative analysis of epigenetic marks. An important concern in designing comparative epigenomic studies is to select specific tissues/cell types, since epigenetic marks are differentiated between tissues and cell types (Villar, et al. 2015; Jeong, et al. 2021; Singh, et al. 2021; Caglayan, et al. 2023; Klughammer, et al. 2023). Most of the current literature is on the evolution of DNA methylation. While some earlier studies used microarrays, bisulfite sequencing is widely used to generate species-specific DNA methylation maps (e.g. [Molaro, et al. 2011; Zeng, et al. 2012; Glastad, et al. 2014; Mendizabal, et al. 2016; Jeong, et al. 2021; Al Adhami, et al. 2022; Hu, et al. 2023; Chen, et al. 2024]). In addition, it is important to consider bisulfite conversion rates, and the presence of C to T SNPs that affect mapping. In the best possible practice, bisulfite conversion rates should be independently estimated in different experiments and C to T SNPs should be masked based on genome sequence data (e.g. [Jeong et al 2021, Wu et al. 2000]).

While it is relatively straightforward to identify DNA methylation differences between species, deciphering whether and how the observed differences contribute to functional differences is challenging, given our incomplete understanding of molecular mechanisms of epigenetics. In terms of DNA methylation literature, one of the most widely applied ideas is the cis-regulatory effect of DNA methylation in silencing gene expression (Schübeler 2015). However, this relationship is applicable to only a subset of promoters, most likely those that use CpG rich sequences, or CpG islands. For other promoters, there is often no relationship or even negative relationship between promoter methylation and gene expression (e.g. [Morgan et al. 2024]). The relationship between DNA methylation and expression in other genomic contexts is even less clear. For example, the role of gene body methylation is still highly debated (e.g. [Jones 2012; Huh, et al. 2013; Hunt et al. 2013; Takuno and Gaut 2013; Bewick and Schmitz 2017; Zilberman 2017; Seymour and Gaut 2020; Wu, et al. 2022]). Candidate gene studies focusing on specific genes of interest show some success in identifying functional epigenetic differences. For example, a study of ADRA2C in primate brains identified previously uncharacterized cis-regulatory regions that could drive gene expression in reporter assays (Lee, et al. 2018). This region harbored divergent epigenetic signatures between primate brains. Specifically, hypermethylation of the newly identified cis-regulatory region in the human and chimpanzee brains coincided with the increase of expression, associated with fight-or-flight response (Lee, et al. 2018). In another study, human-specific hypomethylation of the 5′ UTR region of a CENPJ, a gene associated with brain size increase, was discovered in a comparative study of several human and nonhuman primates (Shi, et al. 2014).

Notwithstanding the difficulties associated with inferring functional mechanisms, comparative epigenetic data themselves can be useful to identify functional regions, especially from noncoding regions of the genome. For example, analysis of DNA methylation across multiple tissues in primates demonstrated that differentially methylated regions (DMRs) between tissues tended to be conserved between primate species, and genes near those evolutionary DMRs tended to encode tissue-relevant functional annotations (Blake, et al. 2020). A recent study of muscle and liver tissues across 13 mammals reported that methylation changes of promoter regions coincided with genes that encode species-specific biological traits (Hu, et al. 2023). Furthermore, comparative epigenetic studies can even provide insights into molecular mechanisms of epigenetic marks. For example, DNA methylation was thought to play important roles in the regulation of X chromosome inactivation by methylating the inactive X chromosome. Comparative studies reveal that the methylation of the inactive X chromosomes is not conserved in mammals. Instead, the loss of DNA methylation of the inactive X chromosomes is widely observed (Weber, et al. 2005; Hellman and Chess 2007; Singh, et al. 2021; Morgan, et al. 2024). Therefore, comparative epigenetic studies hold promise to identify potential regulatory regions, and to provide useful information in understanding the mechanisms of how epigenetic marks influence genome regulation. While these efforts have mostly focused on vertebrates, there is potential to use epigenetic information to identify regulatory positions in invertebrate genomes (e.g. [Jeong et al. 2018]). It should be noted that epigenomic differences observed between species by no means imply that they are independent of genetic differences. Rather, some, if not the majority of the epigenetic differences found between species, may have genetic origins, as there is strong evidence that sequence contexts are important drivers of epigenome patterns, at least for DNA methylation (e.g. [Lienert, et al. 2011; Krebs, et al. 2014; Long, et al. 2016]).

Potential Link between Epigenetics and Phenotypic Plasticity Needs to Consider Genetic Effects and Divergent Molecular Mechanisms

Since epigenetic changes at one point in a lifetime of an organism can affect the target cell populations and its

descendant cells, epigenetic variation can theoretically produce phenotypic impacts at various timescales within an organism's lifespan. For example, epigenetic variation at an early developmental time point could lead to different phenotypic trajectories of adults. One such possibility was raised regarding the polyphenism in honey bees. When a DNA methylation enzyme *dnmt3* was knocked out using RNAi, larval honey bees preferentially developed into queen-like, rather than worker-like, phenotypes (Kucharski, et al. 2008). These results supported the idea that variation of DNA methylation leads to different phenotypes of bees. Another classic example is the "*Agouti viable yellow* (*Avy*)" mouse, where genetically identical mouse individuals exhibit different coat colors. In this system, the coat color difference was caused by variable DNA methylation of a transposable element ~100 kb upstream of the *Agouti* coat color locus (Duhl, et al. 1994; Morgan, et al. 1999). Additionally, the coat color variation was influenced by maternal dietary supplementation of methyl donors and co-factors (Wolff, et al. 1998; Cropley, et al. 2006). These studies emboldened the *Avy* mouse system as an example of an epigenetic phenotypic trait that could be mediated by environmental effects, in this case diet (Bertozzi and Ferguson-Smith 2020). There are numerous studies that have explored epigenetic variation associated with phenotypic plasticity in other contexts (e.g. [Roberts and Gavery 2012; Zhang, et al. 2013; Duncan, et al. 2014; Richards, et al. 2017; Springer and Schmitz 2017; Loughland, et al. 2021]). There are abundant examples where parental experience is inherited to their immediate offsprings, likely in the form of epigenetic marks, and that such epigenetic inheritance may confer selective advantages (Heard and Martienssen 2014; Rechavi and Lev 2017; Fitz-James and Cavalli 2022).

Some authors thus hypothesized that environmentally induced epigenetic variation, independent of genetic variation, could produce adaptive phenotypes (Bossdorf, et al. 2008; Jablonka and Raz 2009). However, there are several challenges to connecting the environment to epigenetic variation, and subsequently epigenetic variation to adaptation. These include the confounding effects of genetics, methodological difficulties, and uncertain molecular mechanisms. A key issue that is widespread in literature is an inadequate consideration of genetic effects. It is becoming clear that epigenetic variation is tightly linked to genetic variation in a variety of taxa (e.g. [Wang, et al. 2016; Petronis 2010; Eitchen et al 2013; Schmitz, et al. 2013; Seymour and Becker 2017; Villicaña and Bell 2021; Hämälä, et al. 2022; Sepers, et al. 2023]). Therefore, any study of epigenetic variation between individuals or populations should take into account the underlying genetic variation as a source of epigenetic variation. Unfortunately, this is extremely difficult to do, because genetic drivers of epigenetic variation are pervasive in the genome, and many species lack detailed genetic data to account for the genetic effects. For example, DNA methylation studies of human populations, where large cohorts and abundant molecular resources are available, observe that there are numerous methylation quantitative trait loci (meQTLs) that affect DNA methylation. These meQTLs can function both in *cis-* and *trans-*, and are found across the whole genome (Villicaña and Bell 2021). Incorporating meQTLs into epigenomic studies reveal that it is notoriously difficult to separate the effect of underlying genetics in the study of epigenetic variation (Do, et al. 2017; Gao, et al. 2017). For example, in a study of genome-wide epigenetic variation in human immune cells including neutrophils, monocytes and T-cells, more than 50% of epigenetic variation was attributed to genetic effects (Chen, et al. 2016). The well-known example of the *Avy* mouse mentioned above also shows genetic background effects, exhibiting less prominent effects in different maternal backgrounds (Wolff 1978). Therefore, we still lack a thorough understanding of the amount of truly "epigenetic" variation that exists independently of genetic variation in nature.

Even if genetic effects could be successfully accounted for, it is challenging to connect an observed epigenetic variant to its adaptation to the environment. At minimum, we should observe reproducible epigenetic patterns independent of genetic variation and the specific epigenetic phenotype of interest having higher fitness than others, both of which have proven difficult to address in many instances. Another difficulty is identifying the molecular loci whose epigenetic difference causes functional differences. For example, in the example of the *Avy* mouse, dietary supplementation (environmental effect) was shown to influence the color phenotype (Cooney, et al. 2002; Waterland and Jirtle 2003; Cropley, et al. 2006). This was taken as a support for the hypothesis that dietary supplementation of methyl donors led to an increase of DNA methylation of the 'causative' transposable element. However, no methylation difference was observed in the said candidate transposable element in mice with or without dietary supplementation (Cropley, et al. 2010). In the case of the aforementioned honey bee example, it was pointed out that the RNAi was performed after the phenotypic fates of larvae were already determined (Duncan, et al. 2022), indicating that the manipulation of DNA methylation was not the only effect measured by the phenotypes.

Therefore, even though there are examples of environmentally induced phenotypes associated with epigenetic differences, whether they are truly independent of genetic effects has not been exhaustively evaluated in many studies, in large part due to lack of genetic resources to enable such analysis in nonmodel species. When genetic resources are available (as in the example of mice), in-depth studies revealed that phenotypic effects were sometimes inconsistent in different genetic backgrounds, and the molecular mechanisms of environmental effects were not necessarily replicated. Furthermore, long-term adaptive consequences of such changes and the underlying molecular mechanisms of such processes remain elusive. Interested readers in this topic should refer to extensive reviews in literature (e.g. [Venney, et al. 2023; Bogan and Yi 2024]). Nevertheless, ecological epigenetic studies have a great potential to reveal

causative mechanisms of gene expression changes and genome regulation (Richards, et al. 2017).

Epigenetic Inheritance is not Universal and its Adaptive Significance is Unresolved

In some species, epigenetic marks can be inherited independently of genetic sequences. Does this mean that epigenetic marks themselves can be considered an additional component of inheritance? If so, can they provide the raw materials of adaptive evolution? These questions have sparked substantial interest in recent years. Researchers in these areas often focus on environmentally induced epigenetic variation. I will discuss a few key questions in this regard.

First, is there sufficient epigenetic variation in nature? Most certainly (e.g. [Carja, et al. 2017; Noshay and Springer 2021]). Variation of epigenetic marks, or epialleles (defined as alleles harboring different epigenetic marks), can arise via several mechanisms, some due to genetic variation, some independent of genetics (see [Bogan and Yi 2024] for more details). For example, in many species, epigenetic variation is known to arise during the normal process of aging, even for young, reproductive individuals (e.g. [Pal and Tyler 2016; Lu et al. 2023; Brink et al. 2024]). Stochastic mutations of epigenetic marks, or "epimutations," also occur. The most well characterized epimutations have been from the studies of *A. thaliana* and *C. elegans*. In *A. thaliana*, DNA methylation studies of carefully curated recombinant inbred lines revealed extensive epimutations (Becker, et al. 2011; Schmitz, et al. 2013) and much higher rates of epimutations than those of point mutations (van der Graaf, et al. 2015). In *C. elegans*, small RNAs in mutation accumulation lines indicated stochastic variations of small RNA abundance, at much higher rates than point mutations (Beltran, et al. 2020). It is worth noting that data from other taxa is currently lacking so we should refrain from extending these observations to broader contexts. In addition, these two well-known examples originate from two highly divergent molecular pathways, once again cautioning against generalizing these observations in a broad category of "epimutations." Second, do environmental changes cause epimutations? There are conflicting reports in the literature. Some studies report an increase of epimutations under environmental stress (e.g. [Jiang, et al. 2014]), while others report epigenomes that are largely stable (e.g. [Hämälä, et al. 2022]).

Third, can the epialleles be transmitted through generations? Or, do epigenetic marks experience the so-called "transgenerational inheritance"? This is a question that many researchers are interested in. It should be noted that many studies propose transgenerational inheritance based on phenotypic observations, but the molecular demonstration of the transgenerational inheritance of epigenetic marks is limited, and current evidence is mostly from *A. thaliana* and *C. elegans*. In *A. thaliana*, epialleles were shown to be transmitted through several generations (Johannes, et al. 2009). The transmission of epialleles to germline may be facilitated by their reproductive biology where the germline cells arise from somatic cells. In *C. elegans*, transgenerational inheritance of small RNAs across multiple generations has been observed (Ashe, et al. 2012; Rechavi, et al. 2014). The inheritance of small RNAs in *C. elegans* is orchestrated by movement of small RNAs between cells including oocytes, as well as RNA-amplification mechanisms (Rechavi and Lev 2017).

In mammals, the presence and/or mechanisms of transgenerational inheritance are still inconclusive. It is important to discuss further details of mammalian studies. In the example of the *Avy* mouse, supplementing mother's diet with methyl donors influenced offspring coat colors. However, this effect was not transmitted for more than two generations, therefore disputing the existence of a "transgenerational" effect (Daxinger and Whitelaw 2012). Rather, this could be viewed as an example of an "intergenerational" effect where epigenetic variation in parents impacts offspring development, of which there are a number of examples (Heard and Martienssen 2014). In another widely cited example, exposing pregnant female rats to a fungicide vinclozolin impacted male reproductive traits in progenies including F3 and F4 generations (Anway, et al. 2005). However, this observation was not replicated in other genetic backgrounds (Schneider, et al. 2008, 2013; Inawaka, et al. 2009). It was proposed that using inbred lines may have obscured epigenetic inheritance (Hanson and Skinner 2016), but the underlying logic is unclear. It is possible that the outbred lines harbored genetic polymorphism that enabled the observed phenotypes. To identify the actual epigenetic marks that underlie the observed transgenerational inheritance, sperm methylomes of these mice were compared, identifying many DMRs between the control and the vinclozolin-treated lines. However, there was no DMR that was consistently found in the F1 and F3 generations, ruling out a consistent differential methylation of specific loci as an underlying mechanism (Beck, et al. 2017).

A recent study took a different approach and started with the molecular marks by generating DNA methylation-edited mice (Takahashi, et al. 2023). The authors induced DNA methylation of a specific CpG island near the *Ankryn repeat domain 26* (*Ankrd26*) locus in mice (Takahashi et al. 2023) by inserting a CpG-free insert near that region of embryonic stem cells. Note that this experimental scheme relied on the cis-effect of genomic sequence influencing DNA methylation. The authors then used sophisticated gene editing tools to excise out the CpG-free insert, theoretically reverting the cells to the previous genetic background while maintaining the gain of DNA methylation of the target CpG island. They then injected the resulting cells to 8-cell embryos of another strain of mouse, and demonstrated that DNA methylation of this CpG island was maintained for three generations in some, but not in all, cells. While this approach is exciting in that it directly followed the transmission of an epigenetic mark, it is still unclear if the induced cells were entirely of the same genetic background as those prior to the induction of DNA methylation. Indeed, DNA methylation of the induced cells were altered not just at the target CpG island, but over two dozen of other genomic regions, including some where DNA methylation was changed by 91% (Takahashi et al. 2023). It is possible that the gene editing tools they used modified other genomic regions

that influence DNA methylation. Even though the authors confirmed the absence of point mutations within 1 million base pairs of the target site, *trans*-meQTLs are known to influence DNA methylation of distant genomic regions (Villicaña and Bell 2021). In fact, the observed transgenerational inheritance was specific to the induction-excision method they used, and was not observed when a different method, dCas9-DNA methyltransferase system, was used to modify DNA methylation (Takahashi, et al. 2023).

In summary, although many studies strive to investigate the presence and/or mechanisms of transgenerational inheritance in mammals, the exact molecular changes prove elusive. So far, well-known examples have been somewhat restrictive to either the specific genetic background or the molecular method used. As a result, we must be wary of generalizing these observations. Furthermore, if these patterns manifest so temperamentally in well-controlled laboratory conditions, it is hard to expect stable long-term epigenetic inheritance in nature.

Fourth, do epigenetic variations confer adaptive potential? This question is difficult to address in nonmodel species due to the tight association between genetic and epigenetic variation, and the relative lack of (epi)genomic resources. Studies using *A. thaliana* reported potential adaptive changes of DNA methylation and transgenerational inheritance (e.g. [Bossdorf, et al. 2010; Latzel, et al. 2013]) but recent studies utilizing whole genome methylomes report more nuanced or negative results (e.g. [Ganguly, et al. 2017; Van Dooren, et al. 2020]). Here it is useful to consider evolutionary consequences if epigenetic variations were indeed functional (which would be implied if they were to be adaptive). Studies of genetic mutations have demonstrated that the majority of functional (i.e. nonneutral) mutations are deleterious, rather than advantageous. Given that rates of epigenetic mutations are much higher than those of genetic mutations, the majority of epigenetic variation in nature is likely to be neutral with little functional consequences, simply because of the high genetic load of frequent functional mutations (Charlesworth, et al. 2017). Population genetic analyses of epigenetic variation may be critically informative to resolve the selective potential of epigenetic variation. Such studies so far have reported extremely weak selective effects of DNA methylation variation in *A. thaliana* (Vidalis, et al. 2016; Muyle, et al. 2021). These observations suggest that much like genetic mutations, a large portion of epigenetic variation may be neutral with occasional rare advantageous epigenetic mutations occurring in specific genetic background and conditions, useful in short-term adaptation.

Concluding Remarks

I discussed several areas of epigenetics research in the context of evolutionary biology. The topics I discussed in this article highlight several key considerations: first, we must strive to adequately consider genetics when analyzing epigenetic variation. This is not only useful to infer the extent of "true" epigenetic variation but also to improve our understanding of genome regulation and adaptation via epigenetic mechanisms. Second, we cannot generalize findings in one experimental system to broader contexts, as there is significant variation within species, not to mention between species. I discussed that even DNA methylation, which is a highly conserved and extensively studied epigenetic mechanism of development, can have different functional consequences in different taxa or even within the same species. The prevalence and the molecular mechanisms of transgenerational inheritance are highly divergent between different species, due to differences in cellular and reproductive biology. Third, a significant portion of the literature relies on phenotypic readouts to infer underlying epigenetic inheritance, but when the detailed molecular mechanisms of epigenetic marks are investigated, they are often much more complex and obscure. This is partly due to the difficulty of inferring the molecular basis of quantitative phenotypic traits, but could also indicate strong genome-epigenome interactions that depend on genetic background and specific conditions.

Needless to say, this article is not meant to be a comprehensive discussion of epigenetics in evolutionary biology and there are many other exciting areas of research. One such area is the study of genome-epigenome interactions. Epigenetic marks such as DNA methylation and histone modification can directly influence genome sequence evolution (e.g. [Makova and Hardison 2015; Yi and Goodisman 2021]). Conversely, genome sequences can affect epigenome configuration. This area of research has a great potential to enhance our understanding of the tempo and dynamics of genome evolution and help functional annotation of genome sequences, especially of noncoding regions. Transposable elements are key components of epigenetic inheritance, and we cannot understand the evolution of epigenomes without understanding the evolution of transposable elements. Transposable elements also influence mutation rates and gene expression of the genomic neighborhoods where they reside (e.g. [Hollister and Gaut 2009; Choi and Purugganan 2018; Choi and Lee 2020]). Given the abundance of epigenetic variation in nature, opportunities may exist for genome-epigenome interactions involving transposable elements, leading to meaningful variation of phenotypes for adaptation.

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Data Availability

There is no new data associated with this article.

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