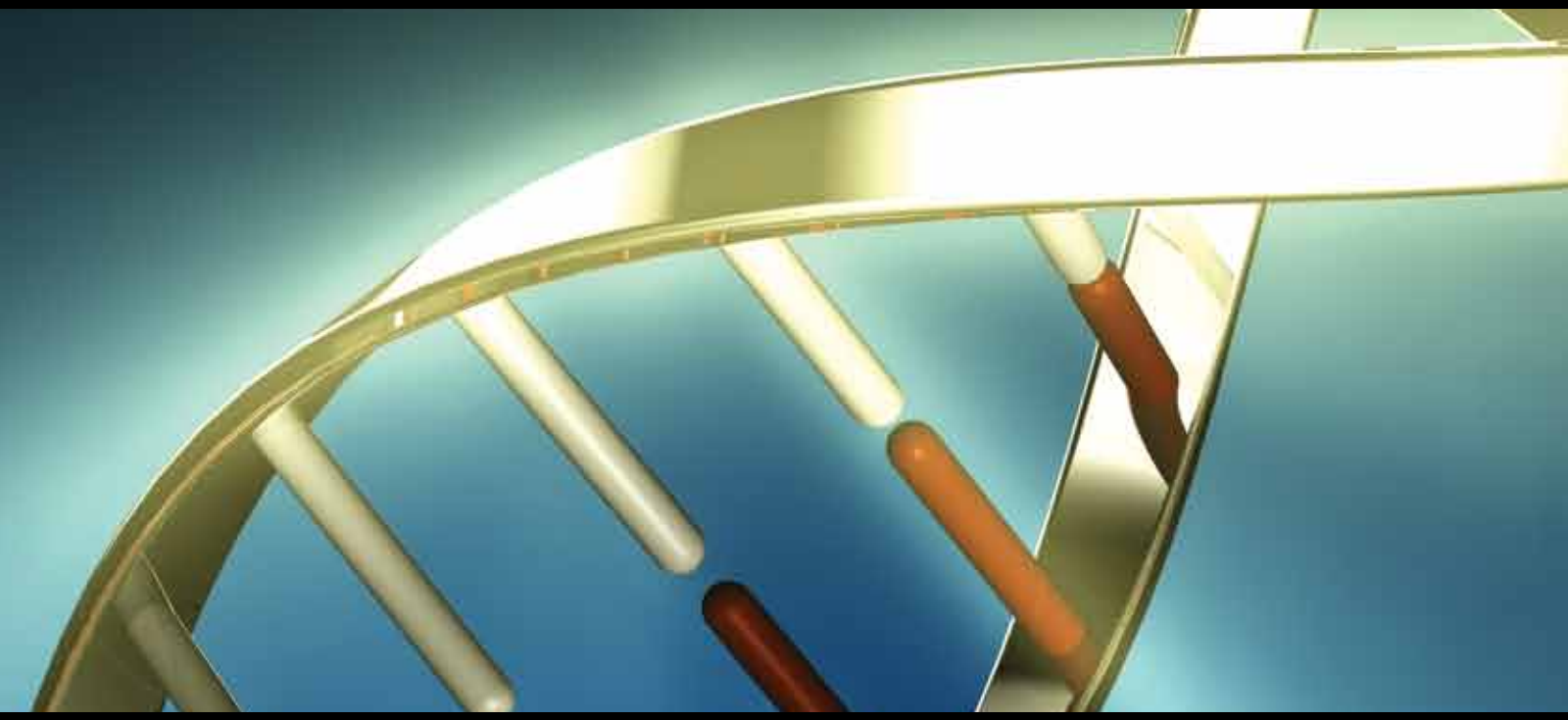
Genetics Research International

The Role of Epigenetics in Evolution: The Extended Synthesis

Guest Editors: Douglas M. Ruden, Victoria Meller, Christina L. Richards, and Vincent Sollars



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Editorial Board

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Editorial

The Role of Epigenetics in Evolution: The Extended Synthesis

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Evolutionary biology is currently experiencing an emergence of several research topics that transcend the boundaries of the Modern Synthesis, which was the last major conceptual integration in evolutionary biology [1]. The Modern Synthesis used the concepts of population genetics to integrate Mendelian genetics with evolution by natural selection [2]. Pigliucci [3, and citations within] identified several major areas of innovation that transcend the Modern Synthesis: epigenetics, evolvability, phenotypic plasticity, evolution on adaptive landscapes, evolutionary developmental biology, and systems biology. Integrating these new ideas with the Modern Synthesis will form a new conceptual framework of evolution, which they termed the Extended Synthesis, as it will extend, rather than refute, the Modern Synthesis [3]. This subject has been the focus of much recent work, and an excellent description is provided in the book *Evolution—The Extended Synthesis* [2].

Epigenetics, one of the emerging areas in the Extended Synthesis, is the focus of this special issue. The importance of epigenetics has long been appreciated at the molecular level (e.g., its role in cell determination and self-recognition). However, the role of epigenetics in evolution and ecology is a more recent focus. Epigenetics has expanded to the study of heritable changes in gene expression and function without alterations in the DNA sequence [4], or the study of stably heritable phenotypes that occur without alterations in DNA sequence [5]. Epigenetic mechanisms interact with genetic, physiological, and morphological systems and may be an important component of organism-environment interactions [6, 7]. Some epigenetic characters can be stably transmitted across generations [8–11]. Thus, epigenetics has a mechanism of heredity that was not considered in the framework of the Modern Synthesis [2]. Epigenetic mechanisms may play critical roles in phenotypic plasticity [12, 13], soft inheritance [4, 14], an individual's response to environmental stressors [6, 8], invasive species biology [15], and conservation biology [16]. Understanding epigenetics will likely provide insights into individual and population processes at both ecological and evolutionary time scales [6, 7, 17–19].

DNA methylation, the most studied molecular epigenetic mechanism [20], is active in DNA imprinting [21], X-inactivation [22], restructuring the genome in response to polyploidy caused by hybridization [23], silencing transposable elements [21], and in response to environmental stressors [8]. DNA methylation is a source of interindividual phenotypic variation [10] and has been shown to cause phenotypic variation in flower shape and fruit pigmentation [24, 25], mouse tail shape, adult body size and coat color [26, 27], and numerous traits differentiating queen and worker honeybees [28].

Epigenetic variation in DNA methylation can provide an evolutionarily and ecologically important source of phenotypic variation among individuals. The violet (*Viola cazorlensis*) has a high level of interindividual DNA methylation variation that differentiated populations from southeastern Spain [29], and variation among individuals was related to

the amount of damage caused by herbivory [30]. The invasive Japanese knotweed (*Fallopia japonica* and *F. x. bohemica*) has significant differences in DNA methylation among populations from the northeastern United States [31, 32], and a portion of the variation could be attributed to different habitats. Allopolyploid orchids (*Dactylorhiza majalis* s.str, *D. traunsteineri* s.l., and *D. ebudensis*) have variation in DNA methylation that was significantly related to environmental variables [33]. Genetically identical dandelion (*Taraxacum officinale*) plants develop variation in DNA methylation in response to stressors, and many of these changes are stably inherited in the next generation [8]. Also, house sparrows (*Passer domesticus*) from North America and Africa introduced into Europe have a higher level of variation in DNA methylation compared with these birds in their native environments, which suggests that DNA methylation may compensate for the decreased genetic variation caused by introduction into a new environment [34].

In this issue, Castonguay and Angers discuss how epigenetic mechanisms are particularly important in asexual organisms, specifically the asexual hybrid fish *Chrosomus eos-neogaeus*, since epigenetic variation allows for phenotypic variation in otherwise genetically identical individuals. Similarly, Flatscher et al. discuss approaches to disentangle the role of DNA-sequence-based and epigenetic polymorphisms in the process of speciation in the *Heliosperma pusillum* and allied taxa (Caryophyllaceae).

Although DNA methylation is the most well-studied mechanism in the context of ecology and evolution, several studies have investigated other epigenetic mechanisms. Histone modifications, small and long noncoding RNAs, and genome structure can regulate gene expression and contribute to phenotypic variation in diverse taxa [35]. In this issue, Bozzetti et al. discuss the role of the *crystal-Stellate* modifiers, which indicate the importance of piRNA pathways in defense of genome integrity against transposons and other repetitive elements in the gonads and are relevant to evolutionary canalization mechanisms. Wells et al. review different mechanism in which modification of the histone H4 tail modulates gene expression for dosage compensation between sex chromosomes and autosomes and between sexes.

Areas of epigenetics outside of DNA methylation and histone modifications are also discussed in this issue. Apte and Meller review the role of homologue pairing in the transmission of information in flies and mammals and show how communication between homologues affects genome regulation in both taxa. Also, Ferree and Prasad discuss the impact highly repetitive, noncoding satellites have on chromosome segregation at different developmental stages and through distinct cellular mechanisms and note their effect on postzygotic reproductive isolation.

While a great deal of work remains, epigenetics has already proven to be very promising in evolutionary biology. Empirical studies that demonstrate the role epigenetic variation has in ecology and evolution will help answer some of the major questions in evolutionary epigenetics, and these empirical studies will allow a development and refinement of a foundational theory of evolutionary epigenetics. In this issue, Maggert cautions about the potential to dilute epigenetics by confounding true cases of heritable nonsequence information with possibly trivial modes of gene regulation, while Bateson argues how the experience of an individual affects the evolutionary potential of its offspring through epigenetic effects. These two papers in particular indicate that it is important to consider if stable inheritance of the epigenetically derived character is a requirement for evolutionary epigenetics. Alternatively, could the Extended Synthesis integrate epigenetic mechanisms that generate variation and respond to the environment, even if the specific changes are not inherited? In certain cases, the presence of an additional source of variation may be most important. In others, the stable transmission of a particular epigenetic state may be important. Ultimately, the increased phenotypic potential of a genotype via epigenetic mechanisms, which in some cases may be inherited, must be incorporated into the evolutionary theory.

Before presenting the papers of this special issue, we would like to alert the reader to a second special issue planned for Genetics Research International: *The Epigenetics of Emerging and Nonmodel Organisms* (edited by Vett Loyd et al.). Together, these two special issues introduce the reader to the importance of epigenetics in evolution and developmental biology.

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Review Article

Environmental Heterogeneity and Phenotypic Divergence: Can Heritable Epigenetic Variation Aid Speciation?

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The dualism of genetic predisposition and environmental influences, their interactions, and respective roles in shaping the phenotype have been a hot topic in biological sciences for more than two centuries. Heritable epigenetic variation mediates between relatively slowly accumulating mutations in the DNA sequence and ephemeral adaptive responses to stress, thereby providing mechanisms for achieving stable, but potentially rapidly evolving phenotypic diversity as a response to environmental stimuli. This suggests that heritable epigenetic signals can play an important role in evolutionary processes, but so far this hypothesis has not been rigorously tested. A promising new area of research focuses on the interaction between the different molecular levels that produce phenotypic variation in wild, closely-related taxa that lack genome-wide genetic differentiation. By pinpointing specific adaptive traits and investigating the mechanisms responsible for phenotypic differentiation, such study systems could allow profound insights into the role of epigenetics in the evolution and stabilization of phenotypic discontinuities, and could add to our understanding of adaptive strategies to diverse environmental conditions and their dynamics.

1. Introduction

Patterns and causes of biological variation have fascinated and challenged natural scientists for a long time. The Darwinian evolutionary theory highlights the importance of natural variation as raw material upon which selection processes can act, thereby increasing the fitness of locally adapted phenotypes [1]. Conceptual and technical developments since the late 19th century have greatly enhanced our understanding of some of the main mechanisms producing and maintaining biological variation, namely, genetic mutation and recombination [2]. However, natural selection acts upon phenotypic variation represented by the individual [3], which is delimited by its genetic constitution, but also shaped by its specific environment [4] and developmental processes [5]. The process of evolution is thus a result of complex interactions between various intrinsic and extrinsic factors [6].

Therefore, current evolutionary investigations should consider several levels of biological variation [7]. First, differences in the DNA sequence account for a great amount of biological variation: the genetic system defines the range of functional possibilities of each individual. However, these heritable differences translate into the phenotype only indirectly via the resulting RNA and protein products which mould the structure and function of an organism. Much progress has been made in recent years in identifying gene functions and candidate genes coding for important metabolic enzymes, but analyses of whole genomes remain a complex challenge. Even in organisms whose whole genome is sequenced, a large number of genes still remain uncharacterized [8]. The second important source of biological variation is fluctuation in rates of gene expression, resulting in phenotypic plasticity [9, 10]. Genes can be up- or down-regulated in response to environmental conditions, such as temperature regimes or water supply, or intrinsic factors such as specific phenological or developmental stages [11]. This leads to temporary modifications of the phenotype, which are generally not passed on to the next generation [12, 13]. The third level, heritable epigenetic variation, via both specialized enzymology inducing structural modifications of the DNA (through DNA methylation, histone acetylation

[14, 15]) and small interfering (si) RNA populations [16, 17], results in (meta) stable chromatin landscape differences. Epigenetic differences determine if and where particular genes or groups of genes are to be expressed, while the underlying DNA sequence remains identical [18]. Most of these differences are reversible developmental effects and they are part of the molecular processes underlying phenotypic plasticity in response to variation in the environment [19]. However, environmental change, severe stress or genomic shock events like hybridization or genome duplication can change the epigenetic configuration of an organism resulting in new phenotypes [20–26], and some of these alterations can be passed on to the next generations [27–30].

The molecular mechanisms underlying these components of phenotypic variation differ in their stability and in the time frames in which they confer phenotypic novelty. The genetic sequence is the most stable, evolving slowly through mutation and gradually accumulating changes over a large number of generations. In contrast, gene expression levels can be rapidly and continuously regulated within a very short time [11], much shorter than the generation length of an organism, and allow an almost instantaneous response of the individual to its environment within limits defined by its genetic constitution. Heritable epigenetic alterations act within an intermediate time horizon, since they can occur as an immediate and multilocus reaction to different kinds of external or intrinsic stimuli [23] but are not as ephemeral as plastic gene regulation and can affect the following generations [18].

It has long been established that mutations in DNA sequence are the primary raw material for evolutionary change [2]. The involvement of environmental influences in generating heritable biological variation is still debated [13, 22], as is the necessity of extending our modern evolutionary synthesis [31]. Accumulating evidence indicates that modifications of epigenetic signals are correlated with phenotypic variation within and among species [25, 32–34], placing epigenetic differentiation even in a macroevolutionary context. Latest developments regarding the potential role of phenotypic plasticity in driving diversification and speciation have been discussed elsewhere (e.g., [13, 35]). We are hereafter focusing on the impact of heritable epigenetic variation on the process of evolution and propose a research plan to address its evolutionary significance.

2. Potential Impact of Heritable Epigenetic Variation on Evolution

Empirical studies have demonstrated high levels of epigenetic variation within natural populations [25, 36–41]. While experiments have shown that environmental conditions can override epigenetic signals (e.g., [26, 42, 43]) and increase this variation, few recent studies indicate that natural selection can act directly or indirectly on epigenetic variation [25, 38, 39, 44], potentially leading to evolutionary divergence and adaptation. Altogether, epigenetic information provides an additional source of natural variation, which may be particularly important for survival of small populations lacking genetic variability [45] and/or occupying a fragmented landscape. Selectable epigenetic variation can enable genetically depauperate lineages to adapt [46] until genetic assimilation occurs (i.e., when environmentally induced phenotypic variation becomes fixed by secondary genetic control, e.g., after deamination of methylated cytosine to thymine [13, 47]). Thus, heritable epigenetic variation could pave the way for genetic adaptation.

The epigenetic sources of variation can be stochastic epimutations, but a major part of the epigenetic variation is triggered by stress or changes in the environment [3, 22, 48], that is, under circumstances when new phenotypes could be crucial for survival. Moreover, if conditions return to their original state, spontaneous back-mutation of epialleles can restore original phenotypes (e.g., in position-effect variegation [27]). In the light of epigenetic variation, the involvement of the environment in evolution becomes twofold: as a stimulant of variation and as the selector of adaptive variation.

At the interface between genotype and environment, the overall rate of epimutations is often much higher than that of genetic mutations [49], resulting in a more dynamic level of variation. Novel epigenetic modifications may originate simultaneously in several individuals in a population under stress, which will facilitate fixation. Despite the potentially high loss of epigenetic novelties by epigenetic reset [19], epimutations can reach equilibrium frequencies within populations rapidly, over less than a dozen generations if the environmental stress is maintained long enough [28]. In stark contrast to the expected incidence of genetic mutations, environmental fluctuations can trigger multiple epimutations in the same individual. This renders fast ecological adaptation affecting (complex) adaptive traits more plausible [50]. Hence, recombination is not necessarily a prerequisite for adaptive change, if the latter is driven from the epigenetic level. In addition, epigenetic mechanisms may partly defy well-understood population processes, such as allelic drift (due to potential maintenance of relatively constant epiallelic frequencies through environmental influence). Being more flexible and dynamic than DNA sequence information, variation in epigenetic signals could therefore act as major driving force in rapid adaptive processes.

Epigenetic variation can have extensive consequences, even in the absence of genetic variability [45, 50, 51]. Epigenetics may introduce, or reinforce in a back-coupling process with environmental stimuli, major changes that lead to strong phenotypic differentiation [52] until becoming a real reproductive barrier. Most phenotypic differences between species are genetically controlled, but epigenetic inheritance can be of particular importance for the initial development of phenotypic divergence [25]. If adaptive and maintained long enough, phenotypic discontinuities can become genetically locked and trigger species divergence [53]. Modelling studies suggest that epigenetic variation can promote population divergence by facilitating adaptive peak shifts, reducing genetic barriers represented by fitness valleys in the adaptive landscape [47]. Therefore, epigenetic novelties have been one of the mechanisms put forward for saltational speciation [29, 54], but

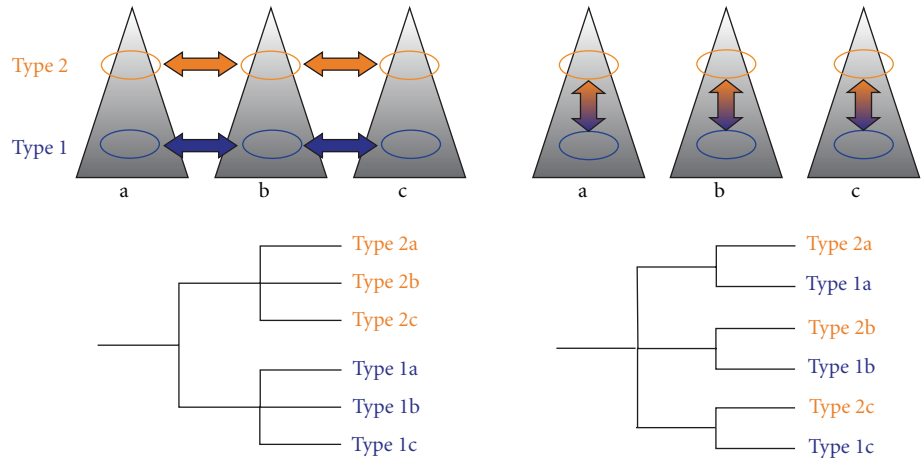

Figure 1: Putative relationships between populations of closely related alternative types (here exemplified with altitudinal differentiation), which lack apparent genome-wide divergence. Below the reflection of the relationships in hypothetical phylogenies is given. Left, single origin of each type, followed by dispersal to other geographical areas. Right, recurrent evolution of the types in several geographic regions under environmental influence.

empirical data is not yet available to support or reject such a hypothesis.

3. A Research Idea

Recently developed tools, in combination with traditional methods, can shed light on the complex interactions between genotype, epigenotype, and environment, and test for their individual contribution to phenotypic divergence and evolution. Evolutionary biologists could address the evolutionary relevance of heritable epigenetic polymorphisms by targeting closely related ecotypes or species (hereafter types) that show phenotypic differentiation without apparent genome-wide genetic divergence. Such types could be identified, for example, within asexual lineages or descendants of recent adaptive radiation events. We suggest a multifaceted research plan using an array of molecular techniques and field experiments to investigate whether epigenetics is involved in speciation by triggering phenotypic diversification.

3.1. Phenotypic Differentiation. As speciation is facilitated by the process of divergence, the first question to be addressed should be whether phenotypic variation in the study group is discrete or continuous. Phenotypic variation is a common feature of populations and species, and only a discontinuity in this variation may indicate incipient divergence and the onset of isolating mechanisms. Therefore, various morphological, anatomical, and physiological traits among populations of different types should be compared to test whether the types form well defined, distinct groups or whether the extreme phenotypes are linked by individuals with intermediate traits or combinations of characters. In addition, measurements and observations of environmental characteristics (e.g., microclimate, geology, soil, biological interactions) could identify limiting environmental factors, and relate them to anatomical, morphological, and physiological specializations.

If main discontinuities in phenotypic variation separate populations along type boundaries (e.g., by morphology or habitat preference), the uniformity within each group and constant difference between the groups might suggest a single origin of each type and subsequent dispersal (Figure 1). However, this seems rather unlikely in absence of genome-wide genetic divergence among the types. An alternative scenario could invoke repeated migration and iterative in situ formation of each type in alternative environments, with very strong and almost identical selection pressures acting upon different populations of each of the types.

3.2. Genetic and Epigenetic Differentiation. Singular versus multiple origin of each type should be tested by investigating the extent and structure of genome-wide genetic and epigenetic divergence within and among populations of both types. If populations cluster genetically in disagreement to the type (possibly determined by other factors, e.g., by geographic proximity), it may be hypothesized that their differentiation is underlaid by epigenetic mechanisms and that types have evolved several times in parallel. Alternatively, local high rates of gene flow combined with strong selection at a few adaptive genetic loci could hypothetically produce a similar pattern of highly porous genomes [55]. In such a case, a small number of adaptive (outlier) genetic loci of large effect should be responsible for the observed phenotypic differentiation. Outlier analyses [56–58] of genetic profiles provided, for example, by DNA fingerprinting techniques such as RAD (restriction site associated DNA) sequencing [59], microsatellites, or AFLP (amplified fragment length polymorphism [60]), could help identifying these loci or closely linked genomic regions. Positive selection will shape at target loci a significantly higher differentiation between populations of the alternative types than the genome-wide bulk of loci, while loci under purifying selection will show much lower differentiation [61]. On the other hand, if individuals of each group share type-specific epigenetic

patterns and/or mRNA transcripts, differentiation could be mediated either by overall differences in the epigenome or by a few epialleles.

As epigenetic variation is not detectable in genomic surveys of sequence variation, dedicated investigations have to be employed to address it. In recent years, a variety of genome-wide approaches, including techniques involving next-generation sequencing, have been developed to comparatively profile epigenetic patterns in nonmodel organisms [62, 63]. Cost-effective comprehensive methods include, for example, fractioning the DNA using C_0t filtration [64, 65] to enrich low-copy regions (mostly genes and their promoters) and sequence this genomic subsample by employing next generation methods and bisulfite sequencing. The latter is a process that converts unmethylated cytosines to uracils, which will then appear as thymines after sequencing [66]. Third-generation DNA sequencers, like the recently released single molecule real-time (SMRT) DNA sequencer could be employed for direct detection of DNA methylation [67] and thus enable much more profound study of both model and nonmodel epigenomes. Alternatively, genome-wide DNA methylation could be studied using isoschizomers [68, 69]. Similarly as for genetic dataset(s), the epigenetic information could be searched for general patterns of differentiation and for signatures of selection on individual (epi)loci [25, 44]. This should clarify if ecological and/or morphological divergence is dependent on just a few loci controlling traits for local adaptation, or if it is triggered by extensive differences. As the alternative types thrive in different environments, the selective pressures and their magnitude may vary across populations. Epigenetic signals will most often suffer from imperfect heritability; therefore, stronger selection will be needed to produce patterns that will be detected as outliers by statistical approaches.

To infer broad, genome-wide regulatory variation, in-depth quantitative gene expression analyses using next-generation sequencing (RNA-seq, [70–72]) could be performed searching for loci with significant expression differences between individuals of different types after growing them under uniform conditions to reduce the momentary-dependent noise in rates of expression. In addition, targeting posttranscriptional regulation, small RNA profiles could be compared using an smRNA-seq approach [63, 73, 74]. The different data types can finally be integrated in functional analyses (i.e., gene annotations) to identify correlated components that are part of the same regulatory network.

3.3. Heritability of Phenotypic Plasticity and Habitat Specificity. If the molecular basis of phenotypic differentiation and/or adaptation to divergent environments is identified within epigenetic rather than DNA sequence divergence, the next research step would be to investigate how stable the phenotypic divergence is. This will also help to assess the stage of speciation in which the group is at present. While facilitating population divergence and speciation [35], nonheritable phenotypic plasticity will trigger speciation only if the environmental conditions are stably different in the alternative localities [35] and gene flow is either infrequent or strongly opposed by natural selection. On the other hand, in the case of heritable phenotypic divergence that is fully stable even in the alternative environment, epigenetically triggered adaptation may have been already assimilated in the genetic code.

Reciprocal transplant experiments together with attempts to grow the different types under the same environment across several generations (i.e., between three and five as a minimum requirement) should be installed to determine the extent of phenotypic plasticity, and the ability of the different types to cope with altered environmental conditions. Growing individuals of the alternative types in a uniform environment across several generations may reveal the heritability of morphological and ecological characteristics within each of the types ("nature versus nurture") [75]. Comparatively investigating relevant (epi)loci in transplanted individuals versus controls will pinpoint those patterns that are immediately disrupted by the environment, and those that persist or, alternatively, are not under the influence of the relevant limiting environmental differences. Integrating this information and comparing morphological, anatomical and physiological traits supplemented by a set of fitness components among transplants and controls will define the links between genotype, epigenotype and phenotype, together with providing additional information on the patterns of selection and their targets.

According to the mechanisms underlying the observed differentiation, at least two possible outcomes can be anticipated. If the morphological and/or ecophysiological differences are triggered by continuous but nonheritable responses to local environments (i.e., as a reaction norm [76]), there should be no phenotypic differences between the progeny of the two types when reared and grown under the same conditions. Such a scenario will not (yet) be relevant for speciation. On the other hand, if heritable epigenetic differences are involved, phenotypic divergence between individuals of the types should at least partly be retained in a common environment. In the latter case the morphology, anatomy, and physiological properties of the transplanted individuals should reflect their origin rather than their current environment. This may go to the extreme that individuals are maladapted and do not survive under alien environmental conditions.

The result of these experiments could simultaneously allow for inferring evolutionary and population dynamics within the study group. If individuals of alternative types can adapt phenotypically to the habitat of the other and develop the habitat-specific syndromes following transplant experiments, the possibility of frequent gene flow between populations of both types should be considered. This might as well explain the lack of overall differentiation, as it prevents lineage sorting and hampers or slows down speciation. On the contrary, low fitness (i.e., poor performance and high mortality) of individuals in the native habitat of the alternative type may point to a differentiation that is strong enough to prevent gene flow between populations. In this case, we may be observing a process of ongoing speciation, where differentiation starts at the epigenetic level, triggering profound changes leading to segregation in terms of habitat, phenology, and/or biological

Figure 2: Low-elevation *Heliosperma veselskyi* and high-elevation *H. pusillum* are differentiated morphologically and ecologically. Particularly conspicuous is the dense indumentum of sticky glandular hairs on *H. veselskyi* in comparison to the glabrous leaves and stems of *H. pusillum* (Photographs: M. Sonnleitner).

interactions. Divergent selection may reinforce this environmentally induced specialization/niche segregation and bring about reproductive isolation. This will eventually result in virtual isolation of gene pools, and ultimately give way to stronger overall differentiation by accumulation of genetic differences due to the stochastic effects of drift.

4. *Heliosperma pusillum* Group: An Example of an Appropriate Study System

Heliosperma pusillum and allied taxa from the carnation family (Caryophyllaceae) contain a variety of morphologically different taxa (Figure 2) with distinct ecology, which are altitudinally or geographically isolated, but genetically intermixed (Figure 3) and do not represent independent evolutionary lineages [78]. Molecular phylogenetic studies based on AFLPs [77] and sequences of several nuclear and chloroplast regions [77–79], show that genetic divergence within the group is generally shallow, many taxa seem to be polyphyletic, and geographically allied taxa often share the same genetic constitution. We hypothesize that they either (i) represent fixed ecotypes, that is, differ subtly in their DNA coding regions with major phenotypic effects, or (ii) result from middle- to short-term adaptive (epigenetic) processes, perhaps under the influence of the environment and independent of actual changes in DNA sequence. All of them are perennial caespitose herbs that inhabit rocky habitats and shallow caves in mountain ranges of southern Europe [78, 80], mostly on calcareous substrates.

Different authors [78, 81] have subdivided this complex into two ecologically and morphologically distinct groups of taxa: a higher elevation group occurring in damp, open habitats and among rocks above the timberline and a lower elevation group inhabiting canyons and gorges as well as shallow caves and cliff overhangs with rather dry soils, high atmospheric moisture and poor light conditions below the timberline. The higher elevation group, including *H. albanicum*, *H. pudibundum,* and *H. pusillum* s.str., differs from the lower elevation group by narrower, glabrous or sparsely hairy leaves and often unicellular glands as well as longer seed papillae [78, 81]. By contrast, plants of lower elevations share a denser indumentum with long multicellular glandular hairs and are often sticky (Figure 2). Generally, morphological variation is much higher in the lower elevation group, which contains several narrowly distributed taxa [78, 82]. Most of them are endemics of the Balkan Peninsula; only *H. veselskyi* is restricted to the southeastern Alps. The origin and evolution of the lower and higher elevation groups and the relationships between them are still poorly understood. Recent molecular phylogenetic studies [78] (see also Figure 3) indicate that neither higher nor lower elevation groups are actually monophyletic, but rather inextricably intermingled with each other, indicating that one of the groups evolved multiple times from the other. Mechanisms involved in the phenotypic diversification of the two groups, the morphological convergence within each group, and the stability of this phenotypic divergence remain unknown, but preliminary evidence suggests that morphological features remain constant in a common garden, at least in the first generation. The *H. pusillum* complex is suitable for (epi) genomic and transcriptomic analyses, because all taxa have a relatively small genome (1C = 1.32 pg [83]) and so far no polyploid cytotypes have been found ($2n = 2x = 24$). In addition, they can be easily grown from seeds and have short generation times, which make them optimally suitable for common garden and transplantation studies.

5. Synthesis and Outlook

Although the possibility of epigenetic inheritance has now been established [7, 18, 27, 30, 84] and we are increasingly understanding the full extent of its role in producing

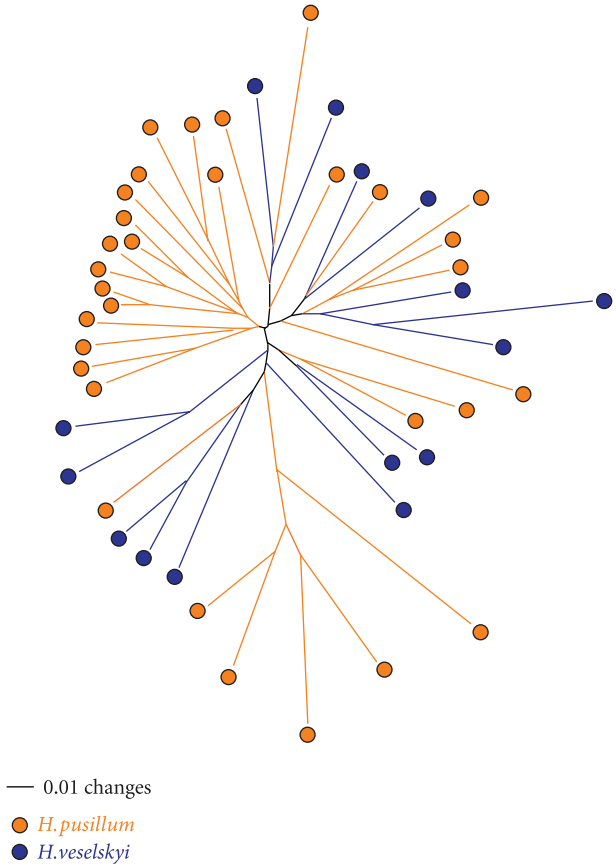

Figure 3: Genetic analyses do not support separation of higher-altitude *Heliosperma pusillum* (orange) and lower-altitude *H. veselskyi* (dark blue), but rather indicate an inextricable relationship between the two taxa. Unrooted neighbor joining tree based on Nei-Li distances calculated with PAUP from AFLP profiles [77].

phenotypic variation [19, 25, 39, 40], little research has been done to systematically study the role of heritable epigenetic variation for speciation. Incorporation of epigenetics into evolutionary models and empirical studies is only now starting to be attempted (e.g., [28, 49]); however, more empirical information from natural populations is needed for accurate modelling of epigenetic dynamics. Indeed, the prevalence of alternative stable epialleles in natural populations, and their significance to phenotypic divergence, ecological interactions and selection in real-world contexts remain too little explored [3, 41, 53]. The limited relevant data available indicate a stochastic nature of epigenetic variation, which is continuously being shaped by the influence of the environment, and further tuned through natural selection [25, 38, 39]. Therefore, the epigenetic aspect of natural variation may contribute to evolution in a fashion similar to genetics, but much more rapidly. Implying heritability of adaptive (i.e., selected) traits, epigenetic inheritance is not a contradiction of the Darwinian evolutionary synthesis [31], but rather a complex augmentation of the classic view on genetic inheritance, particularly as genotype and epigenotype interact to produce a broad array of short- and long-term heritable combinations.

The recently available possibility to profile the epigenome and transcriptome of nonmodel organisms in a high-throughput manner [62, 63, 85] enables thorough investigation of some of the most challenging hypotheses in a modern evolutionary framework, such as achieving and maintaining stable divergence through epigenetic differences. The acquired knowledge also impacts several related domains, from conservation to theoretical evolutionary biology. Investigating recent adaptive radiations with epigenetic markers may be particularly informative. Most traits of ecological significance tend to be continuous or quantitative and appear to be governed by many genes, each of little effect, but with cumulative power [86], resulting in a complex picture of factors and mechanisms acting upon the phenotype. Using appropriate study systems it is now possible to interrogate the links between ecological divergence and many regulatory alterations of small effect or singular major epigenetic switches. In addition, such investigations are expected to pinpoint new loci that are sensitive to epigenetic modification and unravel information on the rates of spontaneous epimutations in natural populations and their stability over time.

Currently accumulating data will offer valuable clues on the establishment of broad regulatory determinants of functional diversity in natural populations. The early evidence we currently hold urges complementing our gene- and genome-centred evolutionary view with a substantial consideration of epigenetic factors when seeking to understand population processes that drive adaptation and divergence [3, 53, 87]. Using modern technologies, future research will identify the exact molecular mechanisms triggering relevant phenotypic divergence and reproductive isolation. We will soon be able to infer the corresponding selection pressures that are responsible for the presence of a particular individual/a particular species in its specific habitat. Understanding how new plant species form and adapt to novel ecological niches is crucial to advance our knowledge of evolutionary processes active at the population level driving adaptation and speciation. An increased knowledge of organismic adaptation strategies is also of outstanding importance in the current context of widespread environmental challenges. It may be a key for predicting effects of climate change and managing biodiversity in a sustainable manner.

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Review Article

The Key Role of Epigenetics in the Persistence of Asexual Lineages

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Asexual organisms, often perceived as evolutionary dead ends, can be long-lived and geographically widespread. We propose that epigenetic mechanisms could play a crucial role in the evolutionary persistence of these lineages. Genetically identical organisms could rely on phenotypic plasticity to face environmental variation. Epigenetic modifications could be the molecular mechanism enabling such phenotypic plasticity; they can be influenced by the environment and act at shorter timescales than mutation. Recent work on the asexual vertebrate *Chrosomus eos-neogaeus* (Pisces: Cyprinidae) provides broad insights into the contribution of epigenetics in genetically identical individuals. We discuss the extension of these results to other asexual organisms, in particular those resulting from interspecific hybridizations. We finally develop on the evolutionary relevance of epigenetic variation in the context of heritability.

1. Introduction

Despite its increased cost relative to asexual reproduction, sexual reproduction is common in multicellular organisms, which can lead to the interpretation that there is an advantage to reproducing sexually. This topic has been the subject of much debate, and, in the last decades, several hypotheses have been proposed to explain why sexual reproduction is maintained in populations. These hypotheses generally can be divided into two classes: (i) sex creates the genetic diversity necessary to cope with environmental variation (Fisher-Muller accelerated evolution theory [1, 2]; Red Queen hypothesis [3]; Tangled bank hypothesis [4]) and (ii) sex allows purging of deleterious mutations [2, 5, 6]. These hypotheses are all based on the assumption that asexual lineages are evolutionary dead ends.

Asexual reproduction is the primary form of reproduction in bacteria, archaea, and protists. It is also not uncommon in multicellular eukaryotes and is found in many phyla, particularly in plants, arthropods, nematodes, and rotifers [7]. In plants and animals, obligate asexuality is a derived character. It often results from the hybridization of two individuals from different sexual species [8–10], producing fertile hybrids no longer capable of reproducing sexually.

Over half the taxa examined by Neiman et al. [10] were represented by asexual lineages estimated to be >500,000 years old. Notably, amongst the oldest asexual lineages are the bdelloid rotifers, reported to have evolved for tens of millions of years without sexual reproduction [11]. These examples constitute a serious challenge to the common view that asexuality increases long-term extinction rate.

Because they generally lack recombination and the possibility to create genetic variation in their offspring, asexual lineages are thought to be limited in their capacity to colonize new environments and respond to environmental fluctuations. However, several asexual lineages have been found to possess a large geographical distribution [7, 12–18]. To explain this observation, based on concepts of the General Purpose Genotype model [19], evolutionary persistent asexual lineages have been hypothesized to be generalists characterized by flexible genotypes that allow them to occupy wide ecological niches [12].

Under this model, asexual lineages would possess an importan capacity for phenotypic variation. Genetic mutation and epigenetic modifications are molecular mechanisms

known to sustain phenotypic variation (reviewed in [20]). Could these mechanisms explain the persistence of these "evolutionary scandals" [21]? As we will explain, this depends largely on the timescale at which they act.

Mutations are long-term acting mechanisms that can create phenotypic variation. Yet many asexual taxa are thought to be particularly efficient in DNA repair, which would allow them to reduce the accumulation of deleterious mutations. There is evidence for this in asexual taxa such as asexual weevils [22], aphids [23], darwinulid ostracods [24], *Daphnia* [25], and oribatid mites [26]. However, the oldest known asexual lineage, the bdelloid rotifers, displays higher accumulation of mutations than related sexual species [27]. While efficient DNA repair will reduce the load of deleterious mutations in asexual populations, they will consequently also possess less genetic diversity to face environmental variation. Therefore, whether this mechanism is prevalent or not, it cannot explain on its own the persistence of asexual lineages since it does not account for how they can respond to environmental variation.

How do asexual organisms face environmental variation without sexual recombination? In bdelloid rotifers, two alleles at a given locus will diverge over time due to their independent accumulation of mutations and lack of recombination, effectively resulting in two genomes within one organism (Meselson effect [11]). However, besides the bdelloid rotifers [11], the Meloidogyne root knot nematodes [28], and Holbøll's rockcress [29], most asexual lineages are not characterized by the Meselson effect [26, 30]. In some asexual lineages, this could be due to the counteracting effect of homogenizing mechanisms such as efficient DNA repair. Alternatively, these other lineages could simply still be too young for mutations to be accumulated.

It appears therefore that many asexuals do not possess any specific mechanism for generating genetic variation. Despite this, these lineages have faced environmental variation for several thousands to millions of years. Even organisms where the Meselson effect is observed have most likely not strictly relied on genetic variation to face environmental variability, as this mechanism is not expected to produce genetic variation at a timescale short enough to be relevant to that at which environmental perturbations occur.

Asexual lineages must therefore possess shorter-term acting mechanisms to face environmental variation. In the absence of genetic diversity, the ability of these organisms to respond to environmental variability will depend on their capacity for phenotypic plasticity ([31] and references therein).

Epigenetic modifications could be a shorter-term acting mechanism allowing the creation of phenotypic variation among genetically identical individuals [32–37]. Epigenetics refers to changes in gene expression stably propagated through cellular divisions that occur without changes in the DNA sequence but through, for example, chemical modifications to the DNA (e.g., DNA methylation) and its associated proteins, the histones [38]. DNA methylation, in particular, is the most studied epigenetic modification. Epigenetic modifications are stably inherited through cell divisions and can underlie phenotypic change at least throughout the lifetime of an individual. The phenotypic differences induced by epigenetic changes can create differences in individual fitness (e.g., [39, 40]). Specific environmental conditions have been shown to induce changes in epigenetic states (e.g., [37, 41–47]). Therefore, epigenetic modifications, unlike mutations, allow the genome to integrate extrinsic environmental signals. Importantly, DNA-methylation-driven phenotypic variation has also been observed to be transmitted across organismal generations [44, 48, 49].

In asexual organisms, epigenetic modifications could cause phenotypic differences among individuals that would affect a single generation of organisms or in some cases that could persist in asexually produced offspring. In the present discussion of asexual organisms, the concept of phenotypic plasticity will be used to describe phenotypic effects of epigenetic modifications affecting a single organismal generation. However, in some other papers, the concept has been expanded to include both single-generation and transgenerational epigenetic modifications (see [33, 35, 50] for further discussion on the relationship between epigenetics and phenotypic plasticity).

Epigenetic modifications might be an important mechanism for creating phenotypic variability in asexual organisms, allowing them to face environmental variability [34, 36, 37]. The role of epigenetics could be especially important in the earlier stages of the existence of asexual lineages, when the effect of longer-acting mechanisms such as mutation is not yet felt. Indeed, epimutations occur at a greater rate than mutations [51–53], and, consequently, epigenetic variation among individuals is likely to precede genetic variation. Also, like mutations, epimutations are not all advantageous, but disadvantageous epimutations have the advantage of being reversible.

Some evidence for the role of epigenetics in asexual organisms comes from studies of asexual dandelions where variation in DNA methylation was detected among individuals of a single apomictic lineage [36, 37]. This variation was transmitted across generations and was sequence independent (see [33, 54] for discussion on the evolutionary significance of different degrees of dependence of epigenetic variation on genetic variation). Moreover, various stresses were shown to induce inheritable variation in DNA methylation [37]. Our group's recent work on the asexual fish *Chrosomus eos-neogaeus* [55] represents to our knowledge the first investigation of variation in DNA methylation associated with the environment in a naturally occurring asexual animal lineage. In the following paragraphs, we will discuss the ways by which epigenetic variation can play a role in the evolutionary success of asexual lineages in light of our results on *C. eos-neogaeus*.

2. Phenotypic Variation in Asexual *Chrosomus eos-neogaeus* Hybrids

Vertebrates are ancestrally sexual and all known (obligate) asexual vertebrates have arisen from hybridizations. Asexual *Chrosomus eos-neogaeus* result from hybridizations between the northern redbelly dace *Chrosomus eos* and the finescale

dace *Chrosomus neogaeus* (Pisces: Cyprinidae) (Figure 1). These all-female hybrids produce unreduced eggs without recombination [56, 57]. They are gynogens so the sperm from one of the two parental species is required to activate embryogenesis, but the paternal genome is not incorporated into the egg. The resulting offspring are diploid individuals genetically identical to each other and to their mother [56, 58].

While parental species and hybrids are common and widely distributed through the northern part of North America, only a limited number of different asexual lineages have been detected [59]. The hybridization events that gave rise to *C. eos-neogaeus* hybrids took place in glacial refuges during the Pleistocene. At the end of the glaciation, the hybrids dispersed throughout North America [59]. The same lineage could therefore occur in different types of environments. This diversity in habitat use of a single diploid clonal lineage has indeed been documented [60, 61].

Chrosomus eos-neogaeus populations appear to possess no interindividual genetic variation. Indeed, in several lakes where these hybrids are found, a single clonal lineage is present and only a few lineages have been detected in every region studied so far [56, 59, 61–63].

A single *C. eos-neogaeus* lineage could therefore be found across a broad geographical and ecological range, indicating the capacity of these asexual organisms to face environmental variability. A number of studies have revealed a substantial amount of morphological variability in hybrids from a single clonal lineage [60, 61]. The diploid hybrids have been found to be at least as morphologically variable as their parental sexual species [61]. The nature of the mechanisms responsible for creating as much phenotypic variation in these asexual hybrids as in sexual species is unclear. Since the hybridizations occurred ca. 50 000 years ago [59], mutation is unlikely to explain the *C. eos-neogaeus* phenotypic variability. In the absence of interindividual genetic variation, we have hypothesized that epigenetic variation was underlying the phenotypic variability observed in *C. eos-neogaeus* hybrids. In the context of the General Purpose Genotype model, epigenetic processes could be regarded as the mechanism for extending the flexibility of their genotype.

3. Variation in DNA Methylation in Asexual *Chrosomus eos-neogaeus* Hybrids

We initially found that epigenetic variation was present in these fish through an MSAP survey that revealed interindividual variation in DNA methylation patterns in individuals from a single clonal lineage [47]. Importantly, the observed epigenetic variation was independent of the genotype. The hybrids came from seven geographically distant lakes characterized by different biotic and abiotic conditions. Based on their methylation profiles, individuals could be grouped according to their lake of origin [55]. The correlation observed between the environment (i.e., lake of origin) and the methylation profile strongly suggests that asexual *C. eos-neogaeus* hybrids respond to environmental variation with DNA methylation. These observations were made on one generation of organisms. We did not investigate the methylation profiles of offspring of these individuals so no conclusion can be made about the heritability of these marks.

4. Epigenetic Variation and Asexual Lineage Persistence

Results of previous studies and ours indicate that DNA methylation could be a viable mechanism for the creation of phenotypic variation in the studied asexual organisms, allowing them to respond to the environment in the absence of interindividual genetic variation. The presence and variation in DNA methylation have not been investigated in most asexual lineages. However, given the widespread occurrence of this modification and its presence in organisms of all the phyla where asexuals are found (except in rotifers, where the presence of DNA methylation has to our knowledge not been investigated), it is likely that many of the unstudied asexual lineages also possess DNA methylation. The ones that do not are expected to rely on other epigenetic mechanisms to regulate gene expression. For example, DNA methylation is absent in the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. Yeast can rely on histone-modifying enzymes to control the packaging of their DNA, therefore regulating the access of their genes to transcription [64–66]. *Schizosaccharomyces pombe* also possesses RNA interference, which is notably involved in the formation of heterochromatin at their centromeres [67, 68].

Contrary to some studies where global undermethylation was observed in interspecific hybrids (e.g., [69, 70]), the methylation levels present in *C. eos-neogaeus* hybrids are comparable to those observed in other sexual vertebrates [47]. It is possible that other asexual lineages possess levels of DNA methylation comparable to those observed in *C. eos-neogaeus* and exhibit interindividual variation in their DNA methylation patterns. Through the creation of phenotypic variability necessary for facing environmental fluctuations, epigenetic processes could play a crucial role in the persistence of asexual lineages. In the next paragraphs, we will discuss the mechanisms by which some asexual lineages could be particularly apt at creating epigenetic variation among individuals and present some of the implications of epigenetic variation in asexual lineages.

5. Mechanisms for Variation in DNA Methylation

The capacity for phenotypic variation through epigenetic processes could explain the success of some asexual lineages. It is possible that these asexual lineages possess particularly efficient mechanisms for generating epigenetic variation.

The enzymes responsible for DNA methylation are the DNA methyltransferases (Dnmt). In mammals, where this epigenetic modification is well studied, the Dnmt3 family is responsible for *de novo* methylation: it establishes new methylation marks on previously unmethylated DNA. The Dnmt1 family of enzymes is responsible for maintenance

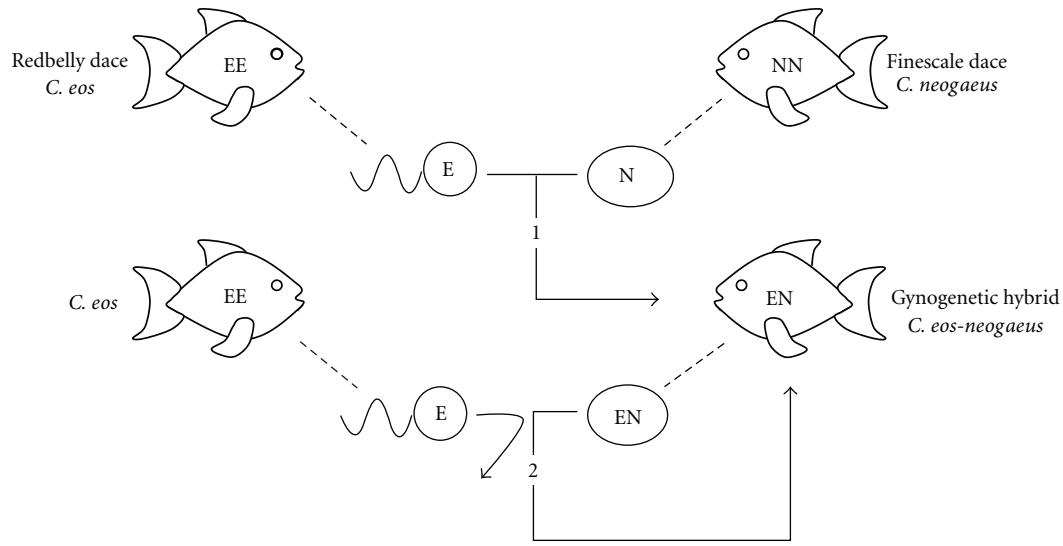

Figure 1: Expected mechanism leading to the natural occurrence of asexual hybrids in *Chrosomus eos-neogaeus*. (1) Gynogenetic hybrids resulted from hybridizations between female *Chrosomus neogaeus* and male *C. eos*. All-female hybrids are composed of one haploid set of chromosomes from each parental species. (2) Asexual reproduction occurs via gynogenesis: the entire genomic constitution of the mother is transmitted to the eggs and sperm from parental species is required only to initiate cleavage. The resulting offspring are genetically identical to the mother.

methylation: it reestablishes the preexisting methylation pattern on the daughter strand after DNA replication. Dnmt1 prefers hemimethylated to unmethylated sites and typically maintains the methylation pattern with 95% accuracy [71]. The error rate of Dnmt1 is therefore much higher than that of DNA polymerase, making epimutations much more likely than mutations. Indeed, the number of epimutations detected in *C. eos-neogaeus* hybrids was much higher than the number of mutations [47].

A mutated copy of Dnmt1 with a decreased preference for hemimethylated DNA would lead to more errors in the propagation of the DNA methylation pattern and an increase in *de novo* methylation at previously unmethylated sites. A byproduct of this would be a greater capacity for creating epigenetic variation among asexual individuals.

Since many asexual lineages result from interspecific hybridizations, genes can be misexpressed due to mismatches between regulatory elements of the genomes of the two species [72]. For example, at a given gene, the interaction between the trans-regulatory elements of one species with the cis-regulatory elements of the other can lead to dysregulation of this gene. Through such dysregulation, asexual lineages resulting from interspecific hybridizations could show, for example, insufficient expression of Dnmt1, leading to a decreased capacity in faithfully copying DNA methylation patterns through cell divisions. Dysregulation could also disrupt the temporal expression pattern of Dnmt3: the enzyme would not only be expressed during the hybrid's development but also throughout its life. New methylation marks could then be established throughout the individual's life, greatly extending its capacity for phenotypic variation.

6. Epigenetics and Asexual Hybrids

When considering how asexual organisms respond to their environment, it is important to take into account that many asexual lineages result from interspecific hybridizations. Global repatterning of DNA methylation can occur upon hybridization and polyploidization. As exemplified by work in plants, methylation patterns can be radically altered [32, 73–76].

Asexual hybrids might not only be able to differentially express their genes but also the specific alleles of their genes, as reported in numerous diseases where heterozygotes exhibit a diversity of symptoms according to the level of expression of the mutant allele [77–79]. *Chrosomus eos-neogaeus* hybrids could achieve this differential allelic regulation through epigenetic modifications such as DNA methylation. These hybrids possess a *C. eos* allele and a *C. neogaeus* allele for every one of their genes. For a given gene, some individuals could have a methylated *C. eos* allele and others a methylated *C. neogaeus* allele, conserving expression of the *C. neogaeus* and *C. eos* allele, respectively (Figure 2). Supposing many of their genes could be regulated this way, the number of ways in which a single genotype could be expressed would be greatly increased (theoretically 3^n, where n is the number of genes where differential allelic expression occurs, 3 refers to expression of alleles from *C. eos* only, *C. neogaeus* only, or from both *C. eos* and *C. neogaeus*). This would greatly increase their capacity for phenotypic variation. It is unclear how this differential allelic silencing would occur, but it could be in response to an environmental cue or randomly. In *C. eos-neogaeus*, Letting et al. [80] have

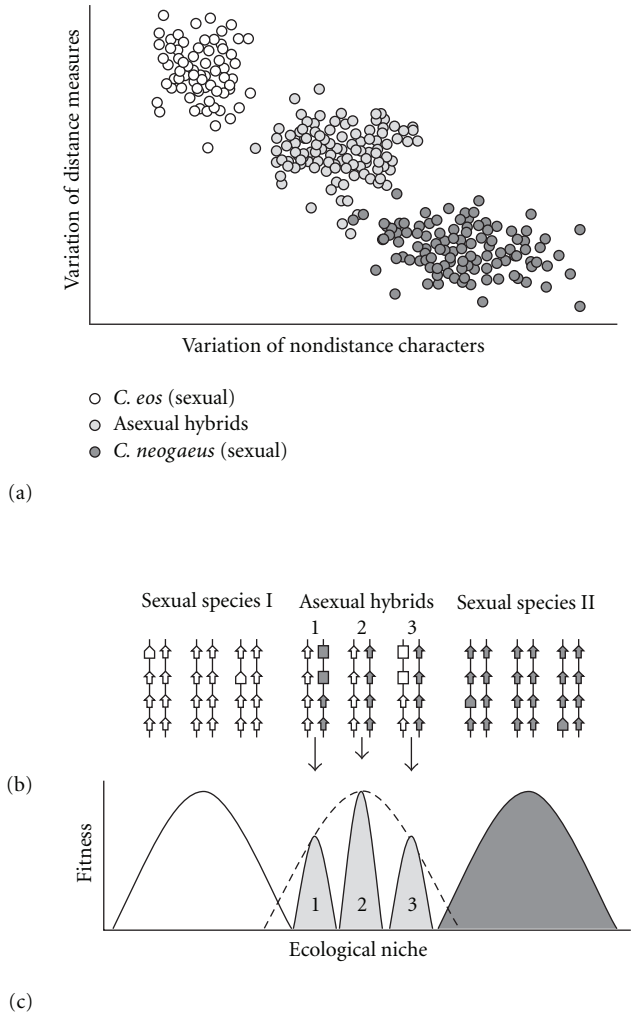

Figure 2: Hypothesis of the epigenetic mechanism underlying the flexibility of a genotype. (a) Phenotypic variation observed in sexual and asexual species. The points represent individual scores of *Chrosomus eos*, *C. neogaeus*, and asexual hybrids from two principal component analyses performed on body distance and nondistance measures (modified from [61]). In sexual species, the phenotypic variation among individuals is mostly the result of genetic variation, whereas, in asexual hybrids, it results from differentially expressed alleles of a same genotype. (b) Putative genetic and epigenetic variation at four genes is represented for three individuals per species. Arrows refer to expressed genes, larger arrows to different alleles of an expressed gene (genetic difference), and blocks to silenced genes (epigenetic difference). (c) Under the General Purpose Genotype model, an epigenetically flexible genotype may provide a wide ecological niche for asexual hybrids, where each different epigenetic variant would occupy a narrower niche.

observed at two different genes that the *C. eos* allozyme was more expressed than the *C. neogaeus* allozyme.

Surveys of the transcriptome of *C. eos- neogaeus* hybrids have also given some preliminary evidence for differential allelic expression. Using cDNA-AFLP [81], we compared among hybrids the expression of (i) alleles common to both parental species (*C. eos-neogaeus* band found in *C. eos* and *C. neogaeus*) with that of (ii) alleles specific to one of the parental species (*C. eos-neogaeus* band found only in *C. eos* or *C. neogaeus*). In case (ii), it is possible to detect differential allelic expression whereas this is not possible in case (i) because of the dominance effect of AFLP. An absence of detection for (i) can therefore only mean that the gene is not expressed. A survey of cDNA fragments was performed on the muscle tissue of 26 genetically identical *C. eos-neogaeus* individuals. Out of 424 cDNA fragments, 75% were common to both parental species (i) while 25% were specific to one or the other parental species (ii). Interhybrid variation for the presence of these fragments was found at 10 species-specific loci (ii) (9.4%) but not at loci shared between species (i) (Fisher Exact Probability Test $P = 0.000003$) [82]. That the variation detected was only at allele-specific cDNAs suggests that, for a given tissue, differential allelic regulation among individuals could be more frequent than differential gene regulation.

As previously mentioned, it is assumed that asexual lineages will accumulate potentially deleterious mutations faster than sexual organisms because they do not possess recombination. Several studies have indeed demonstrated that asexual lineages accumulate potentially harmful mutations at a higher rate than their sexual congeners [83–85]. However, these studies did not demonstrate whether there was a phenotypic consequence to this increased mutation rate. What if it was possible to target these sequences containing mutations with DNA methylation? These potentially harmful mutations would be silenced, allowing asexuals to evade their phenotypic consequences [32, 53]. Silencing of deleterious mutations through DNA methylation could be particularly prevalent in polyploid asexuals. Many asexual lineages resulting from hybridizations are characterized by the presence of polyploids. If a polyploid organism gains a mutation in one of its gene copies, this mutation could be epigenetically silenced and the organism would still retain sufficient levels of expression through its two (or more) other copies.

These epigenetically masked mutations would represent some form of hidden genetic variation. Similarly to the evolutionary capacitance observed with Hsp90 [86], this hidden genetic variation could be exposed under certain conditions, leading to the production of new phenotypes. Such a mechanism could have allowed the accumulation of mutations in bdelloid rotifers characterized by the Meselson effect.

7. Heritability of Variation in DNA Methylation

The existence of environmentally induced epigenetic variation that can be transmitted to offspring poses a challenge to the modern evolutionary synthesis, which is based on the assumption that random genetic variation, impervious to environmental influences, is the only source of heritable variation in natural populations [87]. In this context, it has been argued that epigenetic variation must be heritable to be of evolutionary relevance (e.g., [33, 54]). Organisms from different taxa appear to be uneven in

their capacity for transgenerational epigenetic inheritance. In mammals, methylation reprogramming in mammalian primordial germ cells is quite extensive [88, 89]. Erasure of methylation patterns also occurs in zebrafish development [90]. Therefore, it seems there is a limited potential for DNA-methylation-driven transgenerational epigenetic inheritance in vertebrates. However, this erasure is not always complete and there are a few cases of transmission across generations of variation in DNA methylation in mammals [46, 54, 91].

The extensive reprogramming in DNA methylation observed in mammals is not common to all multicellular organisms. In plants, methylation resetting in the germ line is not as extensive and examples of inheritable variation in DNA methylation are more common [46, 53, 89]. Consistently, the variation in DNA methylation detected in asexual plants by Verhoeven et al. [36, 37] was transmitted across generations.

Even though their potential for epigenetic inheritance through DNA methylation is reduced compared to that of plants, epigenetic inheritance in animals (as well as plants) could be associated with histone marks or small RNAs transmitted in the oocyte and sperm [89]. For example, transmission of phenotypic variation to offspring by nongenetic factors was detected in bdelloid rotifers [92].

As previously mentioned, we did not assess whether the environmentally associated variation in DNA methylation observed in *C. eos-neogaeus* hybrids could be transmitted to offspring. However, even if this variation is restricted to a single generation, it could still be relevant to the persistence of these organisms.

Heritable epigenetic variation is useful if the environment is stable across generations. Environments are however rarely completely stable, and most individuals will have to deal with environmental stresses during their lives. Epigenetic modifications, by increasing the phenotypic spectrum of a given genotype, can provide an alternative way to respond to environmental fluctuations [20]. The relevance of epigenetic mechanisms would in this case lie in their capacity to create phenotypic plasticity, not adaptation. In such cases, it is not the epigenetic mark that is transmitted across generations but the genetically encoded capacity for creating epigenetic variation that can drive phenotypic plasticity. In this case, contrary to the case where epigenetic variation is inheritable, the nature of the heritable material remains genetic, which is not in contradiction with the modern evolutionary synthesis.

In this paper, we have argued that epigenetic modifications are an important mechanism for asexual organisms to face environmental variability. We have highlighted examples in genetically identical asexual organisms where variation in DNA methylation corresponded to environmental variation. Different taxa present different susceptibilities to transgenerational epigenetic inheritance. Epigenetic modifications do not need to be inheritable to be of relevance. In fluctuating environments, it could be favorable to wipe out at least some epigenetic marks every generation. Finally, epigenetic mechanisms, though they play a crucial role in the response to environmental variation, are most likely not the only factors involved in asexual persistence. Long-term survival is likely to be due to a combination of short-term epigenetic and long-term genetic processes.

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Review Article

How Can Satellite DNA Divergence Cause Reproductive Isolation? Let Us Count the Chromosomal Ways

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Satellites are one of the most enigmatic parts of the eukaryotic genome. These highly repetitive, noncoding sequences make up as much as half or more of the genomic content and are known to play essential roles in chromosome segregation during meiosis and mitosis, yet they evolve rapidly between closely related species. Research over the last several decades has revealed that satellite divergence can serve as a formidable reproductive barrier between sibling species. Here we highlight several key studies on Drosophila and other model organisms demonstrating deleterious effects of satellites and their rapid evolution on the structure and function of chromosomes in interspecies hybrids. These studies demonstrate that satellites can impact chromosomes at a number of different developmental stages and through distinct cellular mechanisms, including heterochromatin formation. These findings have important implications for how loci that cause postzygotic reproductive isolation are viewed.

1. Introduction

Decades ago when researchers began purifying DNA from eukaryotes using cesium chloride gradients, they observed bands of DNA that were distinct from the major genomic bands. The sequences comprising these ancillary bands were named satellites—a term from Greek meaning "followers of a superior entity"—and were found to separate from the other sequences due to their adenosine- and thymine-rich base pair compositions. Since their discovery, satellites have proven to be one of the most intriguing parts of the genome, owing to their high abundance, rapid evolutionary change, and a growing body of evidence indicating that they can impact speciation.

The abundance of satellites varies widely in eukaryotic genomes, from effectively 0% in yeast species such as *Schizosaccharomyces pombe* to 25–50% or more in Drosophila and mammalian species [2–4]. Individual satellite monomers also vary dramatically in their monomer length, from the *D. melanogaster* pentameric monomer, AATAT, to more complex monomers such as the 972-bp centromeric satellite in the Indian muntjac [5]. Satellite monomers such as these are organized into arrays, or blocks, of tens to thousands of tandem copies located in the centromeres, the telomeres, and their surrounding regions. Indeed, the Y chromosome in many higher eukaryotes consists almost entirely of satellites. Despite their abundance, satellites are nonprotein coding and were therefore hypothesized to be genomic "junk" [6] or even selfish genetic elements [7]. Contrary to the former idea, the chromosomal regions consisting of satellites are now known to play important but incompletely understood roles in the structure, stability, and segregation of the chromosomes [8–10]. The idea that satellites are selfish elements remains to be determined.

Given the high abundance of satellites and their involvement in chromosome behavior, it is intriguing that these sequences make up one of the most rapidly evolving parts of the genome. Studies conducted over the last four decades have revealed large disparities in satellite abundance between closely related species within insect, mammal, and plant groups [11–16]. Owing to rapid expansions and contractions in copy number, specific satellite blocks may be either severely reduced in size or altogether absent in close relatives (Figure 1) [1, 13, 17, 18]. Additionally, the monomers of

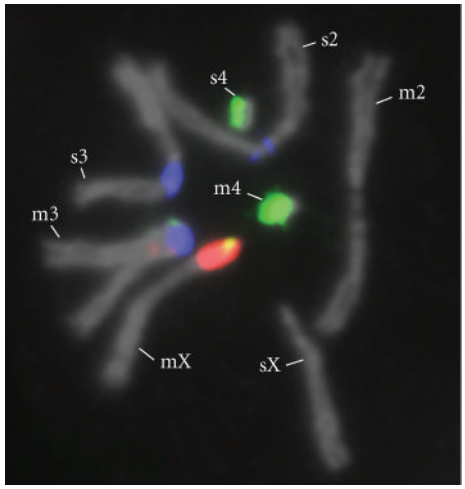

Figure 1: Satellite block divergence between *Drosophila melanogaster* and *D. simulans*. Each chromosome pair, consisting of one homologous chromosome from each species, shows remarkable satellite differences: the *D. melanogaster* X contains a large block of the 359-bp satellite (red) and some AATAT (green) while the *D. simulans* X contains neither of these specific satellite monomers; dodeca satellite (blue) is present on the *D. melanogaster* 2nd chromosome and absent on the *D. simulans* 2nd chromosome; large regions of dodeca satellite are present on the 3rd chromosomes of both species, but only *D. melanogaster* 3rd chromosome has small regions of AATAT (green) and a small region of 359-bp variant (also red); AATAT satellite (green) is more abundant and distributed widely across the *D. melanogaster* 4th chromosome while the *D. simulans* 4th chromosome contains two primary regions of AATAT, which cannot be fully seen in this image, and in smaller amounts. Chromosomes were prepared from mitotic brain cells of hybrid larvae and stained by fluorescence in situ hybridization (FISH) as previously described [1].

some complex satellites can differ in sequence composition between closely related species at levels higher than the average genome-wide divergence [19]. However, certain regions of some centromere satellite monomers and even whole monomers are highly conserved, perhaps out of necessity to maintain their interactions with centromere-associated proteins [20–22].

Various mechanisms, including unequal recombination, gene conversion events, and replication slippage, have been proposed to explain how individual satellite blocks can evolve rapidly [23, 24]. These processes can generate satellite blocks of widely varying sizes (i.e., those containing different copy numbers) within a given species. This variation can influence chromosome dynamics and individual fitness in a number of different ways. For example, large blocks of the *D. melanogaster* Responder (*Rsp*) satellite can be deleterious under certain genetic conditions. Located on the *D. melanogaster* 2nd chromosome, the *Rsp* block is highly variable, ranging from ~10 to over 3,000 monomers per block among individuals [25]. Second chromosomes carrying large *Rsp* blocks are targeted for destruction during spermatogenesis if the other 2nd chromosome carries a selfish allele of the Segregation Distorter (*Sd*) gene and a small *Rsp* block. This effect results in the loss of half the sperm—those carrying the large *Rsp* block—and, thus, high transmission frequencies of the *Sd*-carrying chromosome. In contrast, variants of other satellite blocks may be functionally important for chromosome function and the fitness of the individual. One such case is the 359-bp satellite block on the X chromosome of *D. melanogaster*, which is located immediately adjacent to the rDNA locus and may play a role in regulating expression of the rDNA genes [26]. Finally, satellites can expand without affecting chromosome function. This trend appears to be true for satellites present on supernumerary B chromosomes, such as the Paternal Sex Ratio (PSR) chromosome in the jewel wasp, *Nasonia vitripennis* [27, 28]. Since this chromosome is not essential for the viability of its host, the satellites on them may be free from functional constraints and, therefore, able to expand and contract rapidly without effect.

These observations raise a compelling question—how can rapid changes in satellites affect the biology of their resident chromosomes and, ultimately, the organisms in which they reside? One context in which this question can be addressed is the impact of satellite divergence on interspecies hybrids. Early studies demonstrated that certain reproductively isolated species—that is, those that fail to produce fertile or viable hybrid offspring when they intermate—can exhibit large differences in composition and organization of their satellite blocks [1, 11–14]. These observations led to the suggestion that satellite divergence may contribute to speciation by causing reproductive isolation between species [11, 29]. Is there any validity to this idea, and if so, how might such an effect occur?

In addressing these questions, we describe three general ways in which satellite differences between species could affect chromosome behavior in hybrids: (i) by disruption of chromosome pairing, (ii) by alteration of the chromatin structure of the satellites themselves or their surrounding sequences, or (iii) by involvement of satellites in meiotic or post-meiotic chromosome drive systems. We cite data from previous studies, primarily in Drosophila but also other organisms, that either support or argue against these possibilities. We also describe plausible molecular mechanisms that may underlie these effects. These examples provide new ways of viewing the types of loci that cause reproductive isolation and how they can evolve and operate at the molecular level in hybrids.

2. Disruption of Chromosome Pairing

One process that satellite divergence may affect in hybrids is homolog pairing, whereby similar sequences associate together in close proximity across homologous chromosomes. Pairing is a key aspect of meiosis, and much of what is known about pairing during meiosis derives from studies in *D. melanogaster*. During meiosis I in this organism, pairs of homologous chromatids align side by side at the metaphase plate before they segregate into daughter nuclei. The pairing of homologous sequences occurs before entry into meiosis and is ultimately important in Drosophila and other eukaryotes across the phyla for proper segregation of

chromosomes and, therefore, the formation of functional gametes [30].

There are, however, fundamental differences between male and female meiosis in flies that reflect to what degree satellite divergence may affect homolog pairing. In the pure species *D. melanogaster*, the involvement of repetitive sequences in pairing varies depending on the sex of the individual and the particular chromosome pair. For example, recombination occurs only in the female sex. Thus, synaptonemal complexes and chiasmata, or stable crossover junctions that help to hold the recombining homologs together before segregation, do not form in males [31]. The lack of these structures in males originally suggested that sequence specific interactions must instead dictate chromosome pairing in this sex [32, 33]. Years of work on this topic have shown that small "pairing sites" mediate homolog pairing in males. These sites include sequences found in the gene-containing regions of the autosomes and a single cluster of rDNA spacer repeats on the X and Y chromosomes [33, 34]. However, no data has been found to link satellite DNA or the pericentric regions where they are located with homolog pairing in male meiosis.

In contrast to male flies, satellites may play an important role in meiotic homolog pairing in female flies. Experiments in which recombination, and thus, chiasmata are prevented from forming either through mutations abrogating recombination or through chromosomal inversions revealed that pairing occurs without these structures (reviewed in [35]). Additionally, the 4th chromosomes are largely achiasmatic. Thus, pairing in females is determined not by recombination-mediated structures but instead by sequence-specific interactions. Deletions of the satellite-containing X and 4th pericentric regions, but not the gene-containing regions, were shown to disrupt meiotic homolog pairing in females [35]. Thus, unlike in males, pericentric repetitive sequences may play a strong role in homolog pairing in females.

The fact that the pericentric regions do not influence homolog pairing in pure species *D. melanogaster* males leads to the strong expectation that interspecies divergence of satellite DNA would not affect pairing in Drosophila hybrid males. However, the involvement of these regions in female meiosis legitimizes early speculation that substantial differences in satellites may inhibit meiotic homolog pairing in Drosophila hybrid females [29]. Is there any experimental evidence for these predictions? *D. melanogaster*/*D. simulans* hybrids of either sex normally do not produce gonads, thus precluding the analysis of homolog pairing in these individuals. In order to circumvent this problem, partial male hybrids—those carrying small chromosomal regions or single chromosomes from one species in the genetic background of the other species—were produced [36]. Of particular interest was one type of partial male hybrid containing both the *D. melanogaster* and *D. simulans* 4th chromosomes. These interspecific homologs were found to pair and segregate normally during meiosis [36] despite substantial differences in their satellite DNA content [13]. This result is consistent with the lack of involvement of repetitive sequences in meiotic homolog pairing in *D. melanogaster* pure species males.

Currently, only a few other animal and plant hybrids have been examined. These analyses have focused primarily on the male sex, and while mispairing has been observed in some cases, the findings generally do not support a role of satellite divergence as a cause. In mice, male hybrids produced from *Mus musculus* and *M. poschiavinus* showed normal homolog pairing despite substantial, genome-wide differences in repetitive sequences [37]. In another case, *M. domesticus*/*M. spretus* male hybrids exhibited defective X-Y pairing [38]. The causal locus was mapped to a region near the cytological point of pairing between these chromosomes in the pure species. This finding suggested that a single pairing site, similar to the one that determines pairing of the X and Y in *D. melanogaster* males, is solely involved. In plants, crosses between species belonging to the Paeonia genus revealed incomplete homolog pairing in several different species combinations [39]. Because no major chromosomal inversions were found between these species, it was concluded that mispairing likely resulted from interspecies divergence of pairing genes. However, divergence of repetitive sequences was not discussed as formal possibility.

Taken together, the above results suggest that satellite divergence does not affect meiotic homolog pairing in hybrids under certain species-, sex-, and chromosome-specific contexts. However, additional experiments are needed in other contexts, such as X or 4th homolog pairing in Drosophila hybrid females, in which there is a strong precedence for expecting such an effect. Studies employing specific mutations that allow *D. melanogaster*/*D. simulans* hybrid females to develop functional gonads [40, 41] will be helpful in more fully addressing the impact of satellite divergence on meiotic homolog pairing.

Homolog pairing also occurs in the somatic tissues of Dipterans [42]. It has been proposed that somatic homolog pairing may play a role in the repair of double strand DNA breaks, the transitioning of premeiotic cells into meiosis, or transchromosome gene interactions [34, 42, 43]. Similar to meiotic pairing in females, pairing in somatic cells occurs between the pericentric regions in *D. melanogaster* [44]. What drives these interactions is not clear, but one possibility is high similarity of repetitive sequences between homologous chromosomes. This idea was argued against, however, by the results of one study in which a ~1.6 megabase pair block of AAGAG satellite located on the tip of the rearranged *D. melanogaster* 2nd chromosome, bw^{D}, was recombined onto the *D. simulans* 2nd chromosome and placed into the *D. simulans* genome [45]. In the *D. melanogaster* pure species, this satellite block associated with the pericentric region of the same 2nd chromosome, which also contains several blocks of AAGAG. When placed into the *D. simulans* genome, the bw^{D}-derived AAGAG block associated with the pericentric region on the 2nd chromosome of this species, despite the fact that it does not contain AAGAG satellite DNA. Moreover, the bw^{D}-derived AAGAG block did not associate with either of the *D. simulans* sex chromosomes, which do contain AAGAG satellite DNA. It was concluded from these results that pairing in somatic cells might not result from similarity of homologous sequences, but instead,

through sequence-independent attractive forces between large regions of repetitive DNA.

This conclusion may only partially explain somatic homolog pairing. Sequence-independent pairing alone would be expected to result in inappropriate associations of nonhomologous chromosomes during mitosis, and their missegregation, since all chromosomes in flies contain large amounts of repetitive sequences in their pericentric regions [11, 13]. A more likely scenario may be that both sequence-dependent and independent interactions govern pairing in somatic cells. Previous experiments have demonstrated that somatic pairing in the *D. melanogaster* pure species occurs at specific pericentric regions, such as the *Rsp* locus as well as AACAC and AAGAC satellite blocks [44]. Interestingly, the *Rsp* block is not present on the 2nd chromosome in *D. simulans* [46], and other pairing sequences may also be unique or substantially different between these species. Thus, the *D. simulans*/*D. melanogaster* hybrid is a promising system for taking advantage of these satellite differences in order to more fully explore the effects of satellite divergence on somatic homolog pairing.

3. Alteration of Chromatin Structure I: Satellite DNA/Protein Interactions

Another fundamental aspect of chromosome dynamics is the formation of chromosomes from chromatin. Occurring at entry into mitosis and meiosis, this process involves a number of structural proteins including Condensins and Topoisomerases [47]. These factors become distributed across the entire axes of the chromosomes as they condense at prophase. Other proteins, however, localize to discrete chromosomal regions, such as satellite blocks. For example, the *D. melanogaster* GAGA factor binds to AAGAG and AAGAGAG satellite monomers located in discrete regions on all of the chromosomes in this species [46]. GAGA factor and other satellite-binding proteins, such as Prod, are also transcription factors [48, 49].

The nature of these satellite DNA/protein associations is not well understood. However, it has been proposed that satellite-binding transcription factors may play a role in bending or packaging satellite DNA [26, 50, 51]. This idea is supported by the observation that loss-of-function mutations in the gene encoding GAGA factor result in severe chromosome decondensation and segregation failure [52]. Additionally, this result is consistent with the fact that GAGA associates with the FACT complex, which together may play a more global role in chromatin packaging of repetitive sequences [53].

A potential effect of satellite divergence is that it can drive coevolutionary changes in satellite-binding proteins within the pure species [21, 54]. According to this model, the sets of satellites and their binding proteins will evolve independently from those of different species. A consequence of these independent evolutionary trajectories is that a diverged protein from one species may not properly bind a satellite variant of another species in the hybrid background. This loss-of-function effect may occur particularly in cases in which satellite-binding proteins from only one parental species are expressed in hybrids, such as proteins encoded by X-linked genes in hemizygous males or proteins that are maternally contributed in the egg cytoplasm. Similar effects might also be expected to result in cases where a protein from one species is expressed at low levels or not at all so that satellite DNA is insufficiently packaged. Such a case has not yet been demonstrated in hybrids, but is a formal possibility and might resemble chromatin defects caused by mutational loss of GAGA factor in *D. melanogaster* [52]. Alternatively, deleterious gain-of-function interactions may occur, such as if a satellite-binding protein from one species associates inappropriately either with a diverged or functionally unrelated satellite or with a chromatin-modifying enzyme of another species.

Compelling evidence of a satellite DNA/protein incompatibility was revealed through studies of the Odysseus-site homeobox (OdsH) protein in Drosophila hybrids. Crosses between *D. simulans* males and *D. mauritiana* females produce F1 hybrid males that are sterile. Interspecies cloning strategies identified *D. mauritiana* OdsH (OdsHmau), located on the X chromosome of this species, as a causal locus [55]. Although its function is unknown, OdsH is homologous to Unc-4, a known transcription factor, and is expressed in the apical end of the testes where the mitotic divisions preceding meiosis occur [56, 57]. Transgenic analysis revealed functional divergence between OdsH orthologs and the satellite DNA sequences to which it binds in each of these species. When expressed transgenically in *D. simulans* cells, OdsHsim and OdsHmau associated with similar satellite DNA regions on the X and 4th chromosomes [58]. However, OdsHmau bound to many additional regions on the *D. simulans* Y chromosome [58]. The specific amino acid changes between OdsH orthologs that give rise to their different binding patterns are not known, although substantial sequence divergence was discovered in the OdsH DNA-binding homeodomain [55]. OdsHmau recognizes only a small region of satellite DNA on the *D. mauritiana* Y-chromosome, suggesting that the sequences to which it binds have undergone expansion across the *D. simulans* Y chromosome [58]. Thus, interspecies divergence of both OdsH and its associated satellite DNAs appears to underlie these different binding patterns between *D. simulans* and *D. mauritiana*.

It is currently unclear if hybrid sterility in this case results directly from differential OdsH binding to Y chromatin or to malfunction of an additional role of OdsH in the male germ line. However, several observations support the former possibility. First, deletion of the OdsH gene in *D. melanogaster* has little or no measurable effects on male fertility, demonstrating that OdsH is not an essential gene [56]. Second, the *D. simulans* Y becomes abnormally de-condensed in the presence of OdsHmau [58]. This effect could prevent the other chromosomes from segregating properly in the divisions preceding meiosis, thus leading to improper formation of sperm.

How might OdsHmau induce Y decondensation? One possibility is that this protein may bind satellites on the *D. simulans* Y that it normally binds on the *D mauritiana* Y, but expansion of these sequences in the former species may lead

to a chromosomal overloading of OdsHmau. Alternatively, OdsHmau may associate with expanded sequences on the *D. simulans* Y that are distinct from those that it normally binds in *D. mauritiana*. In either case, high concentrations of OdsHmau may disrupt normal localization of other essential chromatin proteins. Identification of OdsH polymorphisms that cause differential DNA binding, and the specific satellite DNA sequences and other chromatin proteins that OdsH interacts with in each species, will be helpful in exploring these possibilities.

4. Alteration of Chromatin Structure II: Heterochromatin-Related Effects

Another potential effect of satellite divergence in hybrids is disruption of heterochromatin. This term describes the exceptionally dense form of chromatin that packages satellites and other highly repetitive sequences during interphase (for a full review, see [59]). Two primary molecular features that define heterochromatin and govern its compact nature are (i) specific posttranslational Histone modifications and (ii) a small set of associating non-Histone proteins. The basic unit of chromatin is the nucleosome, consisting of DNA wrapped around an octamer of the Histone proteins H2A, H2B, H3, and H4. In heterochromatin, the C-terminal "tail" of Histone H3 carries methyl groups on Lysine residues 9 and 27. Added by Histone Methyltransferases (HMTs), these methyl groups serve as binding sites for non-Histone proteins such as the heterochromatin protein 1 (HP1) and its protein family members [60, 61]. It is believed that the association of HP1 with nucleosomes leads to the compact nature of heterochromatin [62, 63]. In addition to binding methylated Histone H3, HP1 also binds SU(VAR)3-9, a HMT, thereby recruiting this enzyme to chromatin where it can insure methylation of Histone H3 [64, 65]. Thus, the interactions of these proteins with one another and with the nucleosomes constitute a self-regulatory system that maintains the heterochromatic state, which can be epigenetically transmitted through cell lineages.

Support for the idea that satellite DNA divergence can disrupt heterochromatin stems from studies of the *D. melanogaster Zygotic hybrid rescue* (*Zhr*) locus. Crosses between wild type *D. melanogaster* males and *D. simulans* females produce hybrid daughters that die during the cleavage divisions of early embryogenesis [66]. Previous genetic studies mapped a causal locus, *Zhr*, to a position near the centromere of the *D. melanogaster* X-chromosome [67]. Based on these and other genetic experiments [68, 69], it was proposed that *Zhr* consists of repetitive sequences in this region, a novel idea given that many of the known loci involved in reproductive isolation are protein-coding genes [55, 70–72]. More recent cytological analyses have supported this idea, demonstrating the presence of highly stretched region of 359-bp satellite DNA located on the *D. melanogaster* X during anaphase of mitosis in dying hybrid embryos [1]. This satellite region was found to prevent separation of the *D. melanogaster* sister X chromatids, inducing chromosome bridges and mitotic arrest (Figure 2).

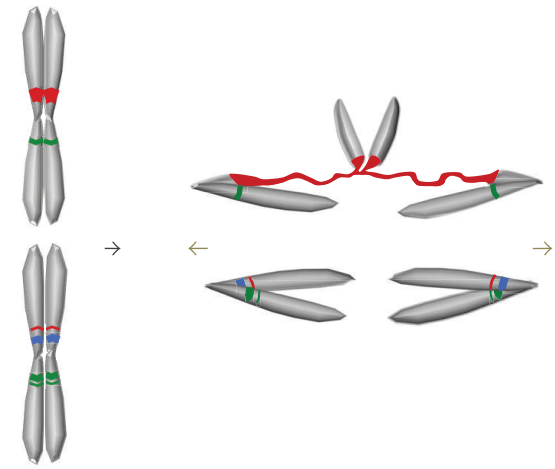

Figure 2: Disruption of mitotic chromosome segregation in hybrid embryos caused by satellite chromatin defects. Chromatid pairs line up at the metaphase plate for segregation at anaphase (left of arrow). The top chromatids fail to segregate due to defective chromatin structure of the red satellite block (right of arrow). This phenotype is analogous to that involving the 359-bp satellite block in *D. melanogaster*/*D. simulans* hybrid embryos [1] and results from an incompatibility between a *D. melanogaster*-specific satellite and a putative chromatin-related factor in the *D. simulans* egg cytoplasm.

Two specific findings support the idea that these defects are due to improper heterochromatin formation. First, Topoisomerase 2 (Top2) was found to accumulate abnormally on the stretched 359-bp satellite block [1]. In addition to its enzymatic role in relieving supercoiled DNA, Top2 is a structural chromatin protein [73, 74]. In *D. melanogaster*, this protein is normally enriched on 359-bp satellite DNA at interphase and becomes evenly distributed across the chromosomes during mitosis [1]. In hybrids, however, Top2 remains abnormally localized to 359-bp satellite DNA throughout the cell cycle [1]. It is unlikely that *D. simulans* Top2, which is the only form present in the hybrid maternal cytoplasm, is the proximal cause, since this protein is highly conserved between *D. melanogaster* and *D. simulans* [1]. Moreover, hybrid females of the reciprocal cross are fully viable. Although only *D. melanogaster* Top2 is present in the egg cytoplasm of these individuals, *D. simulans* Top2 is expressed during later developmental stages while in the presence of the 359-bp satellite block, without deleterious effect.

Second, the observed chromosomal defects occur at the developmental period when heterochromatin forms. In Drosophila, heterochromatin formation is marked by visible changes in chromatin density during early embryogenesis. The first 14 rounds of mitosis in this organism occur in a common cytoplasm derived from the egg before the nuclei individualize through the acquisition of their own plasma membranes [75]. These early divisions proceed under the control of factors present in the maternal cytoplasm until the beginning of zygotic gene expression, which occurs during mitotic divisions 12–14. Heterochromatin formation is marked by the appearance of dense regions of chromatin known as chromocenters during mitotic divisions 9-10

[76, 77]. It is precisely during these divisions when the first chromosome bridges appear in hybrid female embryos [1].

Why might heterochromatin of the 359-bp satellite block fail to form in hybrids? One possibility is that some component(s) of the general heterochromatin machinery present in the *D. simulans* maternal cytoplasm are incapable of recognizing this *D. melanogaster*-specific satellite block. Although there is some precedence for this scenario in other systems [78], it is unlikely in this case for several reasons. First, the chromosome bridges in hybrid embryos appear during mitotic cycles 9-10, before HP1 and methylation of Histone H3 normally appear on the chromocenters [77]. Another general heterochromatin protein, SU(VAR)3-3, which is a homolog of the yeast demethylase LSD1, was recently shown to form foci in interphase nuclei as early as mitotic cycle 8, before bridge formation [79]. To our knowledge, however, this protein has not yet been examined for involvement in hybrid lethality. Second, the known protein components and posttranslational modifications to Histone H3 in heterochromatin, with few exceptions, are highly conserved from yeast to vertebrates [80]. This pattern stands in sharp contrast to the wide range of different satellite DNA sequences that exists within the genomes of most individual eukaryotic species, in all of which the heterochromatin machinery must properly package the entire sets of these sequences. It is, therefore, unlikely that the 359-bp satellite block poses challenges to the general heterochromatin machinery encoded by *D. simulans*.

An alternative explanation may involve small, noncoding RNAs. Studies in *S. pombe* demonstrated that small RNAs derived from centric and pericentric repeats and the proteins that produce these small RNAs are essential for normal heterochromatin structure and centromere function [81]. It was proposed that these small RNAs facilitate heterochromatin formation and maintenance by recruiting the heterochromatin machinery to their complementary sequences for proper packaging. Experimental evidence for this model has since been documented in a number of additional organisms including *Arabidopsis thaliana* and *D. melanogaster* [82–85]. Small RNAs derived from the 359-bp satellite have been detected in the maternal cytoplasm of young *D. melanogaster* embryos [84, 85]. It was proposed that these small RNAs facilitate heterochromatin formation of the 359-bp satellite block in *D. melanogaster* [1, 82–84]. Moreover, the lack of the 359-bp small RNAs in the *D. simulans*-derived maternal cytoplasm of lethal hybrids may lead to mispackaging of this satellite block [1, 86]. One appeal of this model is that it takes into account the specificity of the observed defects, which appear confined to the 359-bp satellite block; all other sequences in hybrids appear normally packaged [1]. The fact that only this satellite block exhibits packaging defects in hybrids may be due to its large size, comprising nearly one half of the pericentric region on the *D. melanogaster* X. Other satellite DNAs unique either to *D. melanogaster* or *D. simulans* may incur problems in heterochromatin packaging but they may not be present in enough copies to alter chromosome segregation.

Finally, the effects of 359-bp satellite DNA in hybrids may be tied to heterochromatin through parental imprinting. Best studied in mammalian eukaryotes, imprinting is a phenomenon that results in differential expression of certain genes when inherited from either the mother or father. In Drosophila, parental imprinting does not affect protein-coding genes, but instead involves the heterochromatic regions of the X- and Y-chromosomes (reviewed in detail in [87]). Imprinting effects in flies include differential levels of silencing of visible genetic markers that are located near these particular regions of heterochromatin. For example, the *scute* gene, located near the pericentric heterochromatin of the inverted X chromosome, *In* (1) sc^8, is expressed at lower levels when paternally inherited compared to transmission from the mother [88, 89]. Similar parental effects of reporter genes located within Y heterochromatin have also been observed [90, 91]. The nature of heterochromatic imprinting is not understood but may involve sex-specific differences in H3K9 methylation of heterochromatin that are established during gamete formation and/or early development [87].

It is possible that the imprint of specific heterochromatic regions like the 359-bp satellite block may not be properly "interpreted" by the *D. simulans* maternal cytoplasm, resulting in the observed heterochromatin defects of this satellite in hybrids. One possible scenario is that the *D. simulans* cytoplasm fails to recognize *D. melanogaster*-specific Histone methylation or another unknown epigenetic mark on this satellite, which might be needed for proper heterochromatin packaging. Currently the Histone methylation state of the 359-bp heterochromatin has not been studied in hybrid embryos. However, a prediction based on the above hypothesis is that transmission of the 359-bp satellite block through the *D. simulans* maternal cytoplasm would result in suppression of packaging defects. Consistent with this prediction is the fact that hybrid females of the reciprocal cross, between *D. melanogaster* females and *D. simulans* males, are completely viable. In this case, the 359-bp satellite block should be imprinted maternally through the *D. melanogaster* egg cytoplasm. However, it is important to point out that the viability of reciprocal female hybrids is also consistent with mechanisms involving diverged satellite-binding proteins or repeat-derived small RNAs outlined above.

5. Release of Meiotic and Postmeiotic Drive Systems

Under normal circumstances, homologous chromosomes are segregated equally into gametes. However, some loci are capable of altering chromosome segregation during or after meiosis in order to selfishly transmit themselves at unusually high frequencies. In these cases, satellite variants can be either the targets of drive or the driving elements themselves (Figure 3).

One well-known example of postmeiotic drive involving satellites is the Segregation Distorter (SD) system in *D. melanogaster*. The selfish component of SD is a duplicated gene on chromosome 2 encoding a truncated RanGAP protein [92]. In males that are heterozygous for this mutant allele, *Sd*, and the wild type allele, Sd^+, the entire half of the spermatids containing the Sd^+ allele exhibit chromosome

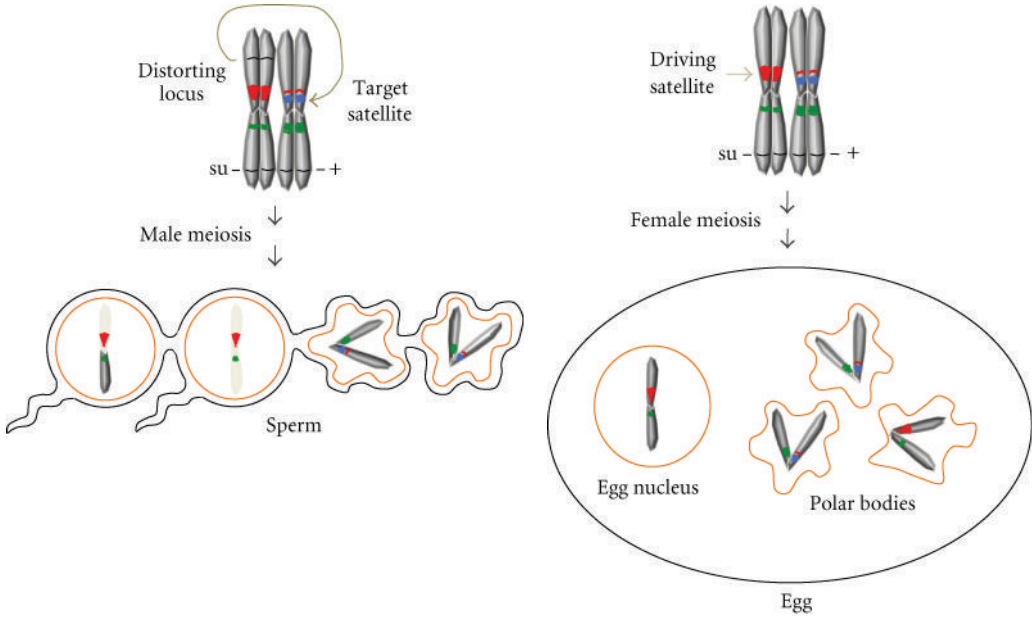

Figure 3: Segregation distortion in hybrid animals. (a) Postmeiotic release of segregation distortion in hybrid males. A recessive suppressor of distortion (su) in one species becomes inactive in the heterozygous hybrid. This allows the distorting locus to target a satellite block on the chromosomes of the other species (top). This effect results in spermatid bundles (bottom) in which spermatids inheriting the targeted chromosome fail to individualize. The spermatids carrying the chromosome with the distorting locus develop normally. (b) Release of meiotic drive in hybrid females. A recessive suppressor becomes heterozygous in the hybrid female. This enables a chromosome from one species, which carries a "selfish" satellite, to outcompete the homologous chromosome from the other species. As a result, the egg nucleus will carry a chromosome with the selfish satellite, and chromosomes lacking these satellites will end up in the unused polar bodies.

condensation defects and they fail to mature. Thus, only chromosomes carrying the selfish *Sd* allele are transmitted. *Sd* does not target the Sd^{+} allele itself, but instead, a closely linked satellite block consisting of a 240-bp monomer known as Responder (*Rsp*). *Rsp* satellite blocks consisting of ~200 to 3,000 or more monomers (termed Responder-sensitive or Rsp^{S}) are targeted, whereas smaller blocks (Responder-insensitive or Rsp^{I}) are unaffected [25]. This effect favors *Sd* since it is linked to Rsp^{I} blocks, whereas Sd^{+} is often linked to Rsp^{S} blocks. It is currently not known how *Sd* targets Rsp^{S} satellite blocks at the molecular level, but may involve mislocalization of *Sd*-encoded RanGAP that leads to chromosome decondensation through a number of possible mechanisms [86, 93, 94].

Distorting loci like *Sd* may eventually harm individuals and populations, such as when distorters are closely linked to deleterious alleles, or if distortion involves the sex chromosomes, thus affecting the sex ratio balance in populations, respectively. As a counter, unlinked suppressors of distortion may evolve. Suppressors are effective until mating occurs with individuals that do not carry them, in which case suppression is lost and the driving phenotype is unleashed (Figure 3(a)). In agreement with this idea, several different masked distortion systems have been identified through both interstrain and interspecies Drosophila crosses [94, 95]. In these cases, the targets of distortion are not known, but may involve species-specific satellites since defects in spermatogenesis are highly similar to those present in *Sd* distortion [94].

Distorting loci can also be the satellites of centromeres or their adjacent regions. One process in which these sequences are thought to be particularly prone to non-Mendelian segregation is female meiosis. This is due primarily to the fact that meiosis in females is asymmetric; four meiotic products are produced but only one becomes the egg's hereditary material, while the other three products form polar bodies and are eliminated. It has been proposed that certain centromeric satellite variants can take advantage of this asymmetry by outcompeting other sequences for extraordinarily high rates of transmission into the egg's nuclear material (Figure 3(b)) [96–98].

Non-Mendelian segregation of certain alleles during female meiosis has been detected genetically in a number of organisms [99–102]. However, the most direct evidence for meiotic drive of repetitive elements stems from one study in Mimulus (monkeyflower) species hybrids. Crosses between *Mimulus guttatus* and *M. nasutus* resulted in release of a suppressed meiotic driver locus on the *M. guttatus* chromosome 2 that approaches transmission of 100% [103]. Genetic and cytological mapping revealed that the driving element is located in or immediately adjacent to the centromere, consistent with the possibility that the element is a satellite [102]. Interestingly, this driving allele is associated with a fitness cost in hybrid males. In the pure species, such deleterious effects may prevent selfish elements from reaching fixation before driving suppressors can evolve. Future molecular and cytological studies in this system will help to test existing

models that predict how meiotic drive might occur at the molecular and cellular levels [98, 104].

6. Satellite Divergence and the Dobzhansky-Bateson-Muller Model of Hybrid Incompatibility

Early work by Dobzhanksy, Bateson, and Muller provided the foundation for a genetic model that explains the evolution of hybrid sterility and lethality [105]. The simplest form of this model involves a pair of loci, each of which has diverged functionally between sibling species. The products of these loci malfunction when expressed together in hybrids, leading to developmental defects that cause sterility or lethality. Such interspecies molecular interactions that reduce hybrid fitness are referred to as hybrid incompatibilities (HIs). Over the past decade, a number of HI loci have been identified. Some of these loci encode proteins [106]. It was proposed that HI loci encoding transcription factors cause large-scale misregulation of gene expression in *D. simulans*/*D. melanogaster* hybrids [70], although this was later shown to not be the case [107]. Other models implicate deleterious interactions between proteins encoded by HI loci [108]. In general, much remains to be uncovered mechanistically regarding the majority of HI cases that involve protein-coding genes.

A number of studies discussed here have documented the negative effects of satellite divergence on chromosome behavior in hybrids. The results from these studies have demonstrated that satellites, like protein-coding genes, can operate as HI loci. The biology of satellites is complex, with a diverse array of associated factors including general and specific heterochromatin proteins, small RNAs, and epigenetically modified histones that are often developmentally regulated. This complexity offers researchers new ways to envision how HI might occur in hybrids and new HI candidates to test.

At the core of the evolution of such HI cases may be a scenario in which rapidly evolving satellite sequences force their packaging or associating proteins to evolve equally rapidly in order to preserve chromosome function in the pure species. However, proteins—or perhaps other factors—adapted to satellites from one species may interact inappropriately with diverged satellites from another species in hybrids, thus causing HI. The complex nature of satellite heterochromatin is consistent with previous speculation that most HI interactions may be more complex than the two-locus model [109]. Reciprocally, however, the existence of satellite HI loci may also offer more simplified views of HI, such as an HI locus pair consisting of satellite DNA in one species and the absence of complementary small RNAs in the other species. Indeed, satellite DNA may even be regarded as a special type of HI locus because it can direct its own packaging by generating small RNAs, thus operating as both the cause and suppressor of HI [86].

Given the functional involvement of satellites in chromosome dynamics and their evolutionarily labile nature, it is no surprise that these sequences make up a common type of reproductive isolating locus. Further exploration will, no doubt, be challenging due to difficulties in manipulating sate-llite sequences and the epigenetic states of heterochromatin, but they will progressively reveal a more detailed picture of how these hybrid incompatibilities occur at the molecular level.

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Review Article

Homologue Pairing in Flies and Mammals: Gene Regulation When Two Are Involved

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Chromosome pairing is usually discussed in the context of meiosis. Association of homologues in germ cells enables chromosome segregation and is necessary for fertility. A few organisms, such as flies, also pair their entire genomes in somatic cells. Most others, including mammals, display little homologue pairing outside of the germline. Experimental evidence from both flies and mammals suggests that communication between homologues contributes to normal genome regulation. This paper will contrast the role of pairing in transmitting information between homologues in flies and mammals. In mammals, somatic homologue pairing is tightly regulated, occurring at specific loci and in a developmentally regulated fashion. Inappropriate pairing, or loss of normal pairing, is associated with gene misregulation in some disease states. While homologue pairing in flies is capable of influencing gene expression, the significance of this for normal expression remains unknown. The sex chromosomes pose a particularly interesting situation, as females are able to pair X chromosomes, but males cannot. The contribution of homologue pairing to the biology of the X chromosome will also be discussed.

1. Introduction

One of the most intriguing aspects of somatic homologue pairing is that such a basic condition has enormous variability between species. Homologues pair vigorously in *Drosophila*, as illustrated by the remarkable alignment of polytene chromosomes. In fact, homologue pairing is pervasive throughout the Diptera, but in other organisms the occurrence and extent of homologue pairing is often unknown [1, 2]. Close association of homologous chromosomes in vegetative diploid budding yeast has been reported, but a careful reexamination suggested that little, if any, pairing occurs [3]. In diploid fission yeast both homologues occupy the same chromosome territory and centromeric pairing is observed in most cells [4]. Early studies suggested somatic homologue pairing in numerous plant species (Reviewed in [2]). Recent work supports the idea of homologue pairing in some grains and fungi, but also casts doubt on other reports of pairing in plants [5–8].

2. Mammals: Pairing to Share Information

Mammals have perhaps the most elaborate manifestation of homologue pairing. While complete pairing of the mammalian genome is not reported outside of the germline, somatic pairing of specific chromosomal regions does occur, but is tightly regulated. For example, homologous association of pericentromeric regions of human chromosome 1 is detected in cerebellar, but not cerebral, tissue [9]. Heterochromatic regions of chromosomes 8 and 17 also pair in parts of the brain (Figure 1(a)) [10, 11]. Chromosome-specific pairing of chromosome 7 and 10 is also seen in case of cell line derived from follicular lymphoma [12]. Several cell lines derived from renal carcinomas display an abnormal pairing of one arm of chromosome 19 and misexpress genes within the paired region (Figure 1(b)) [13]. This suggests that modulation of homologue associations may be necessary for normal gene regulation. The mechanism of pairing in these examples has not been investigated. However, this type of pairing is

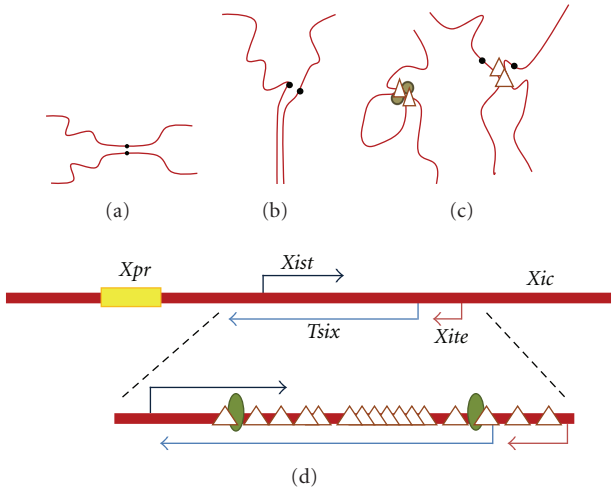

Figure 1: Modes of somatic pairing in mammalian tissues. (a) Pericentromeric homologue pairing in parts of the brain. Centromeres are depicted by black dots. (b) Abnormal pairing of chromosome 19q in renal carcinoma. (c) Looping between two sites on a chromosome (left) and interchromosomal contacts (right) are mediated by sequence-specific DNA-binding proteins such as CTCF (triangle) and cohesin (brown circle). (d) Pairing of the *X inactivation center* (*Xic*) initiates X chromosome inactivation in females. Sequences that participate in *Xic* pairing are depicted. The *X-pairing region* (*Xpr*, yellow) initiates *Xic* pairing. *Tsix* (light blue) and *Xite* (pink) pair transiently, enabling counting and choice to occur. Oct4 and CTCF are necessary for contact and communication at the *Xic*. Oct4-binding sites (green ovals) and CTCF-binding sites (triangles) within the *Tsix* and *Xite* regions of the mouse *Xic* are depicted.

very tissue specific and limited to portions of particular chromosomes. It therefore must depend on chromosome-specific features, as well as developmental cues.

The best understood somatic homologue associations in mammalian cells are transient and occur at individual loci, rather than encompassing extensive chromosomal regions. These contacts appear to be a subset of long-range interactions between chromosomes, which includes looping and interactions between nonhomologous regions (Figure 1(c)) [14, 15]. One notable function of these interactions is their role in establishing inactivation of one of the two female X chromosomes and in controlling monoallelic expression of imprinted genes.

The long-range contacts made by mammalian homologues overlay a general nuclear organization that seems designed to discourage interaction. Mammalian chromosomes occupy nonoverlapping regions, termed chromosome territories, in the nucleus. These territories are organized by specific rules (Reviewed by Spector [16]). For example, gene-poor regions tend to be close to the nuclear membrane, while gene-dense chromosomes localize in interior of the nucleus [14, 17]. The territories of small and early replicating chromosomes also tend to be interior. Interestingly, in human epithelial cancer cell lines and mouse primary lymphocytes the territories occupied by the homologues are more widely separated than expected from a random distribution [18, 19]. One function of chromosome territories may be to keep the homologues apart.

The properties of the molecules that mediate long-range contacts between allelic and nonallelic loci suggest strategies that facilitate specific interactions. One of these molecules is CTCF (CCCTC-binding factor), a highly conserved, DNA-binding protein with a multitude of seemingly disparate regulatory functions (Reviewed by Philips and Corces [20]). Depending on context and binding partners, CTCF can be a transcriptional repressor or an activator [21–24]. Adjacent CTCF binding sites are often drawn into chromatin loops, insulating promoters from nearby regulatory regions [25–30]. One of the best-understood examples is found at the imprinted Igf2/H19 locus. Imprinting, established in the parental germline, produces an allele-specific difference in genetic properties (Reviewed by Verona et al. [31]). The Igf2/H19 locus has a CTCF-binding site that is differentially methylated in the parental germlines [32–34]. Methylation of the paternal allele blocks CTCF binding, preventing formation of an insulator that would otherwise separate Igf2 from an enhancer [33, 35–37]. On the maternal allele, CTCF binds between Igf2 and this enhancer, silencing Igf2 by insulation and through recruitment SUZ12, a member of the Polycomb Repressive Complex 2 (PRC2) [29]. On the maternal chromosome CTCF binding adjacent to H19 is necessary to induce expression of this transcript [38].

CTCF also mediates interactions between Igf2/H19, on chromosome 7, and other regions throughout the genome. Igf2/H19 contacts the Wsb1/Nf1 locus on chromosome 11 [26, 39]. This interaction is dependent upon binding of CTCF to the maternal Igf2/H19 allele and is required for monoallelic expression from Wsb1/Nf1. Additional interactions between Igf2/H19 and several other imprinted loci have been identified, and these findings are consistent with the idea that Igf2/H19 coordinates the epigenetic status of imprinted regions throughout the genome [40].

Some imprinted homologues pair transiently, an activity that may be necessary for normal developmental regulation. In lymphocytes, transient association at 15q11–q13 occurs in late S phase [41]. This region is imprinted, containing several monoallelically expressed genes. Loss of expression, or lack of normal imprinting at this locus, causes Prader-Willi and Angelman syndromes, both of which display developmental and neurological abnormalities (Reviewd by Lalande [42]). Interestingly, lymphocytes from Prader-Willi and Angelman syndrome patients do not pair [41]. Homologue communication at 15q11-q13 may be a factor in normal brain development, as this locus pairs persistently in normal brain, but not in brains from patients with some autism-spectrum disorders [43].

Homologue pairing also plays a central role in orchestration of X inactivation in mammalian females. Mammalian females randomly inactivate one X chromosome, thus maintaining an equivalent ratio of X to autosomal gene products in both sexes [44, 45]. Each cell of the early embryo counts the number of X chromosomes and inactivates all but one (Reviewed by Royce-Tolland and Panning [46]). Counting, and choice of the inactive X, relies on a transient pairing of the *X inactivation center* (*Xic*), a locus on the X chromosome

(Figure 1(d)). Pairing is believed to enable XX cells to coordinate inactivation of a single X chromosome. Deletion of regions engaged in pairing led to skewed or chaotic X inactivation [47]. The process of pairing is complex, involving multiple elements within the *Xic*. The *X-pairing region* (*Xpr*) may support initial interactions, and its deletion diminishes *Xic* pairing [48, 49]. Several genes within the *Xic* produce noncoding RNAs that participate in counting and inactivation of the X chromosome. *Xist*, a long noncoding RNA, initiates the process of X inactivation and coats the inactive X (Reviewed by Chow and Heard [50]). *Tsix*, transcribed antisense to *Xist*, and a nearby gene *Xite* contribute to pairing of the *Xic* and also produce noncoding RNAs (Reviewed by Lee [51]). Following pairing, transcription of *Tsix* and *Xite* is necessary for orderly X inactivation, suggesting that communication might occur by an RNA-protein bridge between two X chromosomes [52]. CTCF plays a central role in pairing at the *Xic*. The *Tsix* promoter contains numerous CTCF binding sites (Figure 1(d)) [52–55]. Pairing at the *Xic* is disrupted upon the loss of CTCF [56]. Initiation of inactivation occurs during a narrow window in early development [57]. Oct4, a transcription factor key to the maintenance of stem cells, forms a complex with CTCF at *Tsix,* and is required for transient association of *Xics* [56]. After this transient pairing, the X chromosomes separate, assume different fates and localize to distinct nuclear compartments.

The examples above illustrate the idea that CTCF fulfills disparate functions in a developmental and cell type-specific manner. The proteins mentioned above, Oct4 and SUZ12, are among many CTCF partners that enable modulation of CTCF effects [58]. An additional CTCF binding protein that contributes to its localization and function is nucleophosmin, a component of the nucleolus [59]. Some loci that bind CTCF are anchored at the nucleolus, leading to the idea that the nucleolus functions as a hub where long-range interactions occur. While recruitment to the nucleolus appears to be a factor for some CTCF-bound loci, it does not contribute to X chromosome pairing [59, 60].

Another protein that contributes to CTCF function is cohesin, a multisubunit complex that regulates sister chromatid cohesion during meiosis and mitosis. Cohesin, consisting of SMC1, SMC3, Scc1, and Scc3 subunits, is believed to encircle sister chromatids to maintain their association [61, 62]. The C-terminus of CTCF interacts with the cohesin subunit Scc3, and cohesin and CTCF are often colocalized on mammalian chromosomes [63–65]. Depletion of CTCF results in loss of cohesin binding but, at most sites, loss of cohesin does not affect CTCF binding to DNA [66, 67]. CTCF thus appears to recruit cohesin to specific DNA sequences. Cohesin recruitment facilitates long-range interactions, either by securing aligned regions or by inducing looping. For example, cohesin plays a regulatory role in CTCF-mediated intrachromosomal contacts between sites in the interferon-γ locus [65, 66]. Loss of cohesin or CTCF also leads to misregulation of expression from Igf2/H19 [39, 64].

While cohesin colocalizes with CTCF on mammalian chromosomes, the association of these molecules is not universal. In *Drosophila*, cohesin and CTCF have not yet been shown to colocalize. In spite of this, in flies CTCF performs many functions similar to those in mammals. For example, it localizes to insulators and contributes to looping between boundary elements [68, 69]. *Drosophila* CTCF also plays a role in imprinting in flies [70].

3. Flies: Always in Touch

In contrast to the carefully orchestrated pairing of specific loci in mammals, complete homologue pairing is the default condition in *Drosophila*. Pairing is evident from the mitotic cycle 13 of embryogenesis onwards [71, 72]. Cellularization occurs during cycle 14, which marks a dramatic reorganization of the nucleus [73]. Heterochromatin becomes detectable at cycle 14, and transcription of zygotic genes begins in earnest [74]. While pairing is persistent throughout the cell cycle from this point onwards, it is relaxed, but still apparent, during replication and mitosis [75, 76].

Homologues might encounter each other by directed movement, or by random diffusion [77]. Analysis of chromosomal movements preceding pairing in embryos supports the idea that random motion leads to homologue encounters and suggests independent initiation at numerous sites, rather than a processive zippering along the length of the chromosome [71, 75]. Space constraints within a chromosome territory or an underlying chromosome arrangement could speed the search. Early studies by Rabl and Boveri revealed the nonrandom organization of the interphase nucleus. The centromeres cluster at one pole of the nucleus, while the chromosome arms extend across the nucleus towards the other pole. This polarized pattern of chromosomal arrangement, known as Rabl configuration, is not apparent in some species (rice, maize, mouse, and humans) but is observed in a wide range of organisms (*S. cerevisiae*, *S. Pombe, Drosophila*, and several grains) (Reviewed by Spector [16] and Santos and Shaw [78]). The Rabl configuration is reminiscent of the arrangement of chromosomes following mitosis, where the centromeres lead the chromosomes into the daughter cells. While the anaphase movement of chromosomes does promote this arrangement, cell division is not essential for the Rabl conformation in yeast [79]. Regardless of how formed, homologous chromosomes in the Rabl configuration are roughly aligned, more or less parallel, placing alleles closer together than predicted by chance distribution.

While pairing of imprinted loci and the *Xic* is necessary for correct regulation of developmentally important genes in mammals, there are no examples of flies utilizing chromosome pairing to count X chromosomes or to regulate monoallelic gene expression. However, homologue pairing in flies does affect gene expression through a mechanism known as transvection [80]. Pioneering work by Lewis on the *Ultrabithorax* (*Ubx*) gene showed that the mutant phenotype was stronger when pairing between two loss-of-function *Ubx* alleles was disrupted by chromosomal re-arrangements. When paired, *Ubx* expression was elevated, enabling complementation between the two mutations. A well-supported model for transvection is that pairing enables regulatory elements on one chromosome to drive (or silence) expression from an intact promoter on the other chromosome [81]. Confirmation of transvection is obtained when the phenotype is

sensitive to disruption of pairing, for example, by inversion of one chromosome [80, 82]. Transvection has been demonstrated for numerous genes in *Drosophila*, and it appears able to operate throughout the genome [83]. Transvection has also been observed in the diploid stages of *Neurospora* [5]. A few examples of transvection have been described in mammals, and the term is often used to describe nonallelic regulatory interactions *in trans*, such as the CTCF-mediated long-range interactions that were described in preceding sections [84, 85].

A limitation of our understanding of transvection is how alleles communicate. Communication may differ from gene to gene. For example, transvection at *Ubx* is disrupted by breaks anywhere within a large critical region between *Ubx* and the centromere, but transvection at the *yellow* gene is only sensitive to breaks very close to the gene. This is consistent with different mechanisms of pairing or communication at these loci, but could also reflect the length of the cell cycle, and thus the time available for homologue association, at the time of gene expression [86]. For example, expression of *Ubx* is required in rapidly cycling embryonic cells. In contrast, the critical period for *yellow* expression is in pupal cells that have ceased dividing. In accordance with this idea, extension of the cell cycle in *Ubx* mutants with inversions reduces phenotypic severity, presumably by allowing extended time for chromosome pairing [86].

One molecule that affects pairing-dependent gene regulation is encoded by *zeste* (*z*). Zeste is a DNA-binding protein that affects pairing-dependent expression at many genes that display transvection (Reviewed by Pirrotta [87] and Duncan [88]). The Zeste protein polymerizes, leading to the suggestion that it might bridge homologues, but loss of Zeste does not affect homologue pairing [89]. Zeste binding sites are found in promoters, and the Zeste protein interacts with the activating *Trithorax* chromatin regulatory complex, as well as the repressing *Polycomb* PRC1 complex [90, 91]. Thus it appears likely that Zeste is a transcription factor able to interpret the state of homologue pairing.

An RNAi screen in tissue culture cells identified Topoisomerase II (Top2) as necessary player in homologue pairing [76]. Topoisomerases play pivotal roles by solving topological problems associated with DNA replication, transcription, recombination, repair, and chromosome segregation (Reviewed by Nitiss [92]). Type II topoisomerases introduce double-strand breaks, pass an intact DNA duplex through the cut, and rejoin the cut ends. Top2 also makes up a large fraction of the insoluble nuclear matrix and contributes to chromosome architecture [93, 94]. It preferentially binds scaffold-associated regions, which anchor chromatin loops during interphase. There are several potential mechanisms through which Top2 might contribute to pairing. Because it plays a central role in chromosome organization, loss of Top2 could lead to a general disruption that abrogates homologue association. It is also possible that Top2 engages in protein/protein interactions that stabilize pairing.

One protein that interacts with Top2 and also affects pairing in *Drosophila*, is condensin. Condensins function in chromosome condensation, induction of DNA supercoiling, and anaphase chromosome segregation. Metazoans have two paralogous condensin complexes, condensin I and II. Each contains conserved SMC2 and SMC4 subunits, but different non-SMC subunits: Cap-H, Cap-G, and Cap-D2 or Cap-H2, Cap-G2, and Cap-D3 [95, 96]. Condensins influence the activity of Top2, and Top2 interacts directly with the *Drosophila* Cap-H homologue Barren on mitotic chromosomes [97]. Both proteins are necessary for chromosome segregation, and loss of either produces a similar mitotic defect. Condensin I is also required for localization of Top2 on mitotic chromosomes in flies, yeast, and humans [98–100].

In spite of the dependent interactions between condensin and Top2, condensin acts to antagonize homologue pairing in *Drosophila* [101]. Most dramatically, ectopic expression of Cap-H2 in salivary glands separates the aligned polytene chromosomes. Increased condensin reduces transvection at two loci, revealing the dissociation of paired homologues in diploid cells. The involvement of Top2 and condensin reveals that homologue pairing in flies is regulated by conserved proteins necessary for the maintenance of chromosomal architecture and stability in all eukaryotic organisms. It will be fascinating to see if Top2 or condensin levels affect pairing in other organisms.

4. Pairing and Sex Chromosomes

An unanswered question is whether pairing-dependent regulation contributes to the expression of wild-type genes in *Drosophila*. Analysis of *Ubx* revealed that expression from a wild-type allele was increased when it could pair with a gain of function mutation [102]. Homologue pairing might also contribute to expression of other unmutated genes in a wild-type context. The phenotypic normality of flies with inverted chromosomes would suggest that transvection makes little contribution to expression, but a functional assay for homologue association demonstrated that alleles on inverted chromosomes can pair surprisingly efficiently, when given sufficient time [86]. But there are situations in which homologue pairing cannot occur, including the single male X chromosome and regions made hemizygous by deficiency. If pairing influences expression of wild type genes, the regulation of the entire X chromosome might differ between the sexes. This could contribute to sexually dimorphic expression or influence the biology of the X chromosome.

Flies have a dedicated regulatory system that accommodates hemizygosity of the X chromosome in males. Males produce the chromatin-modifying Male-Specific Lethal (MSL) complex, which is recruited to the X chromosome at 3 h after fertilization [103]. The result is increased expression of virtually every X-linked gene. Surprisingly, RNA sequencing of single-sexed embryos has identified partial dosage compensation at mitotic cycle 13, an hour before the MSL complex localizes to the X chromosome [104]. One mechanism proposed to explain this is that pairing of X chromatin in females inhibits transcription from X-linked genes. This idea deserves to be tested, as it could explain several situations in which dosage compensation occurs in the absence of the MSL complex. For example, X-linked genes are dosage compensated in the male germline, where the MSL complex is not formed [44, 105]. Autosomal deficiencies are partially

compensated by an unknown mechanism [106]. In addition, considerable evidence supports the idea that the MSL complex does not fully compensate X-linked genes in somatic cells. If formation of the MSL complex is blocked, expression of X-linked genes is reduced by 25%–30%, rather than the predicted 50% [107, 108]. These observations support the idea that differences in gene copy number are buffered by mechanisms that operate throughout the genome (Reviewed by Stenberg and Larsson [106]).

A copy number buffering mechanism would differentially affect X-linked gene expression in males and females. Over time, this could be a factor in creation of the striking differences in gene distribution observed when comparing the X chromosome and the autosomes in some species (Reviewed by Vicoso and Charlesworth [109] and Gurbich and Bachtrog [110]). For example, the mammalian X chromosome appears enriched for genes with a male-biased expression, including those expressed in the premeiotic testes [111]. This is postulated to reflect the fact that hemizygosity of the male X chromosome enables rapid selection for beneficial recessive alleles. The same argument should apply to other species with XY males, including flies. However, the X chromosomes of *Drosophila melanogaster* and related species are depleted for genes with male-biased expression in somatic tissues and testes and enriched for genes with female-biased expression [112]. These notable differences in the distributions of sex-biased genes in mammals and flies have yet to be adequately explained. A recent study revealed that the fly X chromosome was also depleted for developmentally regulated genes, with the notable exception of those expressed in the ovary [113]. The authors propose that demasculinization of the X chromosome was due in part to the fact that male-biased genes tend to be developmentally regulated and suggest that chromatin modification by the MSL complex may be incompatible with developmental regulation, making the X chromosome an unfavorable environment. However, a genome-wide buffering system that contributes to X chromosome dosage compensation could also influence the distribution of developmentally regulated genes. Analysis of expression in flies with autosomal deficiencies and duplications lends support to the idea that such a system exists, but constitutively expressed genes and those with highly regulated expression respond differently [114]. A speculative model for the role of homologue pairing in buffering gene dose is presented in Figure 2. A key feature of our model is that homologue pairing is repressive. The absence of pairing of the male X chromosome, and autosomal deficiencies, leads to a modest increase in expression from these regions.

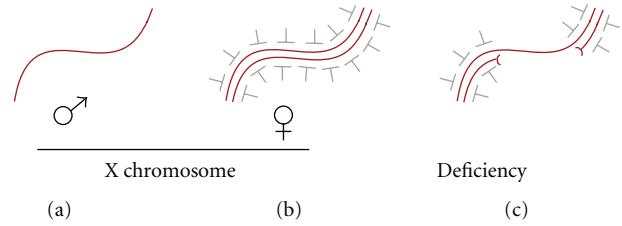

Figure 2: Hypothetical model for pairing-dependent buffering of gene dosage in flies. (a) The unpaired X chromosome of males escapes repression. (b) Paired female X chromosomes are subject to repression. (c) Paired regions of an autosome are repressed, but an unpaired region created by deficiency escapes repression.

5. Conclusions

Somatic chromosome pairing obeys strikingly different rules in mammals and flies. Mammals sharply limit contacts between homologues. When homologues do make contact it often serves to coordinate regulatory mechanisms, such as imprinting and X inactivation, that are essential for normal development. It seems ironic that mammals use pairing to communicate critical information, yet flies, with constant homologue pairing, appear to make little use of this feature of genome organization. Recent studies of early dosage compensation and buffering of copy number variation in flies suggest that additional regulatory mechanisms exist to accommodate variation in gene dosage. A pairing-based regulation of gene expression could account for many of the findings of these studies. A broader question is why homologue pairing exists in some species, but not in others. The precise control of homologue association in mammals, and inappropriate pairing in some cancers, suggests that homologue association can be dangerous. What this danger is, and how flies evade it, remains to be discovered.

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Review Article

The "Special" *crystal-Stellate* System in *Drosophila melanogaster* Reveals Mechanisms Underlying piRNA Pathway-Mediated Canalization

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The Stellate-made crystals formation in spermatocytes is the phenotypic manifestation of a disrupted *crystal-Stellate* interaction in testes of *Drosophila melanogaster. Stellate* silencing is achieved by the piRNA pathway, but many features still remain unknown. Here we outline the important role of the *crystal-Stellate* modifiers. These have shed light on the piRNA pathways that defend genome integrity against transposons and other repetitive elements in the gonads. In particular, we illustrate the finding that HSP90 participates in the molecular pathways of piRNA production. This observation has relevance for the mechanisms underlying the evolutionary canalization process.

1. The Stellate-Made Crystals in Spermatocytes Are the Phenotypic Manifestation of a Disrupted *crystal-Stellate* Interaction in Testes of *Drosophila melanogaster*

The history of the *crystal-Stellate* system started in 1961 when Meyer and collaborators discovered the presence of crystalline aggregates in primary spermatocytes of *D. melanogaster X/O* male testes. They also described the morphological differences between needle-shaped and star-shaped crystals [1].

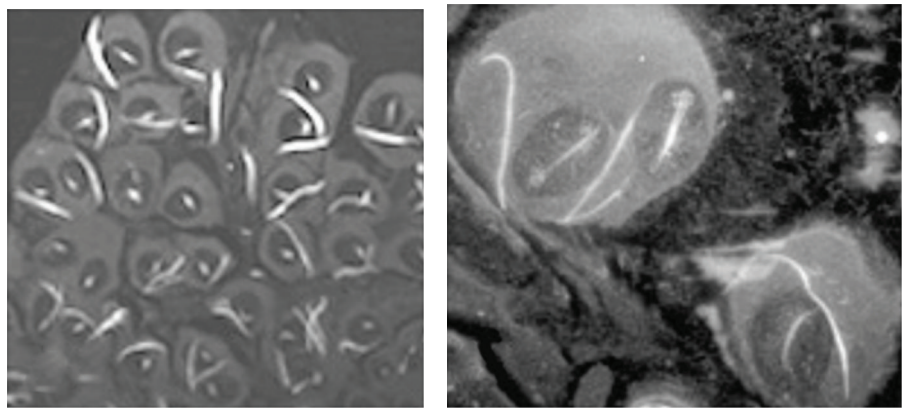
In 1983, Gatti and Pimpinelli provided a detailed cytological description of the *Y* chromosome. They showed that the *hll* region contains the genetic determinants for normal chromosome behavior during male meiosis and for the suppression of Stellate-made crystals formation in spermatocytes [2]. This region was called the *Suppressor of Stellate* [*Su*(*Ste*)] locus, also referred to as *crystal* (*cry*) [3]; in this paper we use "*crystal*."

Afterwards, different groups established that both the morphology of the crystalline aggregates and the severity of the meiotic defects in *X/O* and X/Y^{cry-} males depend on the *Stellate* (*Ste*) locus on the *X* chromosome [4–6]. Two regions containing clustered *Stellate* elements have been identified on the *X* chromosome: *12E1* in euchromatin and *h27* in heterochromatin. *Stellate* and *crystal* are both repetitive sequences and they share sequence homology [6–8].

At the molecular level, the loss of the *crystal* region results in the production of a testes-specific *Stellate* mRNA of 750 nucleotides in length. The product of this mRNA is the Stellate protein [8, 9]. In 1995 there was a fundamental discovery: the Stellate protein is the main component of the crystals in the primary spermatocytes [10] and Figure 1.

2. The Regulation of the *crystal-Stellate* Interaction

The first indication about the mechanism that regulates the interaction between *crystal* and *Stellate* sequences was



(a) (b)

Figure 1: Testes of X/Y^{cry-} males immunostained with anti-Stellate antibody, (a) magnification 20x; (b) magnification 40x.

obtained in 2001; the *Stellate* silencing was associated with the presence of small RNAs, 24–29 nt long, homologous to *crystal* and *Stellate* sequences [11]. These were named rasiRNAs (repeat-associated small interfering RNAs) [12].

The detailed analysis of the *crystal*-rasiRNAs in fly testes demonstrated the existence of a specific RNAi pathway in the germline that silences repetitive sequences such as Stellate and transposable elements [13]. It was also demonstrated that rasiRNAs show differences in structure compared to other classes of small noncoding RNAs, such as siRNAs and miRNAs and their biogenesis is Dicer-independent [13]. The rasiRNAs work associated with the Piwi subfamily of the Argonaute proteins, Aubergine, Ago3, and Piwi. rasiRNAs were subsequently designated as Piwi-interacting RNAs or piRNAs [13]. The studies on the *crystal-Stellate* system have been therefore crucial for the discovery of the piRNA pathway.

In 2007, two independent groups used a deep sequencing strategy to identify small RNAs bound to each of the three Piwi proteins in fly ovaries. Their expectation was that this approach would reveal how piRNAs were made and how they function. They demonstrated that piRNAs arise from a few genomic sites, grouped in clusters that produce small RNAs that silence many transposons [14, 15]. In fly testes, the most abundant Aubergine-associated piRNAs (~70%) correspond to *crystal* antisense transcripts [16].

3. The piRNA Pathways in the Fly Ovaries

Studies on the sequences of the small RNAs associated to Piwi subclade proteins carried out in 2006 and 2007 by the Hannon, Zamore, and Siomi groups have been crucial to formulation of a model for the biogenesis and the function of the piRNAs in the germline [13–16]. The proposed model, called the "ping-pong" model, requires a primary piRNA, whose biogenesis has not yet been elucidated, bound by Aubergine or Ago3. In particular, Aub binds an antisense piRNA and cleaves the sense transcript from an active transposon; transcript cleavage produces a sense piRNA that is loaded onto Ago3. This Ago3-piRNA complex binds complementary transcripts and initiates the production of piRNAs by an amplification loop [14]. The piRNAs originated by this mechanism are now called "secondary" piRNAs and they exhibit specific signatures consisting of the adenine at the 10th position of the sense piRNAs, which is able to base pair with the initial uracil of the antisense piRNAs [14, 15].

Identification of *ago3* mutants led to the discovery of two different piRNA pathways in the fly ovary: one in the somatic cells of the ovary and the other in the germline cells. The somatic pathway, called "primary piRNA pathway," involves Piwi, and it does not require an amplification loop. This pathway regulates the transposons belonging to the so-called "somatic" group [17, 18].

4. The piRNA Pathways in Fly Testes and Open Questions

Deep sequencing of piRNAs bound to Piwi-subfamily proteins associated to genetic studies, supplied thousands of data about almost all the piRNAs sequence biogenesis and orientation produced in testes [16, 19].

Although the overall structure of the *crystal* and *Stellate* loci remains unclear, regions of homology between *crystal* and *Stellate* piRNAs, and repeat monomers from each of these loci has been summarized in the scheme depicted in Figure 2. The position of several piRNAs on the *crystal* and *Stellate* sequences, their orientation and the Piwi protein(s) to which they are bound are indicated. Detailed information on the sequences of *crystal* (Z11734) and *Stellate* euchromatic sequences (X15799), depicting the location of piRNAs, are shown in Figure 1S (see Figure 1S in supplementary material available online at doi:10.1155/2012/324293). In light of this map we note that almost all the *crystal*-specific piRNAs come from the region, depicted in purple, of homology with *Stellate* sequences. These are predominantly "antisense" as already reported [11, 12, 14, 16, 19]. However, *Stellate*-specific piRNAs, whether euchromatic or heterochromatic, are predominantly in the "sense" orientation (Figure 2).

The majority of these piRNAs do not show the ping-pong signature. There are only 3 pairs exhibiting the A at

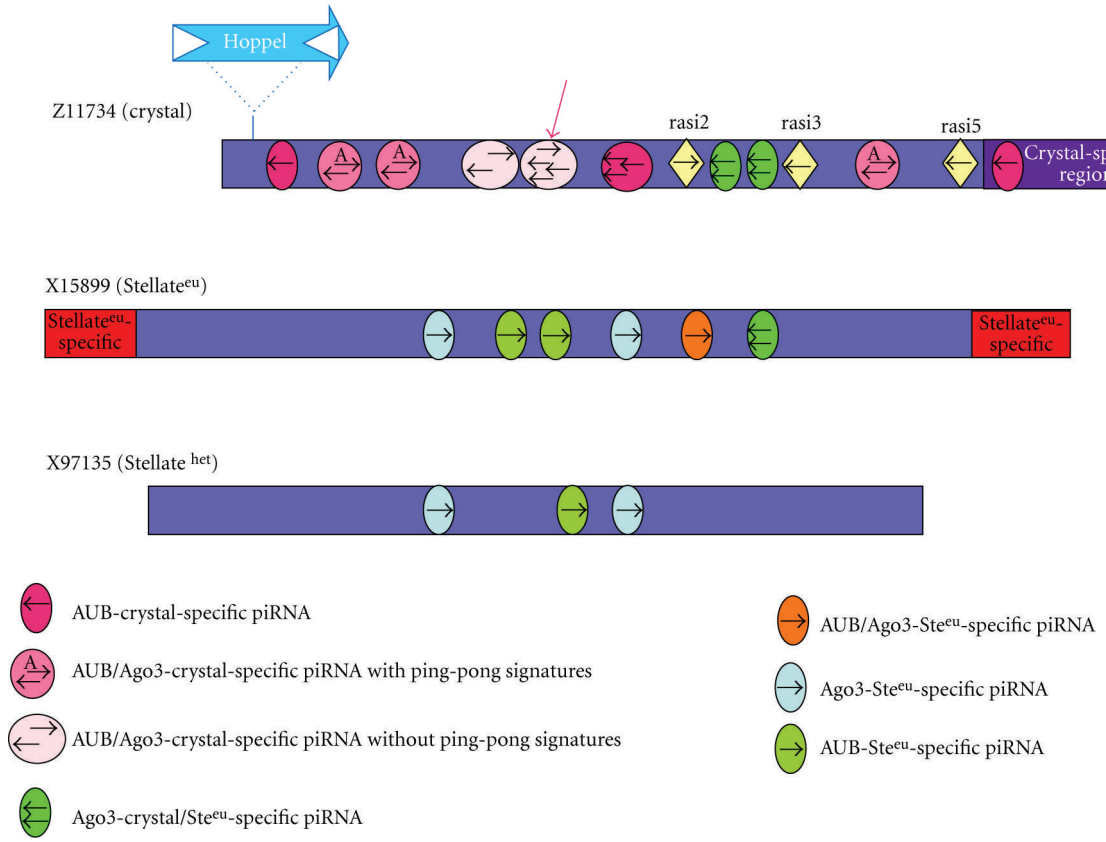


Figure 2: Schematic of the elements of the *crystal-Stellate* system. *crystal* (corresponding to sequence Z11734); euchromatic *Stellate* (corresponding to sequence X15899); heterochromatic *Stellate* (corresponding to sequence X97135). The position and the orientation of the most prominent piRNAs is indicated, on each element, by the colored little circles and rumbles. The sequence and the length of indicated piRNAs can be deduced from the Supplemental Figure 1. The Piwi protein to which each is bound is also indicated. The drawing is schematic and not to scale.

the 10th position of the "sense" piRNA, and these "sense" piRNAs show 2 or 3 mismatches with *Stellate* euchromatic and heterochromatic sequences. Therefore, they cannot be considered canonical ping-pong pairs [Figure 1S(a)]. The *crystal*-specific piRNA, reported to be the most abundant one in testes, is only antisense [19], Figure 2.

For all the reasons reported above, we hypothesize that different though interconnected pathways exist to silence *crystal* and *Stellate* sequences. *crystal*- and *Stellate*-specific piRNAs cooperate in some way to silence the *Stellate* euchromatic and heterochromatic sequences that produce the Stellate protein ("active elements") [10, 20]. These different pathways could be present in both the somatic and germline tissues of testes.

In support of these considerations, we refer to the previous data on the silencing of another class of repetitive sequences in testes. In fact, a second large class of piRNAs associated with Aubergine in the testes is derived from a short repeated region, termed *AT-chX*, on the X chromosome [16]. These piRNAs are predominantly antisense. Only one pair with ping-pong signatures was found among all sequenced *AT-chX* piRNAs. These remarks confirm that the ping pong is not the only piRNA pathways operating in the silencing of these repetitive sequences in testes [19].

5. Mutants Affecting the *crystal-Stellate* Interaction Clarify Unknown Aspects of the piRNA Pathways in Testes

Mutations in piRNA-pathway genes, such as *aubergine*, *ago3*, *spindle E*, *armitage*, *zucchini*, and *squash*, lead to the formation of the Stellate-made crystals in spermatocytes [17, 21–24].

spindle-E encodes a member of the DExH family of ATPases with a Tudor domain. Mutations in this gene are known to impair *Stellate* and transposon silencing in the *Drosophila* germline. In ovaries *spindle-E* acts specifically in germ cells and in the ping-pong cycle [18, 22, 25].

Armitage encodes a homolog of the *Arabidopsis* SDE3, an RNA helicase that is involved in RNAi. Mutations in *armitage* affect translational repression and localization of *oskar* mRNA, block RNAi in *Drosophila* oocytes, and impair *Stellate* silencing in testes [23, 26]. In ovaries, *armitage* acts in the primary piRNA pathway [18, 27, 28]. *zucchini* was identified in a screen for female sterile mutations, and causes dorsoventral patterning defects. This gene encodes a nuclease. Mutations in *zucchini* lead to formation of Stellate crystals [24]. In ovaries *zucchini* mutations specifically decrease the piRNA levels in somatic ovarian cells [18].

Table 1: List of some genes involved in the piRNA pathways.

Genes	Crystals	Function	Ping pong*	References
Aubergine	+	Piwi protein	− −/+	[14–19, 21]
Ago3	+	Piwi protein	− −/+	[17–19]
Piwi	−	Piwi protein	+	[13–18]
Spindle-E	+	RNA helicase	− −/+	[18, 22, 25]
Squash	+	Tudor-domain nuclease	+	[24]
Zucchini	+	Nuclease	+	[24]
Armitage	+	RNA helicase	+	[18, 23, 26–28]
hsp83	+	Heat-shock protein	nd	[29]

* "+" indicates that the ping pong is functional in the mutant.

In Table 1, we listed some of the modifiers of the *crystal-Stellate* interaction that have been related to the piRNA pathways in gonads. Mutants of genes implicated either in the primary piRNA pathway, excepting *piwi*, or in the secondary ping-pong amplification pathway show crystals in their spermatocytes.

After all, we are convinced that the molecular mechanisms, underlying the piRNA pathways, are not completely understood and that there are more players to be discovered in both the somatic and germline-specific piRNA pathways. The genetic characterization of known and still unknown components, combined with the deep sequencing strategy of the piRNAs bound to specific Piwi proteins, will help us in understanding the piRNAs production and function in the *Drosophila* testes. Because Stellate-made crystals are symptomatic of a disrupted *crystal-Stellate* interaction, they allow the identification of new genetic components of the piRNAs pathway. An emblematic example is the discovery that the *hsp83* gene participates in piRNA.

6. *hsp83*^{scratch}, an Unexpected *crystal-Stellate* Modifier

The *hsp83* gene encodes HSP90 protein, a molecular chaperone involved in several cellular processes and developmental pathways [30–33]. We have recently demonstrated that primary spermatocytes of *hsp83*^{scratch} homozygous mutant males exhibit Stellate-made crystalline aggregates, suggesting a role for this protein in the piRNA-mediated mechanisms. We also demonstrated that *hsp83*^{scratch} affects the biogenesis of the *crystal/Stellate*-specific piRNAs and transposon piRNAs in testes. We went on to demonstrate that the effect of HSP90 in morphological variations is due, at least in part, to activation of transposons causing *de novo* mutations [29]. Among the *hsp83* mutant flies showing morphological abnormalities, we selected one exhibiting a *Scutoid*-like phenotype and demonstrated that this phenotype is caused by the insertion of an *I* element-like transposon in the *noc* gene of this fly.

The role of HSP90 in piRNAs-mediated silencing is in addition to the "buffering" role on the genetic cryptic variation initially put forth by Rutherford and Lindquist [34] as the molecular explanation for the Waddington's "canalization" process.

Canalization is the resistance of an organism to phenotypic variation during development, in the presence of genetic and environmental changes. This "phenotype robustness" is due to buffering mechanisms. Severe perturbations, which reduce buffering, produce heritable phenotypic variants that can be canalized by a genetic assimilation process [35]. An interesting aspect to investigate is if, and how, the reduction of HSP90 causes a stress response-like activation of mobile elements, creating a link between environmental changes and genomic variation.

Further mechanisms could be involved in increasing the phenotypic variations underlying evolution. One of these could be related to HSP90-mediated epigenetic chromatin modifications [36, 37].

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Review Article

The Impact of the Organism on Its Descendants

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Historically, evolutionary biologists have taken the view that an understanding of development is irrelevant to theories of evolution. However, the integration of several disciplines in recent years suggests that this position is wrong. The capacity of the organism to adapt to challenges from the environment can set up conditions that affect the subsequent evolution of its descendants. Moreover, molecular events arising from epigenetic processes can be transmitted from one generation to the next and influence genetic mutation. This in turn can facilitate evolution in the conditions in which epigenetic change was first initiated.

1. Introduction

The view that knowledge of development was irrelevant to the understanding of evolution was forcefully set out by the advocates of the Modern Synthesis [1]. They brought the mechanism for the evolution of adaptations originally proposed by Darwin and Wallace together with Mendelian and population genetics. Maynard Smith [2] suggested that the widespread acceptance of Weismann's [3] doctrine of the separation of the germline from the soma was crucial to this line of thought even though it did not apply to plants. Such acceptance led to the view that genetics and hence evolution could be understood without understanding development. These views were, until recently, dominant. Briefly put, genes influence the characteristics of the individual; if individuals differ because of differences in their genes, some may be better able to survive and reproduce than others and, as a consequence, their genes are perpetuated.

The extreme alternative to the modern synthesis is a caricature of Lamarck's views about biological evolution and inheritance. If a blacksmith develops strong arms as a result of his work, it was argued, his children will have stronger arms than would have been the case if their father had been an office worker. This view has been ridiculed by essentially all contemporary biologists. Nevertheless, as so often happens in polarised debates, the excluded middle ground concerning the evolutionary significance of development and plasticity has turned out to be much more interesting and potentially productive than either of the extreme alternatives. This view was developed at length by West-Eberhard [4] who argued that developmental plasticity was crucial in biological evolution. These same ideas are well expressed in Gilbert and Epel's [5] book and developed further in the book edited by Pigliucci and Müller [6].

Bateson and Gluckman [7] have argued that developmental plasticity is an umbrella term for multiple unrelated mechanisms. The term includes accommodation to the disruptions of normal development caused by mutation, poisons, or accident. Much plasticity is in response to environmental cues, and advantages in terms of survival and reproductive success are likely to arise from the use of such mechanisms [7]. An organism that has been deprived of certain resources necessary for development may be equipped with mechanisms that lead it to sacrifice some of its future reproductive success in order to survive. Plasticity includes preparing individuals for the environments they are likely to encounter in the future on the basis of maternal cues; the course of an individual's development may be radically different depending on the nature of these cues. Plasticity may also involve one of the many different forms of learning, ranging from habituation through associative learning to the most complex forms of cognition.

I will not deal extensively with all the various ways in which an individual can affect the evolution of its descendants since I have discussed them recently elsewhere [8]. To summarise my position on this topic, I believe that

the organism's mobility, its choices, its construction of a niche for itself, its capacity for behavioral innovation, and its adaptability have all played important roles in biological evolution. All these activities should be contrasted with the essentially passive role often attributed to the organism by many evolutionary biologists. Modern understanding of an individual's development goes well beyond accepting that interactions between the organism and its environment are crucial. The conditional character of an individual's development emphasises the need to understand the processes of development that underlie these interactions.

2. The Importance of Epigenetics

Epigenetics is a term that has had multiple meanings since it was first coined by Waddington [9]. He used the term, in the absence of molecular understanding, to describe processes by which the inherited genotype could be influenced during development to produce a range of phenotypes. He distinguished "epigenetics" from the eighteenth-century term "epigenesis," which had been used to oppose the preformationist notion that all the characteristics of the adult were already present in the embryo.

More recently, the term epigenetics has been used for the molecular processes by which traits, specified by a given profile of gene expression, can persist across mitotic cell division without involving changes in the nucleotide sequence of the DNA. (Nowadays this usage is also taken to include transgenerational inheritance as discussed below.) In this more restricted sense, epigenetic processes are those that result in the silencing or activation of gene expression through such modification of the roles of DNA or its associated RNA and protein. The term has, therefore, come to describe those molecular mechanisms through which both dynamic and stable changes in gene expression are achieved, and ultimately how variations in extracellular input and experience by the whole organism of its environment can modify regulation of DNA expression [10]. This area of research is one of the most rapidly expanding components of molecular biology. It should be noted, however, that some authors [11], myself among them, continue to use Waddington's broader definition of epigenetics to describe all the developmental processes that bear on the character of the organism. In all these usages, epigenetics usually refers to what happens within an individual developing organism.

Variation in the context-specific expression of genes, rather than in the sequence of genes, is critical in shaping individual differences in phenotype. This is not to say that differences in the sequences of particular genes between individuals do not contribute to phenotypic differences, but rather that individuals carrying identical genotypes can diverge in phenotype if they experience separate environmental experiences that differentially and permanently alter gene expression.

The molecular processes involved in phenotypic development were initially worked out for the regulation of cellular differentiation and proliferation [5]. All cells within the body contain the same genetic sequence information, yet each lineage has undergone specialisations to become a skin cell, hair cell, heart cell, and so forth. These phenotypic differences are inherited from mother cells to daughter cells. The process of differentiation involves the expression of particular genes for each cell type in response to cues from neighbouring cells and the extracellular environment and the suppression of others. Genes that have been silenced at an earlier stage remain silent after each cell division. Such gene silencing provides each cell lineage with its characteristic pattern of gene expression. Since these epigenetic marks are faithfully duplicated across mitosis, stable cell differentiation results. These mechanisms are likely to play many other roles in development, including the mediation of many aspects of developmental plasticity.

A growing body of evidence suggests that phenotypic traits established in one generation by epigenetic mechanisms may be passed directly or indirectly through meiosis to the next, involving a variety of different processes, some involving microRNAs and some involving maternal behaviour [12]. In itself, this evidence does not relate to the thinking about biological evolution because the trans-generational epigenetic effects could wash out if the conditions that triggered them in the first place did not persist. The crucial question is to ask how epigenetic changes that are not stable could lead to genetic changes. I suggest that the answer subdivides into two likely routes for an evolutionary change in the genome.

3. Epigenetics as a Driver of Evolution

The first account of how a phenotypic change induced by a change in the environment could lead to a change in the inherited genome was provided by Spalding [13]. His paper is also historically important because it provides the first clear account of behavioural imprinting with which Lorenz [14] is typically associated.

Spalding's driver of evolution comprised a sequence of learning followed by differential survival of those individuals that expressed the phenotype more efficiently without learning. The same idea was advanced once again by Baldwin [15], Lloyd Morgan [16], and Osborn [17], all publishing in the same year. Seemingly, their ideas were proposed independently of Spalding and, indeed, of each other, although they may have unconsciously assimilated what Spalding wrote 23 years earlier in what was a widely read journal, *Macmillan's Magazine*, the predecessor of today's *Nature*.

Regardless of how they derived their ideas, the evolutionary mechanism proposed by Spalding and then Baldwin, Lloyd Morgan, and Osborn was known at the time as "organic selection" and is now frequently termed the "Baldwin effect," largely because of Baldwin's influential book [18]. Baldwin was not always consistent in how he thought about the process, and, as a result, modern usage is confused [19]. By contrast, Lloyd Morgan's account of the process was particularly clear. He suggested that if a group of organisms respond adaptively to a change in environmental conditions, the modification will recur generation after generation in the changed conditions, but the modification will not be inherited. However, any variation in the ease of expression of the modified character which is due to genetic differences

is liable to act in favour of those individuals that express the character most readily. As a consequence, an inherited disposition to express the modifications in question will tend to evolve. The longer the evolutionary process continues, the more marked will be such a disposition. Plastic modification within individuals might lead the process, and a change in genes that influence the character would follow; one paves the way for the other.

Given Spalding's precedence and the simultaneous appearance in 1896 of the ideas about "organic selection," it seems inappropriate to term the evolutionary process the "Baldwin effect," particularly since it has not been used consistently [19]. Calling the proposed process the "Spalding effect" is not descriptive of what initiates the hypothetical evolutionary process. West-Eberhard's [4] term "genetic accommodation" is more general but makes no inference about the inducing pathway; it would therefore be more appropriate to employ a term that captures the adaptability of the organism in the evolutionary process, and, to this end, I have suggested the term "adaptability driver" [20].

While the focus of Baldwin, as a psychologist, was largely on behaviour as the form of phenotypic response that was, in some way, incorporated over time into the genome, the model also allows for other forms of adaptive or plastic response to be thus incorporated. All that is required is that the adaptability in some way confers advantage in the novel environment, be it a physiological response such as coping with high altitudes by enhancing the oxygen-carrying capacity of the blood, or a change in coloration that improves concealment against predators, or a change in tail morphology in the tadpole that reduces the risk of predation. Over time, genetic accommodation can fix the alteration in the lineage. As the evolutionary change progressed, the population would consist of individuals with the same phenotype but which developed in different ways, some by their capacity to respond adaptively to environmental challenges and some by spontaneously expressing part or all of the phenotype without employing plastic mechanisms.

A clear case of adaptability driving evolutionary change may be that of the house finch (*Carpodacus mexicanus*). In the middle of the twentieth century, the finch was introduced to eastern regions of the USA far from where it was originally found on the west coast. It was able to adapt to the new and extremely different climate and spread up into Canada. The finch also extended its western range north into Montana, where it has been extensively studied. After a period involving great deal of plasticity, the house finch populations spontaneously expressed the physiological characteristics that best fitted them to their new habitats without the need for developmental plasticity [21].

The question remains: under what circumstances will fixation of a previously plastic phenotype occur? The chances that all the mutations or genetic reorganisations necessary to give rise to genetic fixation would arise at the same time are small. To take a behavioural example, if a phenotype expressed spontaneously without being learned is not as good as the learned one (in the sense that it is not acquired more quickly or at less cost), then nothing will happen and fixation will not occur. If the spontaneously expressed phenotype is better than the learned one, evolutionary change towards fixation is possible. If learning involves several subprocesses, as well as many opportunities for "chaining" (the discriminative stimulus for one action becoming the secondary reinforcer that can strengthen another action), then the chances against a spontaneously expressed equivalent appearing in one step are small. However, with learning processes available to fill in the gaps of a sequence, every small evolved step that cuts out the need for a plastic component while providing a simultaneous increase in efficiency is an improvement.

Simpson [22] thought that the proposed evolutionary change would lead to a generalised loss of the ability to learn. Quite simply, it would not. Learning in complex organisms consists of a series of subprocesses [23]. A particular activity can evolve to a point where it is expressed spontaneously without involving plastic process without any more generalised loss of plasticity. It remains to be seen whether similar arguments can be applied cogently to other forms of phenotypic change, where the plastic response has been physiological or anatomical. When a plastic change involves a system that does not have parallel architecture with built-in redundancies, then the cost of losing it could outweigh the benefits of increasing the efficiency of response to an environmental challenge.

4. Epigenetics as a Driver of Mutation

A wide variety of changes in endocrine regulation following developmental stresses are mediated by epigenetic mechanisms in experimental animals [7]. Induced epigenetic changes have also been described in naturally occurring plants [6]. The evidence for transmission across generations in both animals and plants continues to grow [12]. Epigenetic inheritance over at least eight generations has been reported in the plant *Arabidopsis* [24]. One research programme on mice examined individuals possessing a *Kit* paramutation (a heritable, meiotically stable epigenetic modification resulting from an interaction between alleles in a heterozygous parent) that results in a white-spotted phenotype. Injection of RNA from sperm of heterozygote mice into wild-type embryos led to the white-spotted phenotype in the offspring, which was in turn transmitted to their progeny [25]. In another study, mouse embryos were injected with a microRNA that targets an important regulator of cardiac growth. In adulthood, these mice developed hypertrophy of the cardiac muscle, which was passed on to descendants through at least three generations without loss of effect [26]. Furthermore, the microRNA was detected in the sperm of at least the first two generations, thus implicating sperm RNA as the likely means by which the pathology is inherited. The possible involvement of sperm is also supported by observations that transgenerational genetic effects on body weight and appetite can be passed epigenetically through the mouse paternal germline for at least two generations [27].

Male rats were exposed *in utero* to the endocrine disruptor vinclozolin during the sensitive period for testis sex differentiation and morphogenesis. Lowered spermatogenic capacity and several adult-onset diseases were observed over four successive generations; these were accompanied by

altered DNA methylation patterns in the germline [28, 29]. Further analysis of these male offspring revealed that vinclozolin decreased methylation levels of two paternally imprinted genes and increased that of three maternally imprinted enes [30]. The work on *Arabidopsis* and mice suggests that micro-RNA may provide the means for transmission of methylation marks from one generation to the next [25, 31].

In most experimental studies, the environmental stimulus producing an epigenetic change is only applied in one generation. This might be enough since work on yeast suggests that an environmental challenge can permanently alter regulation of genes [32]. In natural conditions, the environmental cues that induce epigenetic change may be recurrent and repeat what has happened in previous generations. This recurring effect might stabilise the phenotype until genetic accommodation and fixation have occurred. Alternatively, DNA silencing may be stable as, for example, in *Linaria* [33] in which the epigenetically induced phenotype does not change from one generation to the next.

A central question in considering evolutionary change driven by the environment is whether the transmitted epigenetic markers could facilitate genomic change [34]. The answer is that, in principle, they could if (a) they were transmitted from one generation to the next, (b) they increased the fitness of the individual carrying the markers, and (c) genomic reorganisation enabled some individuals to develop the same phenotype at lower cost. Epigenetic inheritance would serve to protect the well-adapted phenotypes within the population until spontaneous fixation occurred. That much is exactly the same as has been proposed for the operation of the adaptability driver. However, another process could be at work.

DNA sequences where epigenetic modifications have occurred may be more likely to mutate than other sites. The consequent mutations could then give rise to a range of phenotypes on which Darwinian evolution could act. If epigenetic change could affect and bias mutation rates, such nonrandom mutation would facilitate fixation.

Methylated CpGs are mutational hotspots due to the established propensity of methylated cytosine to undergo spontaneous chemical conversion to thymine and methylated guanine to convert to uracil [35]. As these are functional nucleotides, they are not recognised as damaged DNA and excised or corrected by DNA repair mechanisms. Thus, the mutation becomes incorporated in subsequent DNA replications. DNA mapping shows fewer CpG sequences in the DNA than expected [36], and CpG hypermutability has led to a decrease in frequency of amino acids coded by CpG dinucleotides in some organisms. Indeed, comparison of the human and chimpanzee genomes has shown that 14% of the single amino acid changes are due to the biased instability of CpG sequences, which can be subject to methylation and thence to mutations [37]. The methylation of CpGs is a major contributing factor to mutation in *RB1*, a gene in which allelic inactivation leads to the developmental tumour, retinoblastoma [38].

Further evidence in support of the hypothesis that epigenetic change can lead to mutation is found in the analysis of neutrally evolving strands of primate DNA. The evidence indicates that the phylogenetically "younger" sequences have a higher CpG content than the "older" sequences, due to the reduced opportunity for spontaneous mutation. Intriguingly, the CpG content is strongly correlated with a higher rate of neutral mutation at non-CpG sites [39, 40], which suggests that CpGs play a role in influencing the mutation rate of DNA not containing CpG, perhaps by influencing the chromatin conformation surrounding the CpG and making it more accessible to other modifying processes. Furthermore, CpG content also appears to influence the *type of mutation* that occurs, with a higher ratio of transition-to-transversion mutations observed in parallel with the non-CpG mutation rate [40].

5. Implications for Evolutionary Novelty and Speciation

Major transitions in evolution have been explained in terms of changes in genetic organisation [41], and such changes have been offered as an explanation for the explosion of variety seen in the Cambrian era [42, 43]. Transitions in the rate of evolution can involve the remodelling of existing structure by changes in which part of a regulatory gene is expressed and when in development it is expressed [44]. Some of this might involve epigenetic mechanisms. The occasional appearance of mutations and the reorganisation of the genome permit evolutionary change that would not have previously been possible. Gene duplication provides a substrate on which new features can be added while sustaining existing phenotypic characteristics.

Many years ago, Riedl [45] argued that the structure of an organism made certain types of evolutionary change more probable than others. Dawkins [46] noted that when he introduced the possibility for segmentation within his computer-generated biomorphs, he was able to obtain variation that he had not found without such a developmental capability. This general point about the role of development in evolution has enormously important implications for the understanding of evolutionary processes, and the issue of evolvability continues to excite considerable debate [47]. What makes one lineage evolve more rapidly than another has already opened up the new science of "evo-devo" [42, 43]. The role of epigenetic change in driving novel mutational substrates, as discussed above, provides further opportunities for phenotypically driven evolutionary change. This point is discussed further in the final chapter of the book edited by Gissis and Jablonka [12].

More speciation occurs within a clade when polyphenism occurs within that clade [48]. This suggests that the presence of developmentally induced polyphenism favours adaptive radiation, providing a range of niche-defined phenotypes on which Darwinian evolution can act after fixation of the epigenetically mediated difference. Such a set of processes is likely, for example, to have occurred in a violet, *Viola cazorlensis* [49]. In this case, epigenetic differentiation of populations was correlated with adaptive genetic divergence.

King [50] suggested that speciation often involves a change in chromosome number. The number is known to be under genetic control. Closely related species can be

strikingly different. In horses, for example, the chromosome number ranges from 32 in *Equus zebra hartmannae* and 46 in *Equus grevyi* to 62 in *Equus assinus* and 66 in *Equus przewalski*; all but two of the horse hybrids are sterile. Similar variations in chromosomal number have been found in other mammals and strikingly in Alpine populations of house mice [51]. Humans and chimpanzees have different chromosomal numbers; chromosome 2 of the human is a fusion of two ancestral chromosomes, denoted 2A and 2B in the chimpanzee [52]. How could these differences between closely related species arise in evolution without involving the problems encountered by a solitary "hopeful monster" [53]? A hypothetical example illustrates one way.

Suppose that a herd of zebras wanders away from its usual habitat and enters an area where many of the plants available to the zebras as food contain toxins which they had not previously experienced. These toxins exert a developmental impact on the fetuses carried by the mares, and they form characteristics that are novel. When born, the zebra foals cope through phenotypic accommodation, but this nevertheless occurs at significant cost. In time, and in some individuals, these costs are minimised by genetic changes—perhaps biased by epigenetic change—and the type of evolutionary mechanism proposed by Darwin and Wallace operates to the advantage of these individuals and their offspring. Over time, the reorganisation required by such changes cascades and more and more genetic changes appear as the evolutionary adaptation processes create new order in the regulation of the zebra's development. The final step in this conjecture is that the genomic reorganisation impacts on chromosome number since the number is under genetic control. If this happens, then a reproductive barrier would be established between the new zebra population and the one from which it originated.

My general point is that an individual's adaptability allows a lineage to occupy a new place which can then lead to descendants entering many unexploited niches within that new habitat. The Galapagos finches are a clear example of how, in a relatively short space of time, birds arriving from the mainland were able to radiate out into many different habitats [54]. Tebbich et al. [55] discuss how the finches' capacity to respond to environmental challenges, for which they provide some evidence, could have played an important role in this process. None of this challenges the evolutionary mechanism postulated by Charles Darwin and Alfred Russel Wallace. The evolutionary process requires variation, differential survival and reproductive success, and inheritance. Three questions for the modern study of epigenetics arise from this formulation. First, what generates variation in the first place? Second, what leads to differential survival and reproductive success? Third, what factors enable an individual's characteristics to be replicated in subsequent generations? In answering all of these questions, an understanding of development is crucial.

6. Conclusions

One of the near-universal aspects of biology is that genetically identical individuals are able to develop in such strikingly different ways. Phenotypic variation can be triggered during development in a variety of ways, some mediated through the parent's phenotype. Sometimes phenotypic variation arises because the environment triggers a developmental response that is appropriate to those ecological conditions [56, 57]. Sometimes the organism "makes the best of a bad job" in suboptimal conditions. Sometimes the buffering processes of development may not cope with what has been thrown at the organism, and a bizarre phenotype is generated. Whatever the adaptedness of the phenotype, each of these effects demonstrate how a given genotype will express itself differently in different environmental conditions.

The decoupling of development from evolutionary biology could not hold sway forever. Whole organisms survive and reproduce differentially, and the winners drag their genotypes with them [4]. The way they respond phenotypically during development may influence how their descendants' genotypes evolved and were fixed [7]. This is one of the important engines of evolution and is the reason why it is so important to understand how whole organisms behave and develop.

The characteristics of an organism may be such that they constrain the course of subsequent evolution or they may facilitate a particular form of evolutionary change. The theories of biological evolution have been reinvigorated by the convergence of different disciplines. The combination of developmental and behavioural biology, ecology, and evolutionary biology has shown how important the active roles of the organism are in the evolution of its descendants. The combination of molecular biology, palaeontology, and evolutionary biology has shown how important an understanding of developmental biology is in explaining the constraints on variability and the direction of evolutionary change.

Disclosure

Most of the arguments in this review are developed at greater length in my book with Peter Gluckman [7].

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Review Article

Genetics: Polymorphisms, Epigenetics, and Something In Between

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At its broadest sense, to say that a phenotype is epigenetic suggests that it occurs without changes in DNA sequence, yet is heritable through cell division and occasionally from one organismal generation to the next. Since gene regulatory changes are oftentimes in response to environmental stimuli and may be retained in descendent cells, there is a growing expectation that one's experiences may have consequence for subsequent generations and thus impact evolution by decoupling a selectable phenotype from its underlying heritable genotype. But the risk of this overbroad use of "epigenetic" is a conflation of genuine cases of heritable non-sequence genetic information with trivial modes of gene regulation. A look at the term "epigenetic" and some problems with its increasing prevalence argues for a more reserved and precise set of defining characteristics. Additionally, questions arising about how we define the "sequence independence" aspect of epigenetic inheritance suggest a form of genome evolution resulting from induced polymorphisms at repeated loci (e.g., the rDNA or heterochromatin).

1. Epigenetics and Evolution

The importance of sequence polymorphisms in evolution is fundamental and irrefutable. The contribution of epigenetic gene regulation is considerably less well established. In this perspective, I will not attempt to summarize all the studies that have contributed to our current understanding of epigenetics; instead, I will thread together a handful of salient studies, taken particularly but not exclusively from Drosophila research, to illuminate how common and consequent "epigenetic" gene regulation may result from induced polymorphism. Inclusion of induced polymorphism in the panoply of epigenetic gene regulatory mechanisms may force us to reconsider our definitions, but is in accord with current and historic uses of "epigenetics," and may provide a new mechanism to understand how stable changes in gene expression can be established and maintained.

To understand the role of epigenetics in evolution, it is necessary to consider definitions of both evolution and epigenetics. For the purpose of any discussion linking the two, "evolution" must expand to include the change of frequency of *phenotypic variants* irrespective of underlying allelic variants. This is a mild departure from a sequence-centric view of changes in allele frequencies in evolving populations, but is ironically more aligned with the original use of "epigenetic" to describe the abstract processes that produce a phenotype from a genotype prior to the elucidation of the central dogma, gene regulation, and developmental genetics. Now, "epigenetics" are instances of changes in gene regulation that do not correspond to underlying changes in nucleotide sequence. What one means by "changes in nucleotide sequence" is worth dwelling on, which I will do later. In general, changes in nucleotide sequence are "polymorphisms" although it is common to see them called "genetic" in order to contrast them with "epigenetic." However, this is a misuse, and genetics is the study of inheritance and variation whatever their cause; polymorphism and epigenetics are subsets of genetics, and as I hope to convince you, they are neither exclusive nor exhaustive (Figure 1).

Understanding the joint contributions to evolution of polymorphism and epigenetics, particularly the latter, requires understanding the difference between them. This difference is profound since while polymorphisms are thought to be characterized by random, permanent, and well-understood changes to genetic information, epigenetic gene regulation is more volatile and hence has come to

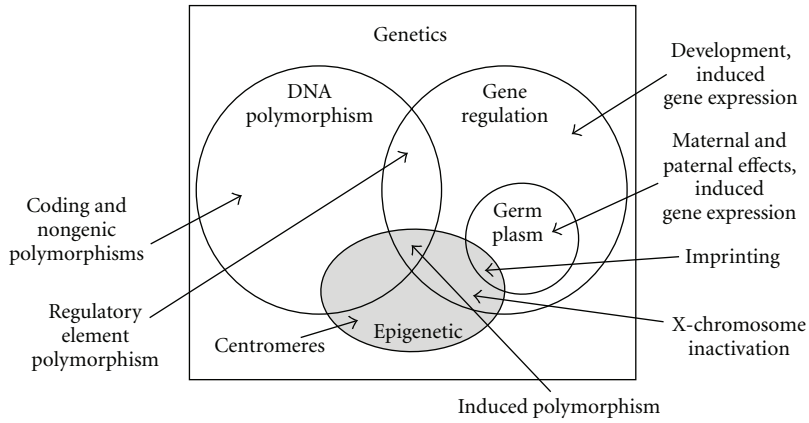

Figure 1: Relationships within genetics: random sequence polymorphisms, epigenetics, gene regulatory mechanisms, and induced polymorphisms.

include induced and reversible alterations in heritable traits. This raises the popular view (however, unfair) of Jean-Baptiste de Lamarck, that our own actions or experiences may come to bear on our offspring. Lamarck envisioned that an organism evolves by passing its experience to offspring. On the surface, the inheritance of acquired characteristics was consistent with slow change in species over time. It was not until Weismann articulated the difficulty in a giraffe's neck discussing its experience with a giraffe's sperm that a Lamarckian mechanism of evolution was cast aside. The resurgence of Lamarckian models of evolution has recently occurred for a number of reasons. First, there are clear examples of inheritance of information outside of DNA sequence, which has opened the possibility of experience affecting gene expression and such changes in expression being transmitted to offspring. Second, not only is this hypothetical model possible, but it is heretical and provocative, and thus exciting. Third, perhaps many of us feel more than a little guilty in heaping ridicule on an otherwise superb scientist who happened to be wrong.

2. What Is/Are Epigenetics?

A clear, concise, and comprehensive definition of epigenetics is tricky to articulate, not because it is difficult *per se*, but because the term has seen an expansion over the last decade and has started to include things that are arguably not epigenetic. To clarify the situation, Youngson and Whitelaw gave a cogent description of the difference between two types of "epigenetics": transmissible changes in expression (which they called "transgenerational epigenetic effects") and transmissible chromosome modifications ("transgenerational epigenetic inheritance") [1, 2]. They were attempting to separate two very different sets of phenomena that are both described as epigenetic. Many cases of "epigenetics" in recent literature fall into the former category and are not epigenetic at all, but rather are examples of germ cell gene regulation. To be meaningfully distinct from simple "transcription factor → enhancer → promoter → expression" forms of gene regulation, epigenetic phenomena must display three characteristics: they must manifest as (1) heritable genetic changes that (2) are associated with chromosomes but (3) are not based on DNA sequence. These are criteria that should not be abandoned, but should be evaluated.

The second characteristic is important because it is the essence of epigenetic inheritance. Why? Because if epigenetics did not require chromosome association, every genetic pathway that included a positive-feedback loop would be epigenetic. Female-specific sex-lethal splicing in Drosophila to form more active sex-lethal splicing factor would be considered epigenetic. Bacterial expression of LacY, the lactose permease, increasing sensitivity to further exposure to lactose would be considered epigenetic. Autophosphorylation of CaMKII upon witnessing a calcium spike would be considered epigenetic. Suppressor-of-Hairless-induced expression of Notch, the Suppressor-of-Hairless activator, would be considered epigenetic. In short, just about every genetic network could be considered epigenetic, and "epigenetics" would not differ in any meaningful way from "gene regulation." Since proteins, lipids, RNAs, metabolic intermediates, and even toxins are passed through cell division in the cytoplasm, it is trivial to say that their effects are "inherited," and it is wrong to conclude that cells retaining consequences of their antecedents' experiences are necessarily epigenetic.

Without requiring chromosomal inheritance of epigenetic phenomena, expression in the germ line would be sufficient to demarcate any genetic pathway as epigenetic, which would serve merely to rename those genes expressed during the creation of eggs and sperm. It should not be surprising that such networks might span multiple organismal generations; after all it is the mother's genetics (and experiences) that create the egg and the father's genetics (and experiences) that create the sperm; alteration in these processes will certainly result in alterations to the next generation. Mammalian biology aggravates the issue even more, since late-term pregnancies can involve three concentric organisms: by the end of gestation, female mammals contain half-genomes of all their potential grandchildren; the oocytes housing those pronuclei are filled with gene products created by their mothers from the nutritional environment provided by their grandmother. Many cases called epigenetic are instead this form of transgenerational gene regulation.

To discriminate broad concepts of "memory" or "potentiation" in gene regulation from specific epigenetic inheritance, it is necessary to show that the epigenetic factors altering gene activity map specifically to the chromosomal locus being regulated. Experimentally, *epigenetic gene regulation is demonstrated when DNA violates the law of mass action*: two identical sequences can act in different ways despite identity in their sequences and in the proteins that bind to them. Conceptually, if a "naive" DNA was introduced into the system, would it behave as do the existing DNAs? If so, it is not epigenetic. Practically, this is most easily shown by showing that identical pieces of DNA (homologs, duplications, transgenes, individuals of repeated gene arrays, etc.) possess different behaviors. This has been shown for centromere identity [3, 4], genomic imprinting in mammals [5], plants [6–8], fungi [9, 10], nematodes [11], and insects [12–14]; it is this requirement that many examples of "epigenetics" do not test. The strong connection between epigenetics and chromatin structure has only contributed to a conflation of these terms. It is not unusual to find the term "epigenetic" associated with studies that merely show changes in histone modifications of a gene, perhaps even acetylation, with no experiments that test for heritability, sequence polymorphism, or chromosome association.

3. Does Epigenetics Exist?

Changes in nucleotide sequence resulting in phenotypic variants are clear, established, and the very foundation of the neo-Darwinian synthesis that married Darwin's theories of variation and selection with Mendel's rules of inheritance. What was, and remains, magical about epigenetics is that substantial variation may be seen with no evident underlying changes in nucleic acid sequence and as such changes are relatively unstable. What first drew attention to epigenetic inheritance was the different behavior of identical genomes, in the variegation as a result of cosuppression which inactivates duplicated gene copies in plants, heterochromatin-induced position effects of Drosophila [15], or somatic mosaicism due to X chromosome inactivation in female mammals. These differences in phenotypes would not be surprising if they were due to differences in DNA sequence.

But how carefully have we tested for sequence identity in these cases? Imagine a hypothetical situation. What if creating a centromere required an enzyme (centromerase?) to cut the DNA and insert a specific sequence necessary and sufficient to establish centromere activity? What if cases of neocentromeres were cases of rare random expression and activity of centromerase? What if loss of centromeric activity in dicentric Robertsonian fusion chromosomes was evidence of the reversibility of centromerase? The hypothetical existence of centromerase is unnecessary, to be sure, given what we know about centromeric histones and chromatin structure, but it is illustrative that in many cases specific induced polymorphism is not even considered. We have a mindset that random mutation is the only mechanism allowed to alter DNA sequence, and therefore that rapid, induced, and reversible changes to chromosome behavior must occur without changes in sequence. But this assumes clearer lines in defining "sequence" than really exist, and it ignores many well-established observations.

Consider mating type switching in Schizosaccharomyces pombe. Switching occurs when a silent cassette of information from a "storage" locus is transferred to the active mating-type locus [16–18]. The mechanism of switching requires a mark, likely a break or ribonucleotide on one strand [19]. Tracing the ancestry of this strand has revealed that the altered strand comes from the switched locus in the previous generation. The result is that switching is limited in frequency and direction. A ribonucleotide in a chain of deoxyribonucleic acid is indeed a surprising way to carry information on a chromosome, but nonetheless it is genetic: it is heritable and consequent. And most surprising, it is inducible.

Consider also genomic imprinting in mammals. Is genomic imprinting really epigenetic? Although perhaps the most accepted form of epigenetics, it may be argued that it is not, for trivial nomenclatorial reasons: do you count 5-methylcytosine as cytosine, or as a fifth base that merely has an additional requirement for incorporation (a replication-coupled DNA methyltransferase)? While your answer may reveal something about your philosophy, it has impact on how we think of epigenetic mechanisms. If we count 5-methylcytosine as a fifth base, then the maternally and paternally derived alleles of genomically imprinted genes are indeed polymorphic. Can we also count dehydroxylation or deglycosylation as a polymorphism? Considering these cases of induced polymorphism would exclude both S. pombe mating type switching and imprinting at the Medea locus (where cytosine methylation induces a strand break on one homolog, alleviating it from silencing) as epigenetic. And why not? Selenocysteine is an amino acid even though a ribosome requires an extensive elaborated system to incorporate it [20, 21]. Methylcytosine is chemically and genetically distinct from cytosine; it merely requires an extensive elaborated system to incorporate it. A nicked DNA strand is again chemically and genetically distinct. It is a fun argument to make but seems overly contrived and unnecessary, and probably a little bizarre. It is not that we need to remove these cases from the list of epigenetics, but rather that we must consider what we mean by "sequence" when using it as the key criterion discriminating "epigenetics" from "polymorphisms." There is a lot of landscape in that gray area.

4. Something In Between

Understanding how and why we define "sequence" and "epigenetic" is important when categorizing modes of gene regulation. But such considerations also reveal insight into how these phenomena might interact and lead us to consider how important induced polymorphism could be in evolution. The above examples—Medea, mating type, and imprinting—are all cases of induced polymorphism which result in changes in genetic activity of the sequence. The fact that they are "sequence independent" is an artifact of our ACGT-sequence bias. Still, it seems doubtful that these handful of examples would by themselves upset our views of

evolution. First, such modes of epigenetic gene regulation are apparently uncommon. It is estimated that there are perhaps hundreds of imprinted loci in mammals, and as few as one in plants. Second, they are not cases of presence/absence of genetic pathways, but rather expression biases of different alleles, and so sibling do not differ markedly because of this mode of regulation; imprinted genes are essentially haploid and so are not much different than sex-linked genes in terms of evolution. Third, they are reset after one generation. It is therefore difficult to imagine that these forms of epigenetic inheritance drive evolution in profound or novel ways.

Are there examples of induced polymorphism that are widespread, consequent, and long-lived and might therefore affect genome evolution?

Almost one-half of the genomes of many popular metazoa are highly polymorphic, but those polymorphisms go unnoticed in genome-wide association, quantitative trait loci, and population genetics studies. This portion, the heterochromatin—alphoid and beta repeats, transposable elements, satellites, repetitive sequence, and so forth, all typically linked to centromeres—are not amenable to our modern approaches to genomics. Heterochromatin can comprise hundreds, thousands, and even millions of copies of simple (e.g., AATAT, AAGAG, and AAGAGAG) repeats [22–26]; hence they cannot be easily cloned, sequenced, or assembled using the techniques directed at whole-genome sequencing. In fact, the definition of "whole" has been altered to ignore this half of the genome [27–29]. Quantifying repeat copy number is cumbersome and imprecise, and stumbling upon rare sequence polymorphisms in otherwise homogenous blocks of satellite DNA is lucky [30]. It is therefore difficult to estimate the degree of differences or rate of polymorphism in this substantial portion of the genome.

Heterochromatin was first described by Emil Heitz in the 1920s and 1930s. At the time, its discriminating feature was heteropycnotic staining, which is still arguably the best definition. Subsequently, it was discovered that heterochromatin is generally late replicating, repressive for gene expression, and enriched in specific modifications of the DNA and the histones that package it although there are exceptions to all of these features [15, 31, 32]. What is agreed is that heterochromatin forms easily on highly-repetitive sequence and exists as a complex with heterochromatin proteins (e.g., histone methyltransferases, HP1, and possibly RNAs). Genetic and mutational manipulations that alter the amount of repetitive sequence or protein components demonstrate a natural balance between the sequence and protein components in forming heterochromatin [33–37]. Excess sequence compromises heterochromatin formation elsewhere by competing for limited heterochromatin proteins. Increases or decreases in heterochromatin proteins increase or decrease the ease of forming heterochromatin or increase or decrease the amount of sequence that can be packaged as heterochromatin.

Malik and Henikoff described their view of a specific example of an evolutionary balance at the centromeric chromatin (or "centrochromatin") [38–40]. They envision a coevolution of sequence expansion and DNA binding by the centromeric histone Cid. Excess centromeric DNA is bound by Cid, and changes in Cid binding (or expression) result in altered centromeric sequence. This may be an example of a broader mechanism of expansion and contraction limited (or promulgated) by the characteristics of DNA-binding proteins that stabilize repetitious sequence. The mix of multiple polymorphic simple repeats in the genome [25, 26, 41, 42] may be stabilized by a mix of dedicated or overlapping heterochromatin proteins [43–48]. The balance between the sequences and proteins that together form heterochromatin is expected to be important because the protein components of heterochromatin play double duty as general transcriptional regulators [49, 50]. Genes shift between "heterochromatin-like" and "euchromatin-like" as they shift between silent and expressed during development or as a response to environmental stimuli. Mutations in the genes that encode these protein components often act dominantly, suggesting that their dose matters [34, 36]. One can easily imagine a three-way balance between heterochromatic sequence, heterochromatin proteins, and euchromatic gene regulatory mechanisms. This predicts that copy number polymorphisms of heterochromatin-forming sequence might impact gene regulation throughout the genome.

It has been very difficult to test whether copy number polymorphisms are consequential because there are few molecular-genetic tools that allow manipulation of copy number in otherwise isogenic backgrounds. We know from classic studies in Drosophila, where the DNA and protein components of heterochromatin are easily manipulated, that the amount of heterochromatic sequence in a cell dramatically affects sensitized variegating genes [33, 51, 52]. At an extreme, multiple supernumerary heterochromatic chromosomes are lethal [53]. Although the reason remains unclear, one can imagine such a disruption in sequence-to-protein balance to cause massive misregulation of many genes. Y chromosomes captured from wild populations vary in their ability to affect heterochromatin-induced position effect variegation and euchromatic gene expression elsewhere in the genome [54–56], yet have very few protein-encoding genes [57–59], strongly suggesting that heterochromatin polymorphisms, perhaps copy number polymorphisms, affect gene expression throughout the genome. Our work has induced copy number variation in one repeat, the ribosomal DNA (rDNA) [60]. The rDNA has precedent for housing-induced phenotypic variation in plants [61, 62], but without being able to induce changes at the rDNA, it had been difficult to test this phenomenon further. In flies, however, induced copy number variation has consequences for heterochromatin-induced position effect variegation and gene expression across the genome [63, 64]. These variations in gene regulation overlap with those seen from isolated natural Y chromosomes [54, 64], suggesting a significant portion of natural variance in rDNA repeat copy number [65, 66] may contribute to phenotypic variance in natural populations. Equally importantly, much of the variance that maps to the Y chromosome does not map to rDNA, suggesting that most phenotypic variance maps to other sequences on the Y, perhaps to the other repeats that are less experimentally manipulable.

Natural variation in repeat sequence copy number may play a role in evolution, but the uniquely dynamic biology of the rDNA implies the more exciting possibility. Changes in copy number may be induced and inherited.

The rDNA contains interspersed active and inactive rRNA genes and thus contains characteristics of both euchromatin and heterochromatin in some cells. The physical manifestation of the tremendous expression and processing of the rRNAs is the nucleolus. The stability of these long stretches of direct repeats in the nucleus is likely due to the heterochromatic packaging of a subset of the repeats. Peng and Karpen observed multiple nucleoli in postmitotic cells of animals carrying mutations of suppressor-of-variegation genes, which encode the protein components of heterochromatin and regulate the rDNA [67]. Their results suggest that repeat sequence not packaged as heterochromatin is unstable and prone to damage/repair or intrachromosomal recombination. Our experiments showed that mutation of suppressor-of-variegation genes resulted in destabilization and reduction of rDNA copy number through mitosis [63]. We further quantified loss in the soma and also showed that loss was seen through the germline, resulting in a permanent decrease of rDNA copy number in a population after exposure to a mutation that disrupts heterochromatin formation. We more recently showed that mutation of a repressor of rDNA expression (CCCTC-Factor, or CTCF) also destabilizes the rDNA, resulting in permanent loss [68]. These results are consistent with heterochromatin-like silencing stabilizing repeated DNA sequence, and a balance between repeat sequences and the protein components that regulate them.

In Drosophila, the ribosomal DNA is a compelling compartment because its dynamism is unmatched. It is the most highly expressed set of genes in the genome [69], coordinates the activity of all three polymerases, shrinks naturally through the formation of extrachromosomal circles [70], can repair itself through meiotic magnification or somatic pseudomagnification [71–75], and can compensate its output through alteration of elongation rate and possibly initiation rate [76, 77]. It possesses side-by-side copies that possess heterochromatic and euchromatic chromatin structures [76, 78–80]. As the central body in protein synthetic capacity, it is also responsive to nutritional status, sensitive to toxins and drugs, and susceptible to instability by alterations of gene products necessary for its regulation [81–87]. Altering regulation of the rDNA through mutation or drug treatment affects not only rRNA output, but also stability [88–90]. Alteration of the activity of a protein component of heterochromatin might therefore affect the copy number of the sequence to which it binds.

Dynamism (of rDNA) and balance (of heterochromatic sequence and proteins) establishes a situation of heterochromatin homeostasis (Figure 2). Sequences are protected from loss by packaging as heterochromatin. Loss of protein (or reduced protein activity, arrow "a") would destabilize repeat DNA (white state) and result in loss, reestablishing an equilibrium (arrow "b" to the gray state). Similarly, excess sequence would revert through loss if there is not sufficient protein to package it for stability. But excess protein is not without consequence, since any heterochromatin protein not bound in constitutive sequence would alter gene expression throughout the genome (dark gray state), favoring either reduced protein expression/activity (arrow "c") or expansion of repeat sequence (arrow "d") to reestablish balance (light gray states). On the whole, the instability of repeat sequence and the consequence of excess heterochromatin proteins creates multiple states that balance the factors and naturally drives the number of repeat sequences and protein expression to equilibrate. Of course, any external factors that influence heterochromatin protein activity would be expected to result in induced and heritable changes in repetitive DNA copy number. The rDNA is particularly sensitive to induced copy number polymorphism, since it is affected by nutritional status throughout the lifetime of an organism and rDNA copy number exists in excess of what is required for translational demands, allowing some plasticity in copy number without being unduly disadvantageous.

On the surface, induced copy number polymorphism is similar to epigenetic modification (particularly if one cannot easily sequence and assemble repetitious DNAs), and the ability of repeat sequences to change in copy number relatively easily adds the degree of volatility common in epigenetic gene regulation. Unlike many forms of transgenerational gene regulatory effects, induced copy number polymorphisms are linked to chromosomes, and thus are both heritable and selectable. Unlike epigenetic regulation of imprinted or inactivated chromosomes, induced copy number polymorphisms can be inherited over multiple generations. But like both transgenerational and epigenetic effects, the role of induced polymorphism is only beginning to be considered in evolution. Such investigation will likely be done in simple organisms, such as Drosophila, that have relatively simple rDNA architecture [91, 92]. By contrast, humans have multiple rDNA arrays which change in size frequently [93], and the complex regulation that renders some arrays active and others inactive means it may be some time before we understand how rDNA polymorphisms and rDNA instability [94] contribute to phenotypic variance in human population or to disease etiology.

5. Is the rDNA Special?

Induced polymorphism of rDNA copy number offers a convenient mechanism by which changes may be inherited although the same objections apply here as they do for the environmentally induced changes in gene expression that craned Lamarck's neck: how is the germline affected? In the case of induced polymorphism, germ cells may be more, not less, sensitive to induced alterations in heterochromatin composition, for three reasons. First, in many cases, gene expression is limited in these cell types. Perdurance of heterochromatin proteins, or the presence of ample gene product to endure fluctuation in gene activity, may be less in these cell types. Second, at least in males, the genome is stripped of most somatic chromatin components in favor of packaging proteins and polyamines. This may increase the sensitivity of such chromosomes to DNA rearrangements or specifically mark some regions for hypervariability. Third,

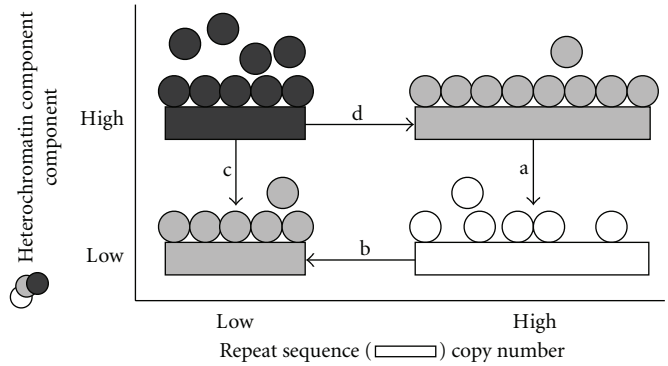

Figure 2: An illustration of a balance between heterochromatic sequences and heterochromatin components (e.g., proteins or RNAs). Repetitious heterochromatin-forming sequences (*rectangles*) are normally in balance with the proteins that bind them (*circles*), package them as heterochromatin, and thereby stabilize them (conditions in *gray*). Since these factors are used to regulate expression of euchromatic genes, the balance must accommodate "excess" factors for that purpose (denoted as circles apart from rectangles). If the expression or activity of proteins is reduced (a), repetitious sequence is exposed, destabilized, and lost through damage-repair, recombination, or extrachromosomal circle formation (b), until a new balance is established. Excess protein has gene regulatory consequence throughout the genome and presses to reestablish balance by altering expression level or activity (c) or perhaps through repeat expansion (d).

germ cells naturally undergo recombination at a high rate. It is well established that changes in microsatellite and rDNA copy number occur in meiosis, while the same sequences are relatively stable in mitosis. The challenge is to understand what identifies a gene as "sensitive to rDNA copy number," because it would be those genes selected for phenotypic variation in response to rDNA copy number changes.

We do not yet understand whether repeated sequences are different from "nonexpressed" sequences in ability to be induced to change, but we do know from mutational and molecular analyses that "heterochromatin" is not monolithic and is more accurately thought of as multiple "colors" [95]. Mutations may affect one chroma of heterochromatin and not another [96]. The five enumerated chromas significantly expand our understanding of chromatin structure, but even those five are likely still a simplification caused by our failure to resolve more subtle differences. Cumbersome work has detected alterations of repeat sequence copy number in few studies, suggesting that this may be a very widespread form of genetic variation [66, 97, 98]. Peng and Karpen showed an increase in DNA damage repair foci in the heterochromatin of suppressor-of-variegation mutants in diploid cells [99, 100]. They did not identify the sequences that were being repaired, but the number and distribution of repair foci in the nuclei indicated that it was not clustered (i.e., limited to the rDNA arrays). This observation suggests that the heterochromatin formed on simple repeats (and not just the highly-expressed rDNA) also is stabilized by packaging as heterochromatin. As our understanding of what heterochromatin is, and as tools become available to probe it in more surgical ways, we may begin to unravel complex interactions between types of heterochromatin as they struggle to keep each other in check or ally to fend off common enemies.

The term "epigenetics" may retain its strict definitions of chromosome-bound nonsequence-based genetic information, or it may be expanded to include induced mutation or gene regulatory networks that impact subsequent generations. In the end, all forms of regulation are genetic, and so are salient in understanding how complex, pleiotropic, and epistatic genetic interactions conspire to create phenotypes. However one defines epigenetics, it's legacy is that we cannot understand the comprehensive synthesis of forces that drive a genome's evolution without understanding how all the alleles within that genome are regulated.

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Review Article

Finding a Balance: How Diverse Dosage Compensation Strategies Modify Histone H4 to Regulate Transcription

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Dosage compensation balances gene expression levels between the sex chromosomes and autosomes and sex-chromosome-linked gene expression levels between the sexes. Different dosage compensation strategies evolved in different lineages, but all involve changes in chromatin. This paper discusses our current understanding of how modifications of the histone H4 tail, particularly changes in levels of H4 lysine 16 acetylation and H4 lysine 20 methylation, can be used in different contexts to either modulate gene expression levels twofold or to completely inhibit transcription.

1. Need for Dosage Compensation

Proper chromosome dosage is essential for the viability and fitness of an organism [1]. Most variations in chromosome quantity (aneuploidies) are inviable [1]. Some aneuploidies are tolerated, but result in severe developmental phenotypes, including Down syndrome, trisomy 21 [1]. However, a difference in sex chromosome copy number must be accommodated across many species. Sex can be determined by sex chromosomes, where one sex is homogametic for the sex chromosome, while the other is heterogametic. In the XY sex chromosome system, females have two X chromosomes, and males are XY or XO. In the ZW system, males are ZZ, and females are ZW. As a consequence of these differences, the heterogametic sex is functionally monosomic for the sex chromosome. The X and Z chromosomes encode genes involved in many processes required for life, not just sex-specific processes. To cope with this disparity, dosage compensation balances the expression of the sex chromosomes to the diploid autosomes and equalizes sex chromosome expression between males and females.

Dosage compensation has been studied in mammals, worms, flies, and birds. These organisms all cope with sex chromosome imbalance between males and females; however the mechanisms and machineries that they use differ widely (Figure 1). In the fly *Drosophila melanogaster*, XY males upregulate their single X chromosome twofold [2]. This process accomplishes both goals: it balances expression of the single X with autosomes and also equalizes X-linked gene dosage in the sexes. Although less well understood mechanistically, X chromosome upregulation is thought to occur in both sexes in mammals [3, 4]. While this balances the genome in XY males, it causes overexpression of the X chromosomes in XX females. A second (and better understood) mechanism then inactivates one of the two X chromosomes in females, thereby equalizing X expression [5]. In the nematode *C. elegans,* the X chromosomes are thought to be upregulated in both XO males and XX hermaphrodites [3] then downregulated two-fold in hermaphrodites only [6]. In birds, dosage compensation occurs regionally on the Z chromosome. This partial dosage compensation increases expression of required genes in ZW females [7].

The dosage compensation strategies outlined above include two-fold upregulation, two-fold downregulation, and complete transcriptional silencing. Interestingly, one feature of chromatin appears to be involved in all of these mechanisms: a difference in the level of histone H4 lysine 16 acetylation (H4K16ac) on the dosage compensated sex chromosome(s). In this paper, we will describe our

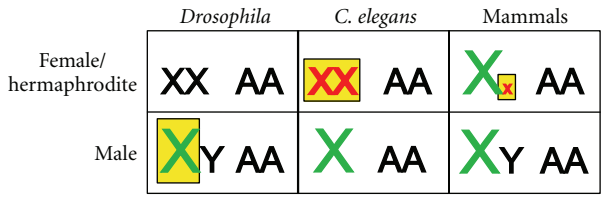

Figure 1: X chromosome dosage compensation. Dosage compensation balances expression of the X chromosomes between males and females and equalizes expression between the X and autosomes. In male flies, the single X chromosome is upregulated. *C. elegans* upregulates the X chromosomes in hermaphrodites and males, and the dosage compensation complex functions in hermaphrodites to downregulate transcription two-fold. The X chromosomes are upregulated in female and male mammals, but one X chromosome is inactivated in females. Green text indicates upregulation, and red text indicates downregulation. Yellow boxes depict chromosomes that are targeted by specific dosage compensation mechanisms.

current knowledge of H4K16ac and its role in modulating the structure of chromatin and regulating transcription. We will then describe how changes in levels of this modification correlate with transcriptional regulation in a diverse array of dosage compensation strategies.

2. Nucleosome Structure and Histone Modifications

Chromatin is a dynamic and flexible structure that not only serves to package DNA into higher-order structures, but also regulates access to the DNA. In the nucleosome, 147-bp of DNA wraps around an octamer of histone proteins, composed of two each of histones H2A, H2B, H3, and H4 [8]. Histones H2A and H3 may be replaced by a histone variant protein [9]. The N-terminal tails of the histones extend from the nucleosome core and can be posttranslationally modified by phosphorylation, methylation, ubiquitination, and acetylation [10, 11]. Modification of the histone tails influences the interactions of neighboring nucleosomes and access of regulatory proteins.

Nucleosome structure affects higher-order folding of the chromatin fiber. High-resolution structure analysis of the nucleosome has provided insights into the interactions between neighboring nucleosomes. Histone H4 tails are highly basic and are thought to bind to an acidic patch in the H2A-H2B dimer in the neighboring nucleosome [12]. Binding across nucleosomes suggests that the histone H4 tail is more important for interactions between nucleosomes than for interactions with other histones within the same nucleosome. Computational modeling has demonstrated that the histone tail forms an α-helix centered around lysine 16 [13]. In its unmodified form, the histone tail α-helix aligns basic charges in one direction, which allows a perfect fit and strong interaction with the acidic patch in the neighboring nucleosome [13].

3. H4K16 Acetylation

Histone H4 can be acetylated on lysines 5, 8, 12, and 16. Studies using site-specific antibodies have indicated that H4K16ac is usually present in the monoacetylated form of the H4 tail [14–16]. The order of acetylation of the other lysines in preexisting H4 tails proceeds in the N-terminal direction, such that K12 is acetylated second, then K8, and finally K5 [17]. In newly synthesized histone tails, K5 and K12 are acetylated first [18]. The pattern of acetylation of the H4 tail is the same in human, mouse, yeast, and *Tetrahymena*, demonstrating the universality of the H4 acetylation mechanism [19].

Regulation of K16 acetylation is unique from the other lysines of histone H4 [20], highlighting the importance of this particular modification. Regulation of H4K16ac is achieved by the balance between MYST domain histone acetyltransferase (HAT) and class III histone deacetylase (HDAc) (Sir2 family) activities [21]. However, recent evidence suggests that this balance is quite complex. Lu and others have shown in HeLa cells that SIRT1 (a Sir2 homolog) activity is needed to limit hMOF (MYST HAT) autoacetylation to allow hMOF to bind DNA [22]. Further, this work suggested that direct regulation of MYST HAT activity is conserved across many species, including additional mammalian systems, *C. elegans*, and *D. melanogaster* [22]. This mechanism suggests that both direct and indirect means are used by the deacetylase SIRT1 to regulate histone acetylation.

H4K16ac is thought to play a central and unique role in modulating chromatin structure (Figure 2(a)). It is unique among posttranslational histone modifications in that it directly affects the structure of the chromatin fiber. Acetylation of K16 decreases the positive charge of the histone tail, destabilizes the α-helical conformation of the tail, and disrupts the interaction of the tail with the acidic patch on the H2A/H2B dimer surface [12, 13]. Therefore, K16 acetylation triggers the unfolding of chromatin by disrupting the interactions between neighboring nucleosomes. Sedimentation assays that evaluate the degree of nucleosome array folding or intraassociation, which mimics formation of the 30-nm fiber, have demonstrated that H4K16ac inhibits nucleosome array folding [23, 24]. Tetra-acetylated H4 dramatically inhibits intraarray folding, more than H4K16ac alone, suggesting that additional acetylation of the H4 tail beyond H4K16 creates an environment even more disruptive to nucleosome folding [23, 24]. Acetylation of K16 also perturbs the divalent cation-induced self-aggregation of nucleosome arrays, thought to mimic higher order folding, or inter-array interactions [23, 24]. Mutation of K16 to a glutamine mimics acetylated lysine but does not cause decompaction of a nucleosome array, indicating that K16 is critical for decompaction [25]. Higher acetylated forms of the H4 tail further prevent self-aggregation of arrays [23].

H4K16ac not only affects nucleosome interactions, but also affects interactions of the nucleosome with chromatin-associated proteins. ISWI is a member of the family of chromatin remodeling ATPases that promotes regularity of nucleosomes and chromatin folding. ISWI binds to amino

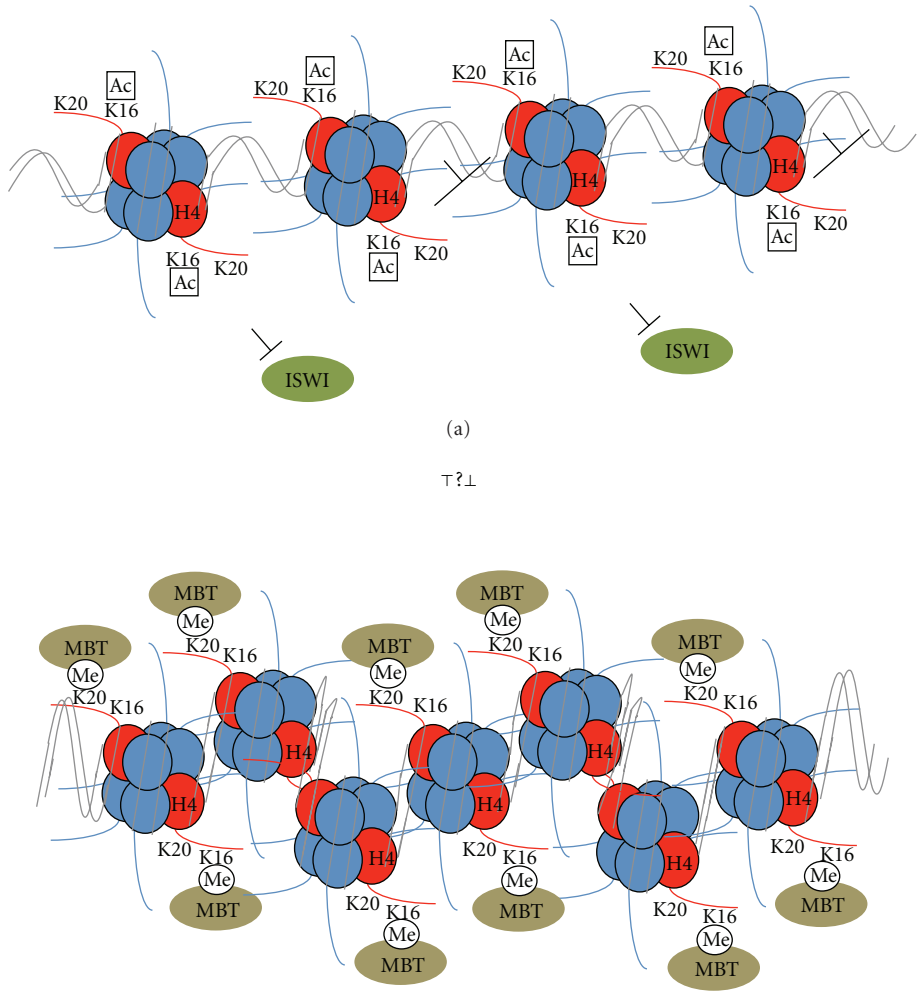

FIGURE 2: A model illustrating the antagonistic effects of H4K16ac and H4K20me1 on chromatin packaging. (a) Chromatin acetylated at H4K16 is loosely packed, due partially to charge neutralization, and partially to effects on interactions with chromatin modifying proteins, such as inhibition of chromatin remodeling by ISWI. (b) Chromatin methylated at H4K20 is tightly packed. In some systems, H4K20me1 and H4K16ac antagonize each other (see text). H4K20me1 also binds to MBT domain containing proteins, which may facilitate chromatin compaction.

acids 17–19 within the H4 tail, and this binding stimulates ISWI activity [26–28]. Acetylation of the nearby lysines 12 and 16 impairs the ability of ISWI to recognize its target binding site to compact chromatin and to slide nucleosomes along DNA [24, 27, 28].

4. H4K20 Methylation Antagonizes H4K16 Acetylation

The fifth lysine residue on the H4 tail, K20, can be mono-, di- or trimethylated. Histone H4 lysine 20 monomethylation (H4K20me1) is established by the histone methyltransferase PR-Set7/Set-8 [29, 30], and Ash1 also monomethylates H4K20 in *Drosophila* [31]. Di- and trimethylation of H4K20 (H4K20me2/3) is accomplished by SUV4-20 [32, 33]. H4K20 methylation antagonizes H4K16ac and is therefore important for controlling gene expression [30, 34, 35]. In *in vitro* assays, H4K20 monomethylation antagonizes acetylation of H4K16 and vice versa [30], and levels of these two marks inversely correlate during cell cycle progression in human cells [35]. However, other studies showed substantial overlap between H4K20me1 and H4K16ac at the β-globin locus, indicating that these marks are compatible in some circumstances [36]. The action of H4K20me1 on chromatin is also context dependent. H4K20me1 correlates with active transcription in some contexts [37–40], while in others it is associated with repressed genes [41–44]. For the purposes of this paper, we will focus on H4K20me1's repressive action because of its role in antagonizing H4K16ac.

H4K20me1 can induce chromatin compaction (Figure 2(b)). The mark is found in the same compartment as other repressive marks in many systems and is proposed to regulate the packaging of chromatin into facultative heterochromatin and serve as an intermediary toward

H4K20me3 enrichment in constitutive heterochromatin [11, 32, 43–48]. Consistent with a role in chromatin compaction, depletion of PR-Set7 results in decondensed chromosomes [49]. Binding of MBT (malignant brain tumor) domain-containing proteins to the H4K20me1 mark contributes to chromatin compaction [50, 51]. The mechanism of chromatin compaction by MBT domain-containing proteins is not completely understood, but it may involve binding to multiple nucleosomes and DNA bending or bridging of neighboring nucleosomes by dimerization of the MBT domain [51–53].

5. The Effect of H4K16ac/H4K20me1 on the RNA Polymerase II Transcription Machinery

In addition to affecting chromatin structure, H4K16ac and H4K20me also regulate the RNA Polymerase II machinery directly. Transcription initiation is a highly regulated process [54]. After initiation of transcription, RNA Polymerase II stalls just downstream of the transcription start site in many highly regulated genes [55]. Stalled polymerase remains at this site until elongation factors, such as P-TEFb, are recruited to facilitate transition to productive elongation [55–57]. P-TEFb recruitment to active loci is an intricate process, involving release of P-TEFb from a sequestration complex by activators including BRD proteins, which are recruited to RNA Pol II and chromatin by H4K16ac [58, 59]. Recruitment of BRD4/P-TEFb to the chromatin occurs by recognizing the combination of H4K16ac and H3S10 phosphorylation, which provide a binding platform for the complex, at least at the FOSL1 gene (this model is shown on Figure 3) [60].

The role of H4K16ac in gene expression has been studied extensively in budding yeast [61, 62]. While H4K16ac is present throughout most of the genome, H4K16 is hypoacetylated at silenced loci, including the mating type loci and telomeric regions [63]. The Sir2, 3, and 4 proteins form a complex essential for transcriptional repression at silenced regions [64]. The Sir complex mediates deacetylation of H4K16 in neighboring nucleosomes through Sir2 action [65, 66]. Deacetylation of H4K16 by Sir2 represses transcription by reducing RNA Pol II promoter occupancy [67] or blocking access of capping enzymes and elongation factors to RNA Pol II, reducing transcriptional elongation [68, 69].

Acetylation of H4K16 is important for transcriptional activation, while H4K20 methylation is suggested to have direct repressive effects on transcription in certain contexts. Trimethylation of H4K20 has been proposed to limit RNA Pol II transcription by blocking H4K16ac and P-TEFb recruitment [70]. PR-SET7 and L3MBTL1 interact directly to repress transcription of a reporter gene, suggesting that H4K20 monomethylation is directly required for transcription repression [71]. Loss of H4K20 monomethylation in multiple studies has indicated the role of this mark in silencing. Deletion of PR-Set7, the H4K20me1 HMT, in flies causes reactivation of genes located in heterochromatin and which would normally be silenced [42]. Furthermore, knockdown of PR-Set7 results in decreased H4K20me1 and an approximately two-fold increase in expression of H4K20me1-associated genes in mammalian cells [41]. H4K20 methylation and H4K16ac have opposing effects on regulation of transcription and transcription machinery, as expected given their mutual antagonism.

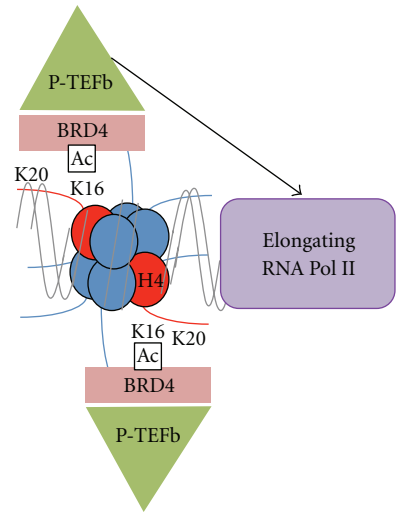

Figure 3: A model of transcriptional regulation by H4K16ac. H4K16ac recruits the transcription elongation factor P-TEFb through the transcriptional coactivator BRD4. P-TEFb phosphorylates RNA Pol II, signaling the transition to productive elongation.

6. Involvement of H4K16 Acetylation in Dosage Compensation Mechanisms

6.1. Upregulation of Gene Expression: Flies and Birds. Fly dosage compensation is accomplished by two-fold upregulation of the single male X chromosome by the *male-specific lethal* (MSL) complex, composed of the proteins MSL1, MSL2, MSL3, MLE, and MOF, and two noncoding RNAs, roX1 and roX2 [2, 72]. The MSL complex specifically binds the X chromosome. The current model of MSL binding to the male X chromosome includes a two-stage process: first, MSL-1 and -2 bind and load at ~150 high affinity (chromatin entry) sites; then, the other proteins localize and facilitate spreading of the complex to many more sites of action across the single male X chromosome [73, 74]. MSL complex loading involves a DNA sequence motif, GAGAGAGA [73]. Models for the spreading of the MSL complex include recognition of cotranscriptionally deposited H3K36 methylation [75, 76], MOF-dependent acetylation/deacetylation cycles tuning MSL-3 activity [77], and binding of specific chromatin features by the MRG domain of MSL-3 [78–80]. The histone acetyltransferase subunit of the MSL complex, MOF, acetylates histone H4K16 leading to an enrichment of this mark on the X [81–84]. By contrast, levels of H4K20me1 are low on the male X [30], although some level of H4K20me1 appears to be necessary for spreading of the MSL complex [79, 80]. JIL-1 kinase, which phosphorylates H3S10 and synergizes with H4K16ac action, also contributes to fly dosage compensation [85–87].

There is also evidence that ISWI, whose binding to chromatin is blocked by H4K16ac, may play a role in fly dosage compensation. X chromosome bloating, which indicates severe decondensation, was seen upon perturbation of the ISWI-containing NURF complex [88, 89]. Blocking H4K16ac in males suppresses X chromosome defects seen in ISWI mutant male flies [28]. Conversely, aberrant overacetylation of H4K16 in ISWI mutant females caused chromosome decompaction defects identical to those seen in ISWI mutant males, especially on the X chromosomes, and broad-reaching gene misexpression [28, 90]. Increased MOF expression also strongly enhances the ISWI loss phenotypes [28].

How does the MSL complex enhance transcriptional output? MSL localization and MOF-dependent H4K16ac are biased toward the 3′ end of gene bodies, which suggests that fly dosage compensation might regulate transcription elongation [75, 91]. Recent work utilizing global run-on sequencing analysis has yielded compelling evidence that dosage compensation in flies is achieved by increased transcription elongation of male X chromosome genes [91]. Other studies have provided further hints that males dosage compensate by increasing transcriptional elongation. The viability of males was greatly affected by knockdown of the elongation factor dELL in flies [92]. The MSL complex chromatin entry site binding motif is a GA-rich sequence [72, 73]. GAGA factor binds to a GAGA motif and helps to release paused polymerase at many genes [93]. Mutations in the GAGA factor gene disrupt dosage compensation in *Drosophila* [94]. JIL-1, the kinase known to play a role in fly dosage compensation, is also involved in transcriptional pause release [60]. The conclusion that fly dosage compensation acts at the level of transcription elongation is consistent with the role of H4K16ac in facilitating release of paused polymerase in *Drosophila* and the other systems described previously.

Like flies, birds regulate expression from the sex chromosome by upregulation. In birds, males (ZZ) are the homogametic sex, and females (ZW) are the heterogametic sex. However, despite the Z chromosomal imbalance between avian males and females, there is no evidence that birds have a chromosome-wide dosage compensation mechanism [95–97]. Rather, it appears that birds use region- or gene-specific methods to balance Z gene expression.

When comparing the expression ratio of genes along the Z chromosome between ZZ male and ZW female chickens, one area displays clear female bias [98]. This region is the MHM (male hypermethylated) locus and is enriched in compensated genes. A non-coding *MHM* RNA is expressed specifically in females [99]. Because the region is hypermethylated in males, it is not transcribed. H4K16ac is strikingly enriched in one area of the nucleus in a female-specific manner [100]. Increased acetylation of H4 at K5, K8, and K12 was also noted in females, although to a lesser extent than acetylation of H4K16. Further analyses demonstrated that the area of increased H4K16ac corresponds to the MHM locus [100]. The enrichment of H4K16ac at the dosage-compensated region in ZW female chickens resembles the enrichment of H4K16ac on the X chromosome in XY male flies, although only at one locus and not chromosome-wide. However, the mechanism of partial dosage compensation may be similar to chromosome-wide compensation, and regional acetylation of H4K16 may allow for increased expression of Z genes sex specifically.

6.2. Transcriptional Downregulation: Worms. Dosage compensation in the worm uses a mechanism different from flies and birds. Upregulation of the X is thought to be non-sex-specific, creating a need to dampen X-linked gene expression in the hermaphrodite. This is achieved by twofold downregulation of each hermaphrodite X chromosome, equalizing expression with that of the single male X [6, 101–107]. This is achieved by the dosage compensation complex (DCC), which is composed of two parts. The first part is condensin I^{DC}, which shares four of five subunits with the canonical condensin, regulator of chromosome structure during mitosis and meiosis [107]. Condensin I^{DC} is composed of MIX-1, DPY-27 (DCC-specific), DPY-26, DPY-28, and CAPG-1 [6, 102, 103, 105–107]. The second part is a recruitment complex, composed of SDC-1, SDC-2, SDC-3, as well as two associated proteins DPY-21 and DPY-30 [6, 101, 104, 106, 108]. The high degree of similarity to condensin has led to the hypothesis that dosage compensation in the worm is achieved by a change in X chromosome structure.

Recent work has identified several connections between chromatin modifications and the DCC. The histone H2A variant, HTZ-1 (H2A.Z), plays a role in DCC localization. Loss of *htz-1* did not alter expression of DCC components, but instead led to spreading of the DCC to autosomes [109]. A survey of histone modifications using ChIP-chip analysis by the modENCODE project found an enrichment of H4K20me1 on the X chromosomes [110, 111]. Using immunofluorescence microscopy, we also observed enrichment of this mark on the X chromosomes in hermaphrodite somatic cells. Furthermore, we see a depletion of the mark antagonized by H4K20me1, H4K16ac. The hermaphrodite X chromosomes show sex- and DCC-dependent enrichment of H4K20me1 and underrepresentation of H4K16ac (Figure 4) (MW and GC, unpublished). Interestingly, worms seem to lack traditional K20 marks of constitutive heterochromatin, H4K20me2 and me3, but retain widespread H4K20me1 [112]. H4K20me2/3 are present in other major eukaryotes, including mammals and *Drosophila* [113]. Therefore, worm dosage compensation uses the same chromatin marks as the ones used in flies, but in opposite ways. In flies, upregulation of the X chromosome involves an enrichment of H4K16ac and may involve a depletion of H4K20me1. By contrast, in worms, downregulation of the X chromosomes may involve depletion of H4K16ac and enrichment of H4K20me1. It will be interesting to investigate in the future how these chromatin marks affect the transcription machinery in worms.

6.3. Transcriptional Silencing: Mammals. Unlike flies and worms, which achieve dosage compensation by modulating transcription of the X chromosome(s) by an average of

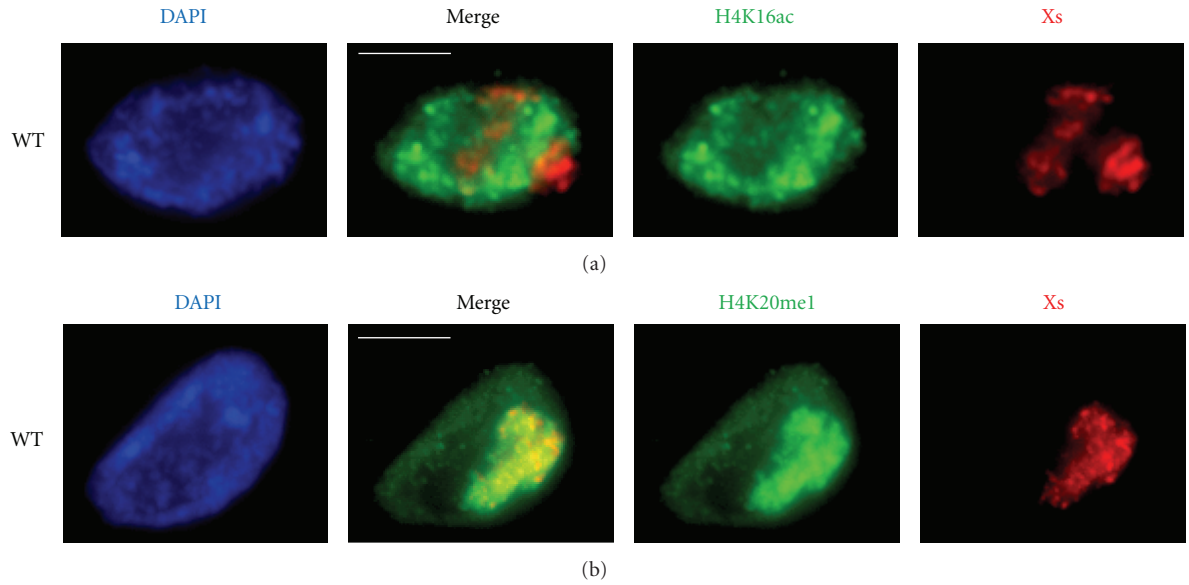

Figure 4: H4K16ac is reduced, and H4K20me1 is enriched, on the X chromosomes in WT hermaphrodite *C. elegans*. Shown are representative immunofluorescence projection images. (a) H4K16ac (green) is markedly reduced on the WT hermaphrodite X chromosomes (red, marked with anti-SDC-3 (DCC) antibodies). (b) H4K20me1 (green) is prominently enriched on the WT hermaphrodite X chromosomes (red, marked by anti-CAPG-1 (DCC) antibodies). DNA (DAPI) is shown in blue. Scale bars are 5 microns in length.

Table 1: Summary of H4K16ac and H4K20me1 modifications on the dosage compensated X chromosomes.

	Levels of histone modification on the dosage compensated X chromosome(s)			
	H4K16ac	References	H4K20me1	References
Drosophila	Enriched on male X	[81–84]	Low levels on male X	[30]
C. elegans	Depleted from hermaphrodite Xs	Figure 4; MW, GC (unpublished)	Enriched on hermaphrodite Xs	Figure 4; MW, GC (unpublished); [110, 111]
Therian mammals	Decreased on the inactive X	[34, 114–117]	Enriched on the inactive X	[34]

two-fold, the mammalian solution to dosage compensation is to silence one X chromosome in females. Many different chromatin marks play a role in X-chromosome inactivation (see below) [118]. X-chromosome inactivation occurs in therian mammals, which includes marsupials and placental mammals, but excludes monotremes. Female monotremes, or egg-laying mammals such as platypus, have stochastic inhibition of genes on the X [119] and no histone H4 modification differences between males and females or X chromosomes and autosomes [114]. Like chickens, monotremes may alter chromatin regionally, rather than chromosome-wide, to achieve gene-specific dosage compensation. Placental mammal and marsupial females have one pair of X chromosomes, and the male has an XY pair. In both placental mammals and marsupials, one X chromosome in the females is inactivated, resulting in both the female and male having one active X chromosome.

X chromosome inactivation in marsupials is imprinted, and the paternal X is always the inactive X. The short arm (Xp) of the X chromosome is gene poor and heterochromatic. The long arm (Xq) is gene rich and is the dosage compensated part of the X chromosome [120]. The active X maintains high levels of H4 acetylation on the long arm, similar to the single male X, while the heterochromatic short arm has low levels of acetylation [115, 121]. Another study examined specific acetylation of H4K8 or H4K16 and discovered reduced acetylation of both chromatin marks on one female X chromosome in the majority of metaphases [114]. Other activating chromatin marks (H2AK5ac, H3K4me2, H3K9ac, and H4K8ac) are also reduced on the inactive X in marsupial females [114, 115, 122]. Therefore, in female marsupials, the inactive X chromosome is globally depleted of activating chromatin marks, and this depletion correlates with RNA Polymerase II exclusion from the X chromosome territory [122].

Unlike marsupials, female placental mammals randomly inactivate one X chromosome around the blastocyst stage of development. Aside from the choice of chromosome to inactivate (imprinted versus random), the overall mechanism of X-inactivation may seem similar between marsupials and placental mammals. However, there are some important differences. In placental mammals, a non-coding RNA *Xist* coats the inactive X chromosome and recruits chromatin modifying complexes that establish epigenetic marks. The

Xist gene is present in all placental mammals analyzed, but is absent in marsupials, suggesting that chromosome-wide inactivation evolved first in a common ancestor, and *Xist* RNA, and the chromatin modifications it recruits added an extra layer of transcriptional repression [122–125].

The mammalian inactive X chromosome is marked by an array of chromatin modifications. Similar to the marsupial inactive X, the inactive X in placental mammals is generally depleted of activating chromatin marks. Histone H4 lysines 5, 8, 12, and 16 are hypoacetylated on the inactive X chromosome [116]. At the gene level, acetylation of specific H4 lysine residues can be detected at the promoters of X-linked genes on the active X chromosome; however there is little to no lysine acetylation of H4 at these genes on the inactive X chromosome [117]. The inactive X is also depleted of acetylation of H3 and H2A [126, 127] and H3 lysine 4 methylation [128]. Unlike the marsupial inactive, the inactive X in placental mammals is also characterized by an *Xist* RNA-dependent accumulation of repressive marks characteristic of facultative heterochromatin. H3K27me3 and the Polycomb complex member Ezh2 are also enriched on, and recruited to chromosomes expressing *Xist* [34, 129, 130]. Other repressive modifications, including monoubiquitination of H2AK119 and dimethylation of H3 lysine 9, also accumulate on the inactive X [131–134]. In a transgenic context, *Xist* RNA expression also triggers an increase in H4K20me1, independent of silencing, and therefore H4K20me1is proposed to be an early mark of X chromosome inactivation [34]. An increase in H4K20me1 was accompanied by a decrease in H4K16ac, consistent with an antagonistic relationship between these two marks [34]. However, a functional role for H4K20me1 or Pr-Set7 in X chromosome inactivation has not been demonstrated. These (or some of these) chromatin changes are thought to contribute to the formation of a repressive nuclear compartment devoid of RNA Polymerase II [135]. Therefore, the depletion of the H4K16ac and other activating chromatin marks in marsupials, as well as the depletion of these marks in combination with the accumulation of repressive marks (including H4K20me1) in placental mammals, leads to transcriptional silencing, an outcome very different from a two-fold modulation of transcriptional activity in flies and worms.

7. Summary and Conclusions

Different mechanisms of dosage compensation have evolved to equilibrate expression of the X chromosomes between females and males and between the X and autosomes. The methods of dosage compensation that are most well understood include two-fold transcriptional upregulation in male flies, two-fold transcriptional downregulation in hermaphrodite worms, and transcriptional silencing in most mammals.

The H4K16ac chromatin mark is either enriched or depleted on the dosage compensated X chromosomes in all three systems (Table 1). Where upregulation is required (in flies), H4K16ac is increased, which is proposed to contribute to chromosome decompaction, preventing chromatin remodeling by ISWI and allowing access of factors for productive elongation. A two-fold downregulation (in worms) may require the opposite: H4K16ac is reduced on the downregulated X chromosomes. Learning from the fly model, one may predict an increased role for ISWI in chromatin remodeling into a more repressive state and subsequently inhibited transcriptional elongation. Mammals sculpt the chromatin of the inactive X more drastically by creating more stable facultative chromatin that lacks activating marks, such as H4K16ac, and is enriched for repressive marks, such as H4K20me1. While the H4K16ac and H4K20me1 modifications are shared by all three mechanisms, mammals achieve more stable silencing when these marks are used in combination with other histone modifications.

How did these diverse dosage compensation mechanisms, with such different transcriptional outputs, evolve? Perhaps the reason for the difference is due to separate evolution of the dosage compensation machineries. The fly dosage compensation machinery coopted a conserved histone acetyltransferase complex [136]. In this organism, H4 acetylation of the X balances X-linked transcription between the sexes. Worms make use of a condensin-like complex for their dosage compensation machinery, suggesting that dosage compensation may involve partial condensation of the X chromosome [105, 107]. Consistent with this idea, reduced H4K16ac contributes to chromatin compaction and results in decreased transcription (as discussed above). Mammals use depletion of H4K16ac in combination with depletion of other activating chromatin marks to achieve transcriptional silencing. In addition, placental mammals acquired the *Xist* long non-coding RNA. Non-coding RNAs have an established role in transcriptional silencing in many processes, including imprinting and X inactivation [137]. *Xist* RNA then serves to recruit chromatin-modifying activities, leading to the accumulation of repressive chromatin marks. Therefore, the same modification, H4K16ac, depending on the chromatin context, leads to vastly different transcriptional outputs.

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