Chapter 4

Non-genetic Transgenerational Inheritance of Acquired Traits in *Drosophila*

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Abstract

It is increasingly recognized that acquired traits may be transgenerationally transmitted through non-DNA sequence-based elements, with epigenetics as perhaps the most important mechanism. Here we review examples of non-genetic transgenerational inheritance in *Drosophila*, highlighting transgenerational programming of metabolic status and longevity, one particular histone modification as an evolutionarily conserved underlying mechanism, and important implications of such studies in understanding health and diseases.

Keywords: aging, *Drosophila*, H3K27me3, metabolic state, PRC2, transgenerational epigenetic inheritance

1. Introduction

Epigenetics is the science of non-DNA sequence-based modifications of gene expression and, subsequently, phenotypic variability at both the genomic and organismal levels [1]. Studies over the past several decades have distinguished DNA methylation, histone modification, and non-coding RNA-based processes as the key mechanisms underlying epigenetic regulation. Epigenetic inheritance has been observed across species, including prokaryotes, plants, and animals [2–8], with an epigenetic trait defined as "a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" [9]. Interestingly, certain epigenetically-regulated phenotypes can propagate across multiple generations, leading to the concept of transgenerational epigenetic inheritance (TEI) [4–8]. This emerging concept has triggered numerous debates and revived old controversies in the scientific community as to whether acquired traits may be transmitted across generations. Nonetheless, it has profoundly reshaped our understanding of biology, particularly human diseases, as

Year	Intervention/treatment (F0 only)	Phenotypic/genomic response	Generation with effect	Authors
2007	Tumor suppressor gene mutation	Tumor risk	F2 but not F3	Xing et al.
2009	Chronic pentylenetetrazole treatment of adult males	Transcriptomic profile in CNS	F2	Sharma and Singh
2010	Old age	Memory loss	F2	Burns and Mery
2012	Low male availability during mating	Number of offspring (to quantify fitness)	F2 & F3	Brommer et al.
2013	Post-eclosion feeding of virgin females with a high-sugar diet	Body composition in larvae	F2	Buescher et al.
2015	Gamma radiation in young adult males	Longevity & rate of development	F2 but not F3	Shameer et al.
2015	Yeast concentration in diets used to raise larvae through development	Somatic rDNA instability & copy number variation	F2 & up to F60	Aldrich and Maggert
2016	Post-eclosion feeding of both virgin males and females with various diets	Longevity & reproduction	F2 & F3	Xia and de Belle
2016	Extended olfactory training with young adults	Approach bias to the same trained odors	F2	Williams
2016	High fat diet to raise larvae through development	Pupal body weight	F2	Dew-Budd et al.
2016	Different food conditions used to raise male larvae and adults	Longevity	F2	Roussou et al.
2016	Post-eclosion dietary, genetic and pharmacological treatments of both virgin males and females	Longevity & H3K27me3 levels	F2	Xia et al.
2017	Epialleles, as defined by differential levels of H3K27me3	Eye color	F5 & up to F10	Ciabrelli et al.
2017	Grandmaternal age	Embryonic & embryonic to adult viability	F2*	Bloch Qazi et al.
2017	Genetic manipulation of parental metabolism	Triglyceride levels & transcriptional profile	F2	Palu et al.

*Potential transgenerational effects were not clearly-defined and quantified.

Table 1. Primary research papers describing TEI in ***Drosophila*** where phenotypic and/or genomic responses were investigated in the F2 or later generations.

stable epigenetic marks may record environmental challenges through modified gene expression patterns and ensure long-lasting, while reversible responses in the absence of the initial triggering events [10–17]. Importantly, the adaptive and reversible nature of epigenetic regulation may offer exciting therapeutic targets to help prevent or treat most, if not all, chronic diseases, including cardiovascular disease (CVD), diabetes, neurodegenerative diseases, and cancers [10–13, 16, 18–20].

The fruit fly (*Drosophila melanogaster*) offers multiple advantages for assaying TEI, in particular to characterize the underlying epigenetic mechanisms, and to identify gene targets for drug discovery. First, the short rearing period and lifespan of fruit flies facilitate transgenerational experiments over multiple generations within a reasonable time scale. Second, various examples of transgenerational inheritance have been established in *Drosophila* (**Table 1**) that enable rapid identification and characterization of underlying epigenetic mechanisms. Third, all major epigenetic mechanisms are present in this model system [1], although DNA methylation in flies appears to be different from many other eukaryotic organisms and is present only at very low levels in adults [21, 22]. Importantly, N^6-methyladenine may complement the function of DNA methylation in flies [23]. Finally, *Drosophila* has been increasingly used for modeling human diseases and drug discovery [24–28]. The *Drosophila* heart has been used to model several different aspects of human CVDs, including congenital heart disease and cardiomyopathy [29–31]. *Drosophila* is a recently-established model system for obesity and diabetes [26, 32, 33]. It has also been widely used to model cognitive diseases [34, 35], and various cancers [36].

TEI has been thoroughly reviewed, focusing mostly on data obtained from mammals [5, 6, 8, 37–39]. Here, such studies from *Drosophila* are discussed, in particular, to highlight transgenerational programming of metabolic status and longevity, and tri-methylation of histone H3 at lysine 27 (H3K27me3) as an evolutionarily conserved epigenetic mechanism underlying TEI.

2. Transgenerational inheritance at the organismal level

2.1. Metabolism

The current Western diet has been defined by increased consumption of meat products, dairy items, grains, and sugar-infused drinks [40]. Having profound effects on glycemic load, fatty acid composition, macronutrient composition, micronutrient density, acid-base balance, sodium-potassium ratio, and fiber content, this diet may underlie the growing prevalence of chronic diseases in Western society, especially CVD, obesity, diabetes, and dementia [41–44]. Often, multiple conditions manifest themselves simultaneously in afflicted individuals, suggesting shared elements in disease pathology. Obesity and other metabolic disorders, for example, are associated with various secondary disease indications as the underlying cellular and organismal metabolism is fundamental to nearly all necessary biological processes [45]. The prominent role of nutrition and other environmental factors in the development of metabolic disorders offers a promising model to identify and characterize the underlying epigenetic mechanisms, leading to diet optimization and nutrition-responsive therapies to combat chronic diseases (cf. [42]). Thus, nutrition has been studied extensively regarding TEI of diabetes and other metabolic disorders across various animal models [14, 46–48]. Metabolic dysfunctions are often measured through development and glucose/insulin homeostasis after nutritional or dietary interventions including overnutrition, high-fat, low-protein (LP), and high-sugar (HS) diets. Typically, the well-controlled application of dietary manipulations and well-established hallmarks of various metabolic disorders offer a tractable yet indispensable approach to studying TEI in many animal models.

Drosophila shares key metabolic pathways and characteristics with vertebrates (cf. [29, 32]). Glucose has been well-studied in the context of metabolic status given its pivotal role in insulin signaling since the 1930s [49]. *Drosophila* utilizes trehalose, the disaccharide of glucose, as its primary form of hemolymph (insect equivalent of blood) sugar [50, 51]. Regulating glycometabolism and maintaining viability in response to shifting external factors [51], trehalose is broken down through the catalyzing activity of trehalase into accessible glucose molecules. Thus, hemolymph trehalose and glucose levels may be quantified to assay glycometabolism in *Drosophila* [29, 32]. As the primary source of body fat from *Drosophila* to humans [52], triglycerides (TAGs) may be quantified for monitoring gluconeogenesis, the metabolic pathway responsible for glucose generation from non-carbohydrate substrates [29, 32]. Both AKT and 4EBP proteins are phosphorylated in response to insulin signaling [53, 54]. AKT is particularly well-characterized as a core component of the PI3K/AKT/mTOR pathway, which is linked to cell cycle regulation, cancer, and longevity [55]. Quantification of phosphorylated-AKT and phosphorylated-4EBP levels has been used to measure insulin sensitivity or resistance [29]. The availability of these assays to characterize both metabolic homeostasis and underlying pathways has supported the use of *Drosophila* to examine TEI of metabolic status after nutritional or genetic manipulations in the founding (F0) generation [32, 56, 57].

Buescher et al. recorded elevated trehalose, glycogen, and TAG levels as well as reduced body weight in adult female F0 flies after feeding on an HS diet for 7 days post-eclosion [32]. Glucose levels were found to be affected by the HS treatment, suggestive of gluconeogenesis dysregulation. Interestingly, trehalose and glucose levels were elevated in the first generation (F1) male larvae, along with a decrease in glycogen levels. Consistently, gene expression analyses demonstrated decreased expression of the genes involved in fat body lipolysis and gluconeogenesis, and increased expression of the ones involved in gut lipolysis, fatty acid synthesis, sugar transport and glycolysis. These results have confirmed the traditional models of insulin signaling, in which impaired insulin sensitivity leads to global increases in circulating blood sugars and decreases in sugar storage. Both glucose and trehalose levels were elevated, with TAG unaffected in the F2 male larvae; trehalose was elevated while TAG was decreased, with glucose unaffected in the F2 female larvae, supporting the existence of gender-dependent differences in transgenerational inheritance of metabolic programming. These results have demonstrated the long-lasting and transgenerational effects of early-life (post-eclosion) nutrition on metabolic status, establishing *Drosophila* as a useful model system to study TEI of nutritional programming of metabolic homeostasis and disorders.

Then, Dew-Budd et al. assayed the effects of gender and genetic lineage on transgenerational inheritance of certain metabolic phenotypes after rearing male (F0; paternal ancestry) and female (maternal ancestry) larvae of 10 (to measure pupal body weight) or 3 (metabolic composition and egg size) independent genetic lines on a high-fat diet [57]. Substantial differences in body weight, metabolic composition, or egg size were observed in both F1 and F2 generations between paternal and maternal ancestries or among different F0 genotypes. Interestingly, phenotypic changes in the F0 flies appeared not to be a consistent predictor of these hallmarks in their untreated F1 and F2 descendants. Therefore, "personalized" consideration of ancestral contributions may be needed to understand and prevent metabolic diseases such as obesity and diabetes.

Palu et al. have employed loss-of-function mutants to induce obesity, assayed with elevated TAGs, in F0 parents and then check TAG levels in heterozygous F1 and wild-type F2 offspring [56]. Loss of *AKHR* (encoding adipokinetic hormone receptor) leads to reduced fat body lipid mobilization and elevated TAG accumulation, as adipokinetic hormone functions analogously to the fasting hormone glucagon in mammals [52]. Mutant *AKHR* F0 and wild-type flies in reciprocal crosses produced heterozygous F1 offspring. These F1 heterozygotes were then crossed to wild-type females or males to generate four types of genetically distinct wild-type F2 (+/+) progeny, corresponding to mutant *AKHR* F0 grandpaternal or grandmaternal and heterozygous F1 paternal or maternal ancestors. Both male and female F0 mutants displayed elevated TAG levels, which were then normalized in the F1 heterozygotes, possessing a functional copy of *AKHR*. Interestingly, this Mendelian model of inheritance was not always followed in the F2 generation with low TAG levels observed in the grandpaternal/maternal group, while normal in the other three groups. Consistently, *ACC*, encoding a conserved Acetyl-CoA carboxylase that acts as the rate-limiting step in fatty acid synthesis [58], was found to be dysregulated in this particular F2 group. These results suggest that genetic manipulation of parental metabolism can provide an effective approach for studying TEI of metabolic state.

2.2. Aging

Aging has been increasingly recognized as a malleable process and the largest risk factor for most aging-related diseases (ARDs). It is no accident that the rapid increase in life expectancy worldwide is concomitant with the epidemic progression of many of these life-threatening and costly diseases [59, 60]. Recent work has demonstrated that many factors, including environmental conditions (e.g., diet) and genetic mutations, can impact the aging process across species [61–63]. In particular, anti-aging interventions often delay or prevent multiple ARDs in animal models [62–64], stimulating the emerging interdisciplinary field of geroscience to study the connection between aging and diseases, and to develop novel multi-disease preventative and therapeutic interventions by targeting the aging process itself [59, 65]. There are clear practical and ethical complications associated with studying aging and its transgenerational inheritance directly in human populations. The timescale of conducting such longitudinal studies would be unreasonable, at best. The shortage of isogenic replicates (e.g., twins) and imprecise environmental manipulation in human models also pose a significant problem in terms of reproducibility and subsequent mechanistic studies.

Drosophila presents itself as an excellent model to study aging, especially its transgenerational inheritance and the underlying mechanisms, owing to its relatively short lifespan, genetic homology with other models and humans, and suite of enriched investigative tools. *Drosophila* has an average lifespan of 2–3 months yet undergoes key parallel developmental stages similar to those of humans [25]. Studies on its life cycle have revealed a number of highly conserved pathways involved in organismal development. The *Hox* genes, for example, which control segment identity during embryonic development, were first identified in *Drosophila* after observation of mutant flies growing legs in the place of antennae [66]. *Hox* genes were later found to be conserved in humans and also linked to congenital disorders, including synpolydactyly and hand-foot-genital syndrome [67–69]. In addition, the key aging pathways, including mechanistic target of rapamycin, sirtuin, and insulin/insulin growth factor 1 signaling, are well

conserved in fruit flies [70–72]. Finally, tissue-specific and time-dependent genetic manipulations may be readily achievable for most genes, for instance, using the 22,270 transgenic lines (currently, covering ~88% of all predicted protein-coding genes) from the Vienna *Drosophila* Resource Center [73]. Therefore, *Drosophila* is well-suited for both correlational and mechanistic studies focusing on transgenerational programming of longevity after nutritional or environmental manipulations in the F0 parents [74–77].

Gamma radiation causes DNA damage and mutations, leading to various health dysfunctions and subsequent lifespan reduction [78, 79]. High doses of gamma irradiation were found to decrease longevity in the F0 flies and further propagate to the F1 and F2, but not to the F3 generation [76]. In contrast, low doses extended longevity across the F0–F2 generations, consistent with the concept of hormesis, by which low exposure to harmful agents (irradiation, caloric restriction, heat stress, and free radicals) improves general health and longevity [79–81]. Related studies have revealed several underlying mechanisms including insulin and glucose metabolism, proteasome activity and histone deacetylation [81, 82]. Histone deacetylation may be particularly relevant in this context as an epigenetic modification involved with many biological processes and human diseases, including CVD, metabolic disorders, and cancers [83–85].

Our recent work has established the first animal model of early-life nutrition-mediated programming of longevity and its transgenerational inheritance [74]. Newly-eclosed F0 virgin flies were reared on one of three different diets (low-protein or LP, intermediate-protein, and high-protein) for the first 7 days post-eclosion. Longevity was assayed for males and females, both virgin and mated, across the F0–F2 generations, allowing us to determine the potential impact of gender and mating on transgenerational inheritance of longevity. Our results suggest that early-life nutrition-induced programming effects on longevity may be transmitted to the F1 generation through intergenerational effects and further to the F2 generation through transgenerational effects, independently of gender and mating. The programming effects, although diminishing, were still present in the F3 generation for the low- and intermediate-protein diets. These observations suggest that early-life nutrition may produce long-lasting and transgenerationally heritable effects on the aging process across multiple generations. Notably, these long-lasting programming effects may be derived from both maternal and paternal contributions, as we treated both newly-eclosed F0 males and females to induce potentially maximal alterations. In contrast, a similar treatment was applied only to the females to examine transgenerational programming of metabolic status [32]. Most rodent studies also used either males or females, instead of both [48]. This design would not distinguish potentially different contributions from males and females, something that requires further investigation. Interestingly, transgenerational glucose intolerance in mice (*Mus musculus*) may be transmitted via the maternal or paternal line through different mechanisms [86, 87], suggesting that transgenerational nutritional programming effects may potentially be additive when induced in both males and females.

A more recent study has demonstrated that distinct dietary manipulations in the larval stage or throughout adulthood may also induce transgenerational programming of longevity [75]. The F2 male offspring were found to be long-lived if F0 male adults were subjected to dietary restriction, but not to starvation, whereas the same outcome was observed if F0 male larvae were exposed to starvation, but not to dietary restriction. The authors also generated two separate

groups of F2 males, from the F1 male (paternal) or female (maternal) offspring of the F0 male larvae exposed to various food media. Extended longevity was observed in both groups of F2 males, but greater extension was seen in the F2 maternal males with one laboratory strain. By contrast, the starvation-induced transgenerational effects were observed only in the F2 paternal males with a different strain. Therefore, cross-generational inheritance of nutrition-mediated longevity changes may be passed through either the male or female line or both, depending on genetic background. Unfortunately, it is unclear whether the observed gender-dependent differences resulted from intergenerational or transgenerational inheritance, as longevity was not assessed in the F0 and F1 generations.

2.3. Fitness

Fitness refers to the reproductive success of an organism over the duration of its lifetime, and has often been linked to genetic regulation. Recent studies, though sparse, have prompted the idea that non-genetic or epigenetic mechanisms may modulate fitness across generations [74, 88–90]. Studying the interplay between genetics and epigenetics through fitness may help us understand various complex traits and disorders [89]. *Drosophila* is particularly suitable for studying TEI of fitness for its rapid maturation following eclosion and high fecundity among model organisms [91].

Brommer et al. have reported that sexual conflict (male availability) may impact the fitness of future progeny up to the F3 generation [90]. Female fitness was quantified by lifetime production of offspring, and male fitness by total offspring produced in a six-day period. For the F0 generation, female flies underwent either a low (one male for 1 day followed by no male for 3 days) or high male (one male for 1 day followed by a different male for 3 days) exposure treatment. This four-day cycle was repeated for the duration of the females' lifespan to measure lifetime fecundity. The same process was repeated for the F1 and F2 generations, thus producing eight groups of F3 flies with distinct ancestral history. All F3 generation daughters experienced the treatment of high male exposure. All comparisons, when made relative to the low versus high male treatments experienced by the F0 females, provided a measure of transgenerational inheritance of fitness. The results indicated that low male exposure treatment in the F0 females did not affect female fecundity across the F1−F3 generations, but increased male fitness in the F1 generation and decreased male fitness in the F2 and F3 generations.

In the same study where we assayed transgenerational nutrition-mediated programming of longevity (see above), we also explored the transgenerational effects of the same early-life diets on lifetime fecundity (egg production) as a measure of fitness and the potential trade-off between longevity and fecundity [74]. Lifetime fecundity was found to be decreased across the F0–F2 generations after raising the F0 virgin male and female flies on the LP diet for 7 days before their mating, while increased transgenerationally after the same treatment with the intermediate-protein diet. Fecundity was also increased in the F0 and F1 generations after the same treatment with the high-protein diet, but the increasing effect was not seen in the F2 generation. These results demonstrate that early-life dietary changes affect fitness of the same generation and the reproductive success of future generations with certain dietary changes. Interestingly, correlation analyses on longevity and fecundity data revealed no evidence for trade-off between them across the F0–F2 generations. This finding argues

that lab-raised flies, with abundant food supplies at all times, may have evolved to abandon such trade-off constraints through hundreds of generations. Therefore, transgenerational nutritional programming of fitness may be achieved independently of longevity, raising the interesting possibility of elevating both longevity and fitness with proper nutrition across generations.

Bloch Qazi et al. recently reported the cross-generational effects of grandmaternal and maternal age on offspring viability and development up to the F2 generation [88]. The study, however, appeared not to distinguish between intergenerational (grandmaternal to maternal and maternal to F2 offspring) and transgenerational (grandmaternal to F2) effects. The complicated design with three interacting factors (i.e., grandmaternal age, maternal age, and stress) and subsequent analyses with mixed-model ANOVAs made it challenging to make conclusions about a straight forward transgenerational effect, although the P value was smaller than 0.05 in three of analyses for the "grandmaternal" factor (in the presence of the intergenerational effect or "maternal" factor).

2.4. Memory

Many behavioral traits, including cognitive functions, may be transgenerationally affected by experiences and environmental factors in mammals, most likely through epigenetic mechanisms [92]. Memory is an essential cognitive function which declines during aging and is impaired in most neurodegenerative diseases such as Alzheimer's disease; it is subjected to various epigenetic regulations, providing novel therapeutic avenues to combat cognitive disorders [12, 93]. Therefore, studying TEI of memory is of immense importance to our understanding of mental health and diseases. A *Drosophila* memory TEI model is established by two recent studies [94, 95] and further corroborated by a similar report in which increased startle responses to the conditioned odor after paternal F0 olfactory fear conditioning was observed in the subsequent adult F1 and F2 mice [96].

A widely-used dual-odor discriminative Pavlovian conditioning assay involves training groups of flies to associate one odor (CS^+; conditioned stimulus) with aversive electric or mechanical shocks (US; unconditioned stimulus), and the other odor (CS^-) as a non-associative control [97–99]. Aged (25-day-old) flies produced F1 offspring with memory impairment detectable in young adults (3–5 days old), and this impairment was transmitted to the F2 generation [95]. The transgenerational effect was specific to short-term memory (STM; as tested 15 min after training), and appeared to be caused by oxidative stress in both F0 maternal and paternal flies. Although the same authors did not evaluate memory in aged F0 parents, an earlier study [100] demonstrated that aging specially impaired middle-term memory (MTM), which starts to form within 15 min after training and is considered to be an aging-sensitive component of STM [101, 102]. In addition to concluding that offspring cognitive ability may be influenced by parental age [95], these studies collectively argue that aged F0 parents may acquire a loss of oxidative stress-sensitive STM, and this acquired memory loss can be transgenerationally inherited at least to the F2 generation. This new explanation also provides a possible mechanistic direction for future investigation, as MTM formation requires normal function of the

amnesiac (*amn*) gene that encodes a precursor neuropeptide encompassing fly homologs of mammalian pituitary adenylate cyclase activating peptide (PACAP) and growth hormone-releasing hormone (GHRH; see below for further discussion) [103].

In a more recent study, F1 and F2 flies, without any training and prior exposure, displayed selective preference toward the same CS odors which were used during 5 days of discriminative training of F0 parents [94]. This preference was selective for the salient CS odors experienced by the F0 parents but not the specific CS-US association, as the F1 and F2 flies did not differentiate between odors that were originally used to train their F0 parents under an aversive (with electric shocks as US) *vs.* appetitive (with corn meal and sucrose as US) conditions. Consistently, discriminative conditioning appeared to increase the perceived salience of the CS^+ odors [104]. Importantly, the observed odor-selective preference in the F1 flies required normal function of *amn* and preserved function of dorsal paired medial neurons in which *amn* is predominantly expressed [105]. Thus, the *amn* gene may be involved with transgenerational inheritance of acquired loss of STM in aged F0 parents [95] and odor-selective preference from discriminative training in the F0 flies [94]. In agreement with this idea, PACAP and/or GHRH stimulate growth hormone release [106], while down-regulation of growth hormone may be involved with cross-generational toxicity [107]. The *amn* gene also plays an important role in the behavioral response to intoxicating levels of alcohol [108], while alcohol abuse has been known to be transgenerationally heritable [109]. Collectively, these studies support *Drosophila* as a useful model to study transgenerational inheritance of memory impairment triggered by environmental factors (e.g., aging) and behavioral traits acquired from experiences (e.g., training), and epigenetic regulation of *amn*-encoded peptides as one potential underlying mechanism.

3. Transgenerational inheritance at the molecular and genomic level

Despite advancement of high-throughput sequencing and the recent surge of research on TEI, there are currently few studies focusing on the transgenerational effects at the molecular and genomic level, and thus the underlying mechanisms remain largely obscure [6, 8, 37–39, 92, 110]. Several recent studies in flies, however, may shed some light on this situation [77, 111–115].

Chronic treatment (7-day feeding and 7-day withdraw) of the F0 males with pentylenetetrazole (PTZ), an FDA-revoked convulsant drug, caused locomotor deficits and long-term alterations in the CNS (central nervous system) transcriptome [116]. A follow-up study from the same group [113] demonstrated that the F0 males (with PTZ treatment) displayed a CNS transcriptomic profile closest to the F2 males; and differentially expressed genes in the F1 males, F1 females, and F2 males showed significant overlap with the PTZ-impacted genes in the F0 males. Interestingly, further clustering analysis of CNS and testis transcriptome profiles and concordant analysis of differentially expressed genes between them implied gametic involvement in the observed transgenerational effect in gene expression. These results suggest that the acquired somatic transcriptomic alteration in F0 PTZ-treated males may be passed via

sperm at least to the F2 generation. This is the first report to study transgenerational inheritance of genome-wide transcriptomic profile as a "phenotype," acquired through drug treatment in the F0 generation.

In another study, a high-protein diet led to somatic rDNA instability and copy number reduction in F0 parental flies [111]. As the insulin/insulin-like growth factor and TOR signaling pathways regulate ribosome biogenesis and rDNA expression for nutrient availability [117], genetic and pharmacological manipulation of insulin/TOR signaling produced similar effects, corroborating the results from dietary treatment. Importantly, rDNA copy number reduction remained in the F2 generation and was still present in flies maintained on standard food for 6 years. These results suggest that the genome rearrangement in F0 flies acquired through feeding on the high-protein diet occurred in both somatic and germ cells, and was transgenerationally heritable for over 150 generations. This outcome revealed a robust and long-lasting transgenerational consequence of adult diets. In a remarkable recent study, early-life protein restriction in mice induced a linear correlation between growth restriction and DNA methylation at certain rDNA copies that lasted into adulthood [118]. These findings, establishing rDNA as a genomic target of nutritional availability across species, are of obvious importance for human health and diseases, as copy number variations have been linked to many chronic diseases such as schizophrenia and Alzheimer's disease [119–121].

Another curious study has shown that a dominant and hyperactive mutation in the *hopscotch* gene (Hop^{Tum-l}), encoding the *Drosophila* JAK kinase, caused epigenetic alterations in F0 parental flies that were transgenerationally heritable and thus influenced tumorigenesis in their F1 and F2 offspring [114]. Interestingly, the transcriptional repressor *Krueppel*, known to repress transcription of the *fushi-tarazu* gene which encodes a homeodomain protein required for embryonic segment number and cell fate [122], is a Hop^{Tum-l} enhancer [123]. *Krueppel* mutations caused increased DNA methylation in the *fushi-tarazu* promoter region. This effect was transmitted across generations in the presence of Hop^{Tum-l} [114]. Therefore, DNA methylation may be altered by *Krueppel* mutations, functioning as heritable epigenetic markings in *Drosophila*. JAK hyper-activation may then interfere with epigenetic reprogramming, allowing the changed DNA methylation (epimutation) to propagate across generations and influence tumor susceptibility.

4. Polycomb repressive complex 2 (PRC2) mediates H3K27me3 as a conserved epigenetic mechanism underlying transgenerational inheritance

Despite decades of intense studies linking all key types of epigenetic regulation (i.e., DNA methylation, histone modifications and non-coding RNAs) to TEI, direct and convincing experimental evidence in support of underlying mechanisms and governing principles is rare [2–8, 37–39]. The difficulties lie in the time-consuming nature of such studies, and lack of well-established epimutations, clearly-defined phenotypic contributions and stably-inherited epigenetic markings

across multiple generations. Here we highlight two recent persuasive studies in *Drosophila* that have characterized one particular histone modification (H3K27me3) as part of an evolutionarily conserved epigenetic mechanism underling transgenerational inheritance [77, 112].

H3K27me3 is a repressive methylation mark on histone H3 established by PRC2 through its core catalytic subunit, the H3K27-specific methyltransferase encoded by the E(z) gene in flies [124] and EZH2 in mammals [125]. PRC2 is evolutionarily conserved across species, including unicellular alga (*Chlamydomonas reinhardtii*) and budding yeast (*Cryptococcus neoformans*) [124–127]. Genes marked with higher-than-normal levels of H3K27me3 in human and mouse spermatozoa continue to show repression during gametogenesis, embryogenesis, and development, suggestive of a role of this histone modification during TEI [128–130]. Furthermore, paternal diet affects H3K27me3 marks at specific loci in their offspring, implying that such nutrition-induced epigenetic modifications may be selectively retained across generations in mice [131]. Finally, TEI of longevity has been reported for H3K4me3 in worm (*Caenorhabditis elegans*) [132], and the bivalent chromatin domains covered by H3K27me3 and H3K4me3 marks have been implicated in aging and ARDs in humans [133, 134]. These results collectively suggest that H3K27me3 may function as an evolutionarily conserved epigenetic mechanism underlying transgenerational inheritance. Our recent work and that of Ciabrelli et al. have directly validated the concept in the context of nutrition-mediated longevity programming, transgene expression, and endogenous genetic variation [77, 112]. Further strengthening the idea, H3K27me3 markings have been found to propagate across generations from the maternal (and likely paternal) germline and survive reprogramming events during early embryogenesis in flies [115].

Our most recent study examined E(z)-mediated H3K27me3 as one potential epigenetic mechanism underlying transgenerational inheritance of longevity [77]. It was prompted by our earlier work to establish nutritional programming of longevity and its transgenerational inheritance [74], and by recent studies supporting the notion that PRC2-mediated H3K27me3 may regulate aging across species. H3K27me3 repressive markings and an epigenomic PRC2 signature marked by EZH2 and SUZ12 (another core component of PRC2) binding have been found to be associated with age-associated differentially methylated regions and aging-associated genes in human embryonic stem cells and various other cell lines, implicating this repressive epigenetic marker as a common mechanism of aging in humans [135]. Consistently, Polycomb repression is associated with healthy aging in humans [136], and replicative senescence of stem cells, an *in vitro* aging model [137, 138]. H3K27me3 and H3K4me3 are also the frequent antagonistic partners found on the bivalent chromatin domains which may be implicated in aging and ARDs in humans [133, 134]. In addition, heterozygous mutations of E(z) increase longevity while also reducing H3K27me3 levels in adult flies, suggesting that PRC2-dependent H3K27me3 may regulate aging in *Drosophila* [139]. Interestingly, E(z)-mediated H3K27me3 is required for paternal transmission of obesity through reprogramming of metabolic genes in flies [140], supporting its potential role in transgenerational reprogramming. Finally, UTX-1 (an H3K27-specific histone demethylase) has been shown to regulate aging, and H3K4me3-mediated TEI of longevity has been reported in *C. elegans* [132, 141].

E(z) protein level was significantly upregulated in F0 flies, and back to normal in F2 flies, after post-eclosion treatment of F0 flies with the LP diet [77]. In contrast, the resulting

increase of E(z)-dependent H3K27me3 was seen in the F0 parents and their F2 offspring. Correspondingly, longevity was reduced in both F0 and F2 flies. These results suggest that early-life dietary insults may trigger E(z)-mediated H3K27me3 changes via misregulation of E(z), and consequently nutrition-induced H3K27me3 dysfunction may be transmitted across generations and underlie TEI of nutritional programming of longevity. First, E(z)-mediated H3K27me3 was found to be necessary for TEI of longevity programming, as early-life RNAi-mediated specific knockdown of E(z) only in the F0 parents extended longevity while reducing H3K27me3 activity, and early-life specific inhibition of E(z) enzymatic function with EPZ-6438 (a highly EZH2-selective inhibitor) also extended lifespan while rendering the H3K27me3 level low across generations. Importantly, the effects of RNAi-mediated knockdown on H3K27me3 and longevity were specific, as (I) similar effects were observed with two independent RNAi transgenes, (II) the E(z) protein level was normal in the F2 generation after its knockdown in the F0 parents, and (III) longevity, E(z), and H3K27me3 levels were not affected without heat shock to induce RNAi transgenes. Similarly, the EPZ-6438-induced effects were specific, as (I) EPZ-6438, as a phase II clinical drug, is highly EZH2 selective and considered safe [142], and (II) E(z) protein was unaffected by EPZ-6438 even in the F0 parents. In addition, H3K27me3 was found to be sufficient for TEI of longevity programming, as EPZ-6438 greatly alleviated the longevity-reducing effect of the LP diet, while counterbalancing its upregulation of H3K27me3 across the F0 to F2 generations. Our data have convincingly demonstrated that E(z)-mediated H3K27me3 activity may play a critical role in the general health of an organism and function as one epigenetic mechanism underlying TEI of early-life nutrition-mediated longevity programming. Our findings have also provided the first proof-of-concept for an epigenetic therapy to confer transgenerational health benefits in a model system, manifested through improved longevity.

Another important aspect of our study was early-life rather than adult-oriented interventions. The critical period refers to a time frame in which an organism's nervous system is especially susceptible to environmental modification. This phenomenon is common to nearly all multicellular model organisms as it primes the organism to environmental stimuli and programs physiological pathways responsible for maintaining general health. Studies have linked abnormalities in the critical period to the development of autism spectrum disorder [143], attention deficit hyperactivity disorder [144], schizophrenia [145, 146], obesity [147], and other ARDs [148]. Indeed, the Developmental Origins of Health and Disease hypothesis (DOHaD) postulates that the current mainstream adult-oriented therapies may be less efficacious than those delivered during the developmental phases of life [149, 150]. Our study has provided direct validation of this concept through the delivery of EPZ-6438 at various time points throughout adult life to alleviate LP-induced longevity reduction. The alleviation effect was found to be greatest, intermediate, or very mild when the drug was delivered within the first 7 days, from day 3–10, or from day 10–17 after eclosion, respectively. The effect was even seen in the F2 generation when the inhibitor was delivered within the first 7 days post-eclosion. These data support the DOHaD approach for studying ARDs in *Drosophila* and the use of a developmentally appropriate time period for intervention. Our follow-up experiments indicated that early-life administration of EPZ-6438 can also prevent multiple LP-induced ARDs (i.e., cardiomyopathy, type 2 diabetes, and aging-related memory loss) throughout adult life.

This represents a novel proof-of-concept of an early-life multi-disease therapy, leveraging epigenetic reprogramming to provide life-long protection against multiple – possibly all – ARDs (Xia et al., unpublished results).

To study epigenetic phenomena in flies, Ciabrelli et al. employed a transgene inserted in chromosome arm *2 L* (*Fab2L*) to establish stable and isogenic epilines that carried distinct epialleles as defined by differential levels of PRC2-dependent H3K27me3 [112]. The *Fab2L* transgene contains the reporter gene *mini-white*, whose expression determines red pigmentation in the eye, under the control of *Fab-7*, a 3.6-kb genomic region that includes a PRE (Polycomb response element). Despite being located on a different chromosome (*3R*), the endogenous *Fab-7* region can affect PRE-responsive repression of the *Fab2L* transgene through long-range 3D chromatin interactions [151, 152], producing variable *mini-white* expression-dependent eye colors among individual flies. These epigenetic differences were somatic and not transgenerationally heritable, but enhancing long-range interactions between *Fab2L* and the endogenous *Fab-7* through removal of one copy of *Fab-7* induced a plastic epigenetic state, allowing the authors to establish the stable and isogenic epilines with the most repressed (white) or the most derepressed (red) eye phenotypes through 15 generations of selection for eye color.

Their subsequent characterization indicated that (I) these epilines carried either silent or active epialleles of *Fab2L*, as determined by high or low levels of PRC2-responsive H2K27me3; (II) these epialleles could be stably and dominantly transmitted to naïve flies, with acquired epigenetic states stably maintained at least until the F10 generations through self-crossing; (III) epiallele maintenance required 3D chromatin interactions, with both epialleles fully and specifically reversed to a non-selective state after complete removal of the endogenous *Fab-7*; (IV) epiallele inheritance also followed the rules of paramutation under natural environment conditions, with environmental factors (e.g., temperature and humidity) affecting the phenotypes of the epialleles; and (V) the paradigm could apply to a naturally occurring phenotype (i.e., antenna-to-leg homeotic transformation [153]) of a spontaneous neomorphic mutation of the homeotic *Antennapedia* gene. This important work, with well-established stable and isogenic epialleles as defined by distinct levels of H3K27me3 markings, has overcome many shortcomings of earlier studies of transgenerational inheritance, such as weak effects fading away within a few generations, ill-defined contributions to the observed phenotypes, and unclear epigenetic markings (cf. [114, 154]). The results have convincingly demonstrated stable transgenerational H3K27me3-mediated inheritance of transgene expression and endogenous genetic variation in fruit flies [112], corroborating our study of establishing the same epigenetic mechanism underlying transgenerational inheritance of nutrition-programmed longevity [77].

In this mode of TEI, PRC2 functions through H3K27me3 repressive markers to acquire specific epigenetic states in response to environmental stimuli or triggers. Alternative states are defined by different levels of H3K27me3 to affect gene expression and epigenetic phenotypes [77, 112, 131]. Polycomb-mediated repression at specific loci and/or long-range chromatin interactions act together to maintain acquired states *in cis* [112], and distinct levels of H3K27me3, as deposited in the maternal oocytes [155], resist epigenetic reprogramming during early embryogenesis and are transmitted across generations, enabling transgenerational inheritance of acquired states and phenotypes [115]. The acquisition and establishment of epigenetic states may occur

rapidly during developmentally appropriate time periods [77, 131] or gradually through phenotypic selection [112]. Deposit of H3K27me3 appears to be locus-specific in response to environmental factors (cf. [131]). The extent and robustness of its inheritance may be environmental factor- and trait-dependent, with the transgenerational effects upon acquired complex traits (e.g., aging) quickly adapting to further environmental changes and decaying away in a few generations (cf. [77]), or upon simple traits (e.g., transgene expression) being relatively resistant to further environmental modifications and transmitting across many generations (cf. [112]).

5. Conclusion

Drosophila as a versatile model organism is profoundly advancing our understanding of TEI and its underlying mechanisms. Short lifespan, well-conserved epigenetic mechanisms, and powerful genetic tools have facilitated TEI studies at molecular, genomic, and organismal levels after various environmental and genetic manipulations (**Table 1**). Many studies have employed dietary interventions at the larval or early-adult life stages, or throughout adulthood, similar to those in mammals [48, 156]. Early-life nutrition in particular has been linked to adult health and diseases, prompting the increasingly-recognized DOHaD approach for studying various ARDs including CVD, obesity, diabetes, dementia, and certain cancers [4, 150, 156]. Importantly, these existing TEI models have enabled exciting investigations of the underlying molecular and epigenetic mechanisms. Here, we have highlighted PRC2-mediated H3K27me3 markings as an evolutionarily conserved epigenetic mechanism underlying transgenerational inheritance [77, 112, 115].

6. Recommendations

TEI research is a relatively new science. H3K27me3-mediated inheritance is providing a platform to address many important questions about TEI in future studies. What are the signals and underlying molecular mechanisms responding to the initial environmental stimuli? How do these signals trigger an epigenetic process and establish corresponding epigenetic states? How can such specific epigenetic states, likely originating in somatic cells, be transmitted to germ cells to enable transgenerational inheritance? What are the molecular mechanisms that maintain transgenerational inheritance? Is H3K27me3 unique in that it may resist epigenetic reprogramming [115]? Is H3K27me3 a common epigenetic mechanism responsible for non-genetic transgenerational inheritance across species? We anticipate that the *Drosophila* model will continue to broaden our understanding of TEI biology and related human diseases in particular.

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