Review Article

Cytogenetic and Genome Research

Cytogenet Genome Res 2016;148:52–67
DOI: 10.1159/000445924

Accepted: January 27, 2016
by M. Schmid
Published online: May 12, 2016

Parallel Universes for Models of X Chromosome Dosage Compensation in *Drosophila*: A Review

James A. Birchler

Division of Biological Sciences, University of Missouri, Columbia, Mo., USA

Key Words
Dosage compensation · *Drosophila* · Inverse effect · MSL complex · X chromosome

Abstract
Dosage compensation in *Drosophila* involves an approximately 2-fold increase in expression of the single X chromosome in males compared to the per gene expression in females with 2 X chromosomes. Two models have been considered for an explanation. One proposes that the male-specific lethal (MSL) complex that is associated with the male X chromosome brings histone modifiers to the sex chromosome to increase its expression. The other proposes that the inverse effect which results from genomic imbalance would tend to upregulate the genome approximately 2-fold, but the MSL complex sequesters histone modifiers from the autosomes to the X to mute this autosomal male-biased expression. On the X, the MSL complex must override the high level of resulting histone modifications to prevent overcompensation of the X chromosome. Each model is evaluated in terms of fitting classical genetic and recent molecular data. Potential paths toward resolving the models are suggested.

Dosage compensation refers to the observation that the expression of genes on a varied chromosome is approximately equal to the total expression from the normal diploid. It is of particular interest for sex chromosomes such as the single X chromosome in *Drosophila*, mammals and *Caenorhabditis elegans*, which have been studied in the most detail [Veitia et al., 2015]. The sex chromosomes are thought to have evolved from a pair of homologous chromosomes in order to maintain divergent alleles or multigenic dosage triggers for sexual differentiation. The viable nature of the single chromosomal copy in one sex compared to 2 doses in the other is a paradox in comparison to the lethal monosomic state of other chromosomes in the karyotype. The case of dosage compensation of the single male X chromosome in *Drosophila* was the first recognized [Muller, 1932]. The basis of the phenomenon has been debated since its first description, and while the focus has changed over the decades, the debate continues to this day with 2 parallel universes of thought about the basic mechanism. Our bias is for one of these hypotheses, but our hope for this review is to detail the issues and suggest some paths forward toward resolution.

The original definition of dosage compensation involved an allele of the *white* eye color gene on the X chromosome of *Drosophila* (fig. 1). This allele, referred to as *white-apricot,* has a low level of function so that the phe-

KARGER

E-Mail karger@karger.com
www.karger.com/cgr

1424–8581/16/1481–0052$39.50/0

James A. Birchler
Division of Biological Sciences, University of Missouri
311 Tucker Hall
Columbia, MO 65211 (USA)
E-Mail BirchlerJ@Missouri.edu

notype is not the null white or the saturated brick red of wild type. Therefore, modulations of the dosage of this allele are reflected in the phenotype. Muller produced a heterozygous deficiency for the *white* locus and *white-apricot* and observed that the color was lighter than in a female with 2 copies of the allele and interestingly also compared to a male that had only 1 copy that exhibits a similar level of pigment as the females (fig. 2). Muller further constructed flies with extra copies of *white-apricot* and found an increased amount of pigment beyond the normal female and an even further increase when the extra copy was present in a male. In other words, a dosage series of *white-apricot* in females gave a proportional amount of pigment in the eyes, but each copy in the male was expressed by about 2-fold greater while still showing a dosage effect.

Richard Goldschmidt questioned whether the X chromosome exhibited any special mechanism with regard to upregulation because, as he pointed out, many autosomal leaky mutations are also more like wild type in males than in females [Goldschmidt, 1954]. This is indeed the case [Smith and Lucchesi, 1969; Birchler, 1984], but the magnitude of the increase and fraction of genes on the autosomes exhibiting this effect is much less than the fraction of the X-linked genes that exhibit compensation. The commonly used *mini-white* transformation phenotypic marker shows this effect when inserted autosomally [e.g. Birchler, 1992; Bhadra et al., 1999] (fig. 3).

Triple X females, or metafemales as they are called, seldom survive to the adult stage but are represented in larval stages to some degree. Muller [1950] rationalized this lethality by suggesting that the total gene expression from the 3 X chromosomes was decreased below the normal female level in the progression from 1 X in males to 2 X in normal females to 3 in metafemales. However, Curt Stern [1960] found that metafemales homozygous for *white-apricot* showed the same amount of pigment as the normal females. In retrospect, previous description of the behavior of the *Bar* eye shape mutation in metafemales is consistent with the same level of dosage compensation [Margolis, 1934]. Below, a further discussion of the situation in metafemales will be detailed.

Triploid Dosage Compensation

Dosage compensation has also been documented in triploid flies that have 3 copies of the genome. A fly with 3 sets of all chromosomes is a triploid female. When gene expression is sampled in triploid and diploid flies, there

Fig. 1. The *white* eye color gene in *Drosophila*. The *white* eye color gene has played a prominent role in understanding dosage compensation of the X chromosome in *Drosophila*. The normal brick red eye color is at the lower left. The null mutant white color is at the lower right. The leaky or hypomorphic *white-apricot* allele for which dosage modulations can be observed is at the top. Photo by James A. Birchler.

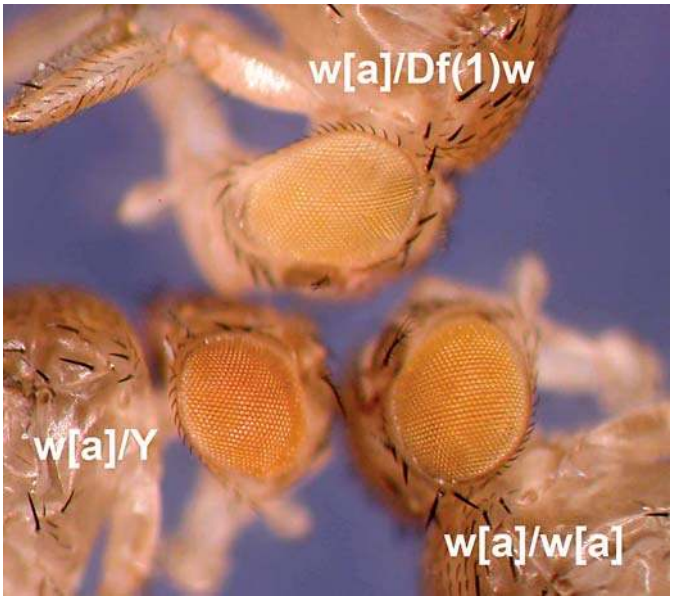

Fig. 2. Dosage compensation of the *white-apricot* allele. The eye color of the 1-copy male *w[a]*/Y (lower left) is approximately the same as of the 2-copy female *w[a]/w[a]* (lower right). When *w[a]* is heterozygous for a deficiency of *white, Df(1)w* (top), the eyes of this fly with 1 copy of *apricot* are lighter in color than in the normal male and female. When the whole chromosome is in 1 copy, there is a 2-fold upregulation that does not occur when only the *apricot* allele is varied – the phenomenon of dosage compensation. Photo by Weiwu Xie.

is basically a directly proportional amount of gene product in relation to the number of genomes present [Lucchesi and Rawls, 1973a; Rabinow et al., 1991]. When the X chromosome is reduced to 2 copies in an otherwise triploid fly, it develops as a mixture of male and female tissues, but the amount of gene expression from the 2 X chromosomes is about equal to that of the triploid females [Lucchesi and Rawls, 1973b; Maroni and Plaut, 1973]. If the dosage of the X chromosome is reduced even further to 1 dose, these flies are referred to as metamales. They usually do not survive to the adult stage, but assays of gene expression in larvae show that the single X chromosome is upregulated to near the triploid female level [Lucchesi et al., 1977]. These results indicate that the magnitude of change for dosage compensation is a 3/2× increase for triploid intersexes and a 3× increase for metamales. When considering all genotypes, diploid and triploid females have a similar level of gene expression per gene; normal males have an approximately 2-fold upregulation per gene; metafemales have an approximately 2/3 downregulation per gene; triploid intersexes have a 3/2 upregulation and triploid metamales have a 3-fold increase per gene. Thus, there are 5 levels of gene expression that need to be explained for a valid model of dosage compensation [Birchler, 1996] (fig. 4).

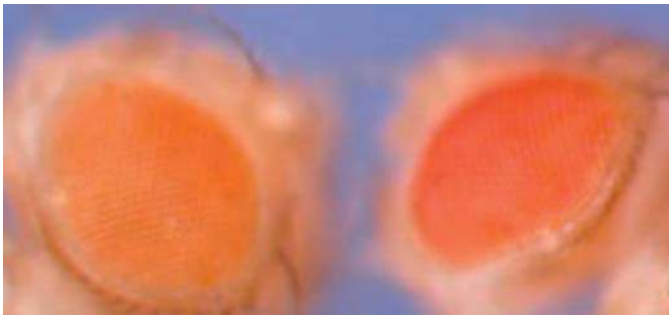

Fig. 3. Male-biased sexual dimorphism of the autosomal *mini-white* transgene. The *mini-white* transgene marker is used extensively for transformation in *Drosophila*. When transgenes are inserted in an autosomal location, the most common effect is that the male (right) shows greater pigment amounts than the female (left) illustrating a male-biased sexual dimorphism in autosomal expression. Photo by Lin Sun.

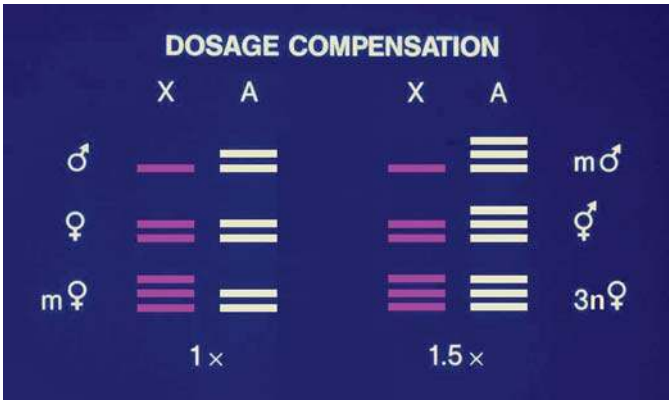

Fig. 4. Dosage compensation in diploid and triploid flies. Dosage compensation not only occurs in normal diploid males and females but also in triple X metafemales (m♀). In triploid flies, 3 copies of all chromosomes is a triploid female, which has a similar 'per gene' expression as a diploid female, but there are 3 copies present resulting in a 1.5× total expression per cell compared to diploids. Triploid flies with only 2 X chromosomes are triploid intersexes, which exhibit dosage compensation. The single X condition in triploids is a metamale (m♂), which also shows compensation. Purple bars represent X chromosome number (X) and white bars represent the collective autosome copy number (A). For these 6 genotypes, there are 5 levels of 'per gene' expression that in the respective cases are the inverse of the chromosomal imbalance.

The *white* Locus and What It Tells Us about Dosage Compensation

The first cases of dosage compensation were defined using the *white-apricot* allele of the *white* eye color gene. The beauty of this system is that it is easy to score and the measure of gene expression can be determined visually as a pigment 'per cell' measure. The full wild-type alleles are saturated for pigment, but those that are partial loss-of-function alleles permit a straightforward determination. Most such leaky alleles show similar expression between males and females indicating dosage compensation. The *mini-white* transgene that is used routinely as a transformation marker has reduced function because part of its regulatory sequences has been deleted. Further deletion to the bare minimum 5′ sites did not reveal a sequence required for dosage compensation – all show it [Hazelrigg et al., 1984; Levis et al., 1985]. Some other alleles are instructive: *eosin*, *apricot-like* and *ivory* all fail to show equal expression between males and females [Karess and Rubin, 1982; Zachar and Bingham, 1982; Rabinow et al., 1991; Birchler, 1992]. They are all lesions in the 5′ regulatory sequence, in the first 2 cases being transposable element insertions that likely provide a different promoter for the *white* structural gene. Another set of alleles are the spotted series. In this case, the eye enhancer is deleted [Zachar and Bingham, 1982]. These alleles exhibit a variable level of

expression, but there is a greater amount of pigment in males than in females. Collectively, the *white* alleles provide a natural deletion series and promoter substitution analysis that suggest that dosage compensation is a transcriptional initiation phenomenon or, at the very least, initiation is a critical step.

Another contribution that studies of the *white* locus have made to the field of dosage compensation involves a comparison of the reaction of these various alleles to dosage compensation in triple X metafemales [Birchler, 1992]. When *eosin* and *ivory* were incorporated into an attached X and then used to generate triple X females, both showed increased expression in metafemales in contrast to the male/female compensating alleles, *apricot* and *blood*, which were both equal in expression between meta- and normal females. With 2 *white-spotted* alleles, the amount of pigment in metafemales was decreased. Thus, noncompensation, compensation and overcompensation, respectively, in males versus females were also found in metafemales. In other words, alleles expressed less in males than in females are expressed even more in metafemales, and alleles with similar expression between males and females extend this relationship to metafemales. Alleles with more expression in males than females have even less in metafemales. Collectively, these results indicate that dosage compensation in males and metafemales has a related mechanism.

Autosomal Sexual Dimorphism in Males

As noted above, Richard Goldschmidt [1954] had pointed out that many hypomorphic mutations of autosomal genes exhibit a more wild-type expression in males than in females. This is obviously the case by observation of mutations in such eye color genes as *purple*, *pink[peach]*, *light*, and *glass,* as examples (FlyBase, flybase.org) [see also Birchler et al., 1989]. All Drosophilists recognize autosomal insertions of *mini-white* as routinely darker in males than in females (fig. 3). Another transgene that can be observed in the eye is the *Alcohol dehydrogenase* promoter-*white* structural gene fusion. Autosomal insertions also have higher expression in males [Birchler et al., 1990; Pal Bhadra et al., 1999]. The molecular nature of these genes is known in some cases (flybase.org) and is quite diverse indicating that this male-biased sexual dimorphism is not specific to a certain class of gene.

Male-Specific Lethal Mutations and Their Hypothesized Role in Dosage Compensation

A popular model invoked to explain dosage compensation in *Drosophila* involves the products of the male-specific lethal (MSL) loci [Belote and Lucchesi, 1980; Lucchesi et al., 2005; Lucchesi and Kuroda, 2015]. These mutations are lethal as homozygotes in males but not in females. The lethality is usually manifested at the transition from the larval to the pupal stage. These mutations include *maleless (mle)* [Belote and Lucchesi, 1980] and the *male-specific lethal 1, 2* and *3 (msl1, msl2, msl3)* loci as well as the *males absent on the first (mof)* [Hilfiker et al., 1997]. Some decades ago, the *maleless* mutation was examined for its effect on transcription by studying the incorporation of tritiated uridine into nascent RNA on the X chromosome versus a portion of the autosomes [Belote and Lucchesi, 1980]. The ratio of incorporation on the X chromosome relative to the autosomal values was reduced. This result was interpreted such that the products of the male-specific lethal genes were responsible for X chromosomal dosage compensation.

When the product of the *mle* gene was identified and localized in the nucleus, it was present on the X chromosome of males [Kuroda et al., 1991]. As the other protein components were identified, they followed the same pattern. One of the later identified protein products, MOF, encodes a histone acetyl transferase that is responsible for acetylation of lysine 16 on histone H4 (H4K16Ac) [Hilfiker et al., 1997]. Accordingly, this histone modification is enriched on the male X chromosome [Turner et al., 1992; Bone et al., 1994]. These findings led to the hypothesis that a complex composed of the MSL proteins brought the histone modifier MOF to the X chromosome in males to cause the upregulation of the single X chromosome. There are also 2 loci *(roX1*, *roX2)* that encode RNAs without protein-coding capacity of which at least one must be present for the integrity of the complex [Meller et al., 1997; Meller and Rattner, 2002]. The JIL1 histone kinase, which phosphorylates H3Ser10, is also enriched on the male X chromosome, although mutations in this gene are not specifically male lethal [Jin et al., 1999, 2000; Wang et al., 2001]. There is an extensive literature on the composition of the MSL complex and its X chromosome targeting that is integral to both models discussed here [see Lucchesi and Kuroda, 2015].

The sex specificity of MSL association with the male X chromosome is determined by the expression of the *Sex-lethal* gene [Kelley et al., 1995, 1997]. This gene is a splicing factor that initiates its own splicing in females, but in males a disruptive exon remains throughout development

because the protein is never expressed sufficiently highly to remove it [Cline and Meyer, 1996]. In females, SXL splices itself to allow continued expression and a cascade of splicing occurs to determine sexual differentiation. The SXL protein binds to the mRNA of the MSL2 protein and blocks its expression in females. The absence of the SXL protein in males allows the expression of MSL2. MSL2 is the critical component of the MSL complex that draws the complex to the X chromosome [Kelley et al., 1995].

This body of work provides an apparently satisfying explanation for dosage compensation of the single male X chromosome. The MSL complex is localized to the male X chromosome. This concentration brings to the chromosome a histone modifier (MOF) that is otherwise known to be involved with increased gene expression. Mutation in one of the components *(mle)* that dissociates the complex from the X chromosome produces a relative change of X and autosomal gene expression. Collectively, these facts seem to provide a simple explanation for the mechanism of dosage compensation.

One line of argument that the MSL complex causes dosage compensation involved the observations that autosomal insertions of the *roX* RNA transgenes will attract the complex, which can spread out from the site of insertion [Park et al., 2002; Kelley and Kuroda, 2003]. One such transgene with *mini-white* being present as the transformation marker shows *white* expression only in the dorsal portion of the eye. However, in males in which the spreading was most extensive, the silenced ventral portion of the eye was derepressed to almost wild type in males. Subsequently, a second site mutation, called *over compensating male (ocm)* [Lim and Kelley, 2013] was recovered that caused strong derepression of this phenotype. These results were interpreted such that this MSL complex association with the *mini-white* reporter caused the acquisition of dosage compensation. However, dosage compensation is a 2-fold modulation rather than a depression of a null state. Another potential explanation of these results is that the MSL complex overrides this type of silencing. In this context, it is interesting to note that the MSL complex appears to override the impact of gene-activating chromatin marks [Sun et al., 2013a], which will be described in more detail below.

Evidence for Genomic Balance and the Inverse Effect

Before delving further into the specifics of dosage compensation models, we review the evidence for genomic balance in general, which is a critical factor for understanding the driving force for dosage compensation and sex chromosome evolution. The idea of genomic balance, namely that varying copies of individual chromosomes has more detrimental effects on the phenotype than does varying the complete set, has a long history of investigation on the phenotypic level [Blakeslee et al., 1920; Bridge, 1925; Blakeslee, 1934]. More recently, parallels have emerged from studies of gene expression and genome evolution enabling the formulation of a broader synthesis that attempts to address the molecular basis of this phenomenon. This synthesis has been referred to as the gene balance hypothesis (GBH) [Birchler et al., 2005; Birchler and Veitia, 2012]. The GBH posits that altering the stoichiometry of members of oligomeric complexes will affect the function of the whole as a result of the kinetics and mode of assembly [Birchler et al., 2005; Birchler and Veitia, 2012]. While the GBH applies to any multisubunited complex or interaction hierarchy of genes, we will focus on how regulatory gene balance operates.

Evidence from gene expression studies in both plants and animals suggests there is a widespread importance for maintaining genomic balance. Aneuploid samples of maize or *Drosophila* exhibit more modulations of gene expression than do balanced changes in ploidy [Birchler et al., 2001]. The modulations of enzyme activities, protein and RNA levels that change in aneuploids usually fall within the range of either direct or inverse correlations with the dosage of the varied chromosome [Birchler, 1979; Birchler and Newton, 1981; Devlin et al., 1988; Sabl and Birchler 1993; Guo and Birchler, 1994; Sun et al., 2013b, c]. *Trans*-acting direct effects show reductions to 50% in monosomics compared to a diploid and increase to 150% of the diploid in trisomics. The more common *trans*-acting inverse effects show increases to 200% in monosomics and reductions to 67% in trisomics. Multiple segments of the genome can modulate any one gene product [Birchler and Newton, 1981; Guo and Birchler, 1994]. Any one chromosome arm can modulate differing numbers of the total detectable proteins [Birchler and Newton, 1981]. Evidence for the inverse effect in several organisms is present in segmental trisomy data [see Birchler, 2010; Nawata et al., 2011; Sun et al., 2013b, c]. Such molecular variations are less often observed for balanced changes in ploidy [Lucchesi and Rawls, 1973a; Guo et al., 1996]. This comparison led to the postulation that altering the stoichiometry of regulatory genes might be responsible for these effects [Birchler and Newton, 1981].

Whenever a regulatory gene producing an inverse dosage effect and a subset of its target loci are varied on the same chromosomal segment, dosage compensation is ob-

served [Birchler, 1979, 1981; Birchler et al., 1990]. In other words, increasing the dosage of an inverse regulator at the same time as a target gene, which otherwise would show a dosage effect, would restore the original diploid expression level [Birchler, 1979, 1981; Devlin et al., 1982; Birchler et al., 1990; Sun et al., 2013a, b] because the 2 opposing effects cancel each other. The first described example involved the *alcohol dehydrogenase (adh1)* gene on the long arm of chromosome 1 (1L) in maize [Birchler, 1979, 1981]. Compensation also operates on the RNA level [Guo and Birchler, 1994]. Similar examples were demonstrated in *Drosophila* [Devlin et al., 1982; Birchler et al., 1990; Sun et al., 2013a, b].

Global studies of gene expression in *Drosophila* using RNA-Seq of trisomy of the X chromosome [Sun et al., 2013b] or of the left arm of chromosome 2 [Sun et al., 2013c] illustrate the ubiquitous nature of the inverse effect. The plurality of all isoforms detected in both trisomy of the X and 2L is inversely affected by the extra chromosome. In parallel, most of the genes on the varied chromosomes are dosage compensated, although some fraction in each case exhibits a dosage effect. Furthermore, there is a sexual dimorphism for the type of effects with one potential explanation being that the reduced dosage of the X chromosome in males intensifies the inverse effect of 2L [Sun et al., 2013c]. Also, the X chromosome responds differently to trisomy of 2L, which was suggested as a reflection of the X evolving to maximize using the inverse dosage effect to bring about dosage compensation of the X [Sun et al., 2013c]. Indeed, genes with sex-biased expression are strongly skewed in their response to trisomy 2L implicating the inverse effect in mediating such sex differential expression.

The inverse and direct effects on gene expression observed in aneuploids could be mimicked at the level of single genes by recovering modifier mutations that, in the heterozygous condition, doubled or reduced by half the expression of a leaky allele of the *white* eye color reporter gene in *Drosophila* [e.g. Rabinow et al., 1991]. The first and most thoroughly studied, *Inverse regulator-a*, produces a near perfect inverse effect on the phenotype of the *white* eye color gene in both diploid and triploid flies [Rabinow et al., 1991]. This gene is synonymous with the *Pcf11* gene, which is involved with transcriptional initiation, elongation and termination reactions [Xie and Birchler, 2012]. A collection of 47 such dosage-dependent modifiers of *white* eye color gene expression was recovered over 2 decades of screening [Birchler et al., 2001] with the majority showing an increased effect on *white,* when the modifier was heterozygous, while others reduced the expression. It is an interesting question of how there can be such a large number of modifiers of a single reporter gene. The answer likely resides in the fact that gene regulatory systems operate in a hierarchy with one regulator controlling downstream effectors, which, in turn, control other regulators ultimately reaching the phenotype [Birchler et al., 2001]. Moreover, regulatory processes are often performed by oligomeric complexes such that modulating different subunits affects the action of the whole. Modeling the kinetics of ordered assembly of multi-subunit complexes predicts such effects [Bray and Lay, 1997]. Other chromatin modifiers are also dosage dependent [Henikoff, 1996].

The molecular nature of many dosage modifiers has been elucidated, which were found to encode transcription factors, chromatin proteins or members of signal transduction cascades [Birchler et al., 2001]. Collectively, this result parallels the realization that most haploinsufficient clinical conditions in human are lesions in transcription factors or other proteins involved with oligomeric complexes [Seidman and Seidman, 2002; Veitia, 2002; Kondrashov and Koonin, 2004]. Gene dosage has an effect on murine phenotypes when mutations in developmental transcriptional regulators are present in a heterozygous state [Boell et al., 2013].

Modeling of haploinsufficiencies has led to the idea that the stoichiometry of interacting subunits would generate a semi-dominant effect on the phenotype based on the action on the target loci [Veitia, 2002]. An experimental demonstration of this principle was provided by evidence from diploid yeast in which the heterozygote fitness was measured for null mutations in essential genes. As the genes were grouped into bins of different heterozygous fitness, a greater proportion were involved in protein complexes as the fitness declined [Papp et al., 2003]. Furthermore, overexpression of genes involved in complexes is also detrimental, which can be corrected by coupregulation of interactors. These results mirror aneuploid effects but on the single gene level.

Gene balance is relevant to how genomes evolve through chromosome doubling and subsequent gene loss. For example, it has been realized that polyploidization events have been quite common in the evolution of eukaryotes [Wolfe and Shields, 1997; Simillion et al., 2002; Bowers et al., 2003; Maere et al., 2005; Aury et al., 2006; Blomme et al., 2006; Makino and McLysaght, 2010; Conant et al., 2014]. In fact, almost all eukaryotes have a history of ancient polyploidization events followed by fractionation to a near diploid state and then repeated polyploidizations and fractionations. In the plant *Arabi-*

dopsis, analysis of gene duplicates provided evidence of at least 3 whole-genome duplications stretching back a quarter of a billion years [for review, see Freeling, 2009]. In *Paramecium tetraurelia*, there are 3 tetraploidization events that can be documented from the analysis of the sequenced genome, each followed by a reduction in gene number [Aury et al., 2006], illustrating the generality across phylogenetic kingdoms.

Interestingly, as chromosomal segments are lost following polyploidization events, the spectrum of retained genes is not random. Indeed, in the independent fractionations in yeast, *Arabidopsis*, rice, *Paramecium* and vertebrates, the classes of genes retained are preferentially represented by those that are members of oligomeric complexes [Blanc and Wolfe, 2004; Aury et al., 2006; Freeling and Thomas, 2006; Hakes et al., 2007; Edger and Pires, 2009; Makino and McLysaght, 2010]. For example, duplicates of transcription factors and members of signal transduction pathways are typically maintained – both categories of gene products are involved with complexes and interacting hierarchies. Thus, the same classes of genes are held in duplicate as those that show dosage effects as regulatory modifiers and quantitative trait determinants. It seems likely that there is selection to retain a stoichiometry of these gene products and that deletion of one member of a duplicate pair might act like an aneuploid effect that is selected against due to reduced fitness. This supports the idea that genes with major regulatory roles are retained to maintain balance in the genome over evolutionary time. These considerations would apply to sex chromosome degeneration as well but have not been examined in detail with some exceptions [Makino and McLysaght, 2010; Pessia et al., 2012; Bellott et al., 2014].

That duplicate pairs are retained in such a manner implies that dosage is important. This fact also illustrates that only a restricted range of perturbation appears to be tolerated. For instance, following a whole-genome duplication involving diploid contributors, there would be 4 copies of a particular transcription factor gene instead of the two present in the original diploid species. The fact that duplicates are retained suggests that a mere reduction to only 3 functional copies causes reduced relative fitness in the population. Furthermore, isolated duplication of genes encoding subunits of complexes are usually selected against due to unbalanced, and therefore detrimental, dosage. Accordingly, these gene classes are underrepresented in genomic analyses of copy number variants in several organisms [Yang et al., 2003; Maere et al., 2005; Freeling and Thomas, 2006; Freeling et al., 2008; Makino and McLysaght, 2010; Schuster-Böckler et al., 2010]. Thus, there is a generalized complementary pattern of gene ontologies present in segmental duplications compared to those that are retained for longer time periods following polyploidization events. These evolutionary trajectories are consistent with the laboratory results that aneuploidy is typically of more detrimental consequence to an organism compared to whole-genome ploidy effects. Understanding how sex chromosome evolution has accommodated these issues is worthy of investigation.

A Critique of the MSL Hypothesis

Despite the simplicity of the MSL hypothesis, there are many biological facts that it does not explain with regard to dosage compensation. First of all, female flies that possess 3 copies of the X chromosome, a genotype referred to as metafemales [Margolis, 1934; Stern, 1960; Lucchesi et al., 1974; Birchler et al., 1989; Birchler, 1992; Sun and Birchler, 2009; Sun et al., 2013b] exhibit dosage compensation. In this case there must be a downregulation of the X chromosomes for dosage compensation to occur. Each gene must be expressed at approximately two-thirds of the level in a normal female. And because there is no MSL complex in females, including metafemales [Sun and Birchler, 2009], this compensation cannot be mediated by the MSL complex. A sampling of endogenous autosomal genes had revealed that many of them are reduced in expression in metafemales relative to the normal female levels [Birchler et al., 1989; Sun and Birchler, 2009]. One can visualize this effect on the phenotypic level using partial loss-of-function alleles whose modulation could be assayed in the fly [Birchler et al., 1989]. These autosomal sexually dimorphic eye color mutations that typically are darker in males than in females show an even greater reduction in metafemales illustrating the continued trend with the dosage series of the X chromosome in males, females and metafemales [Birchler et al., 1989]. Specific endogenous RNA levels were also examined in adults [Birchler et al., 1989] and larvae [Sun and Birchler, 2009] with standardization to ribosomal RNA and in turn to DNA. There was compensation of the X-linked genes and reductions for the autosomal loci. Thus, the gene copies on the 3 X's produce about the same amount as a normal female with 2 X's, and the diploid autosomes in metafemales produce less than they do in normal females.

On the global transcriptome level, an RNA-Seq experiment on larvae compared metafemales to females (and males). When the data are expressed as a ratio distribution, the majority of genes on the X chromosome were in

fact dosage compensated with a small peak that exhibited an expression ratio of 1.5 (a dosage effect) [Sun et al., 2013b]. For autosomal genes, the plurality was reduced to near 67% (the inverse of the 3/2 X dosage) in metafemales compared to normal females with a peak shoulder being unaffected with an expression ratio of 1.0. These data illustrate the pervasiveness of the inverse dosage effect and the generalized X chromosome compensation in metafemales. They also provide a potential explanation of the reduced viability of metafemales. As noted above, Muller suggested that this was due to a multiplicatively reduced X expression, but there are in fact comparable levels of expression for this chromosome in normal and metafemales. The autosomal reductions could potentially provide an explanation for the inviability effects.

Another consideration is that when endogenous genes were sampled for their absolute rather than relative level of expression in the *mle* mutation, most of the genes on the X chromosome in the mutant males did *not* lose dosage compensation, while many of the autosomal genes were actually increased in expression [Hiebert and Birchler, 1994]. In further studies using transgenes for the *white* eye color and the *yellow* body genes on the X and the autosomes, it was found that disruption of the MSL complex with the *mle* mutation did not cause a loss of dosage compensation of either transgene, but the autosomal copies in each case were increased in expression within an about 2-fold range [Bhadra et al., 1999]. As these studies were conducted in larvae, one concern about the results is that they were assayed near the developmental stage at which they normally die. Thus, an RNA-FISH assay was developed for embryos and selected X and autosomal genes were examined for their levels of expression [Pal Bhadra et al., 2005]. As was the case in larvae, the X-linked genes did not show a loss of dosage compensation and expression of the autosomal genes studied was increased. The consistency of these results across the different developmental stages indicates that the concerns about the larval data were unfounded.

The genomic distribution of MOF relates to the gene expression results. In normal females, there is a low level of association with all chromosomes but it becomes highly concentrated on the male X [Bhadra et al., 1999; see also Kind et al., 2008]. In the *mle* mutant males, when compared in a mixed slide preparation with normal males, there is a redistribution of MOF to being uniform across the genome. This result is accompanied by a genome-wide uniform acetylation of H4 [Bhadra et al., 1999]. Considering that when MOF is specifically targeted to a reporter in the absence of the full MSL complex, it conditions a strong increase in expression [Sun et al., 2013a], the redistribution of MOF in the *mle* mutant males to cover the whole genome would be consistent with the retention of X chromosome compensation and any autosomal increases that are observed because more MOF is now associated with the autosomes than in normal males.

Dosage Compensation Does Not Involve the MSL Complex in *Sciara*

The Diptera are divided into 2 suborders. The Brachycera suborder includes *Drosophila* and the Nematocera includes *Sciara ocellaris*, which has also been studied with regard to dosage compensation. In *Sciara*, females are XX and males are XO, i.e. a single X and no Y chromosome. Studies of tritiated uridine incorporation into RNA from larval salivary gland chromosomes suggested similar rates for the single X in males compared to the 2 X chromosomes in females [da Cunha et al., 1994]. However, when the *Drosophila* antibodies to the MSL components, MLE, MSL3, MOF, and MSL1, were examined, they were found equally distributed across all chromosomes [Ruiz et al., 2000]. However, the MSL2 protein was not detected nor was it possible to detect it in *Sciara* DNA via Southern blot analysis. The data suggest that dosage compensation in *Sciara* occurs by upregulation of the single X chromosome, similarly to *Drosophila*, based on the density of tritiated uridine incorporation. However, the uniform distribution of most of the MSL components across the genome would suggest that it does not mediate dosage compensation in this species. A concentration on the X would not necessarily indicate that the MSL complex mediates compensation, in the absence of functional tests, but the failure to be associated specifically with the X would indicate a lack of involvement. The sex chromosomes in Diptera have shifted in numerous ways over evolutionary time [Vicoso and Bachtrog, 2015] and so the relationship of these 2 cases should be interpreted with caution at present. However, these studies indicate that in a sister group to *Drosophila*, X chromosome dosage compensation can clearly occur without the MSL complex and secondly that the MSL complex minus MSL2 likely has an independent function.

Gene Expression in *msl* Mutants

The question then arises as to whether there is a discrepancy between the sampling of X and autosomal genes in the *mle* mutations [Hiebert and Birchler, 1994; Pal

Bhadra et al., 2005], noted above, compared to the original autoradiographic analysis [Belote and Lucchesi, 1980]. The autoradiographic data used a normalization in which the X values were divided by the autosomal values. In the context of the time, this type of analysis seemed reasonable for an internal control. However, when a re-examination of the autoradiographic data is performed, one can recognize that the absolute levels of grain counts on the X chromosome are not reduced in mutant males, and the absolute levels of the autosomal grain counts are increased. In the experiments in question, DNA replication values were also determined. If one normalizes the X and autosomal expression values to the DNA grain counts rather than the X to autosomal RNA synthesis values, as was performed in the study, then there is no loss of compensation of the X, and there is an increase in expression of the autosomes. Similar absolute results but with the same type of normalization were published for *msl3* [Okuno et al., 1984]. Further, phenotypically the *runt* embryo developmental gene does not lose compensation in *mle* mutants [Gergen, 1987]. When viewed in this manner, there is no conflict between the original autoradiographic study and the sampling of X and autosomal gene expression studies. This interpretation of the autoradiographic data, however, is not consistent with the hypothesis that the MSL complex conditions X chromosome dosage compensation in males. Unfortunately, this type of normalization was adopted by other authors [Hamada et al., 2005; Deng and Meller, 2006, among others] reporting studies of gene expression in *msl* mutants or knockdowns with the recurring assumption that the autosomes do not change in expression. However, absolute measures indicate that this does not appear to be the case. Studies of RNA polymerase II occupancy on X-linked genes under different circumstances used the same type of autosomal normalization or reported global modulations of all chromosomes [Larschan et al., 2011; Conrad et al., 2012], and thus the interpretation for these studies is also ambiguous. A recent re-analysis of global studies of dosage compensation when the MSL complex is dissociated concluded that compensation is often independent of the complex [Philip and Stenberg, 2013]. There is a growing realization in other systems of dosage compensation that global effects across the genome occur when the process is disrupted [de Clare et al., 2011; Kruesi et al., 2013] albeit distinctly from those in *Drosophila*. The redistribution of MOF in *mle* mutants and the finding of genome-wide effects of aneuploidy suggest that a global impact of MSL complex disruption is not unreasonable.

Many experiments to examine gene expression with MSL dissociation were performed using S2 tissue culture cells that are tetraploid and have 43 Mb of aneuploidy in an otherwise 120-Mb euchromatic genome [Zhang et al., 2010]. This aneuploid state will no doubt have an impact on global gene expression, and so it is not clear how well this experimental system can be related to what occurs in an organism. Indeed, in the heatmaps of MSL2 knockdown in S2 cells [Hamada et al., 2005], there does appear to be some autosomal increase. However, there is also another consideration: if the amount of MOF is low in S2 cells, then there might in fact be a loss of compensation similar to that found in a *mof*;*mle* genotype in embryos [Pal Bhadra et al., 2005]. This genotype is the only one examined in which the assayed genes show ~50% expression in mutant males compared to normal males and females. These considerations illustrate the importance of conducting RNA-Seq experiments on the *msl* mutants in the organism itself in the context of interacting regulatory machineries.

Another question that arises is whether there is a unifying principle from the gene expression experiments. If one considers the data from the *mle* mutant males and from the metafemales together, it can be realized that there is an inverse relationship between a generalized pattern of gene expression and the dosage of the X chromosome [Birchler et al., 1989; Birchler, 1992, 1996; Sun et al., 2013b]. When coupled with a corresponding change in target gene expression on the X chromosome, then dosage compensation would result. In other words, in males there is a widespread approximately 2-fold increase in expression and in metafemales a two-thirds reduction. For the autosomes that do not change in dosage, this effect is directly manifested but is muted in normal males (see below). But for the X chromosome in which the assayed genes are also changed in dosage, the magnitude of the global effects will cancel the change in dosage of the assayed genes. This principle will also explain the inverse relationship of the change in expression of each X chromosome copy in triploid intersexes and triploid metamales. Such a response is reminiscent of the expression of genes in a dosage series of chromosome arms in maize [Birchler, 1979, 1981; Birchler and Newton, 1981; Guo and Birchler, 1994]. Similar cases of 'autosomal dosage compensation' occur in flies in experimentally generated aneuploids and have a similar basis [Devlin et al., 1982, 1988; Birchler et al., 1990; Sun et al., 2013c]. In a recent RNA-Seq study of trisomy 2L in flies compared to diploids [Sun et al., 2013c], the inverse effect is also present similar to the trisomic X meta-

females [Sun et al., 2013b], illustrating that this effect is the prevalent response to changes in chromosomal dosage in *Drosophila*.

Ectopic Expression and Targeting of the MSL Complex

Returning to the MSL hypothesis, a straightforward prediction would be that ectopic targeting of the MSL complex to the X chromosome or a transgene would produce a 2-fold increase in expression given that this would mimic the situation in normal males, which this hypothesis suggests causes the 2-fold increase in expression. Indeed, in experiments conducted in yeast, appending the GAL4 DNA-binding domain to the MOF protein targeted the fusion protein to a reporter that was preceded by the upstream activating sequence recognized by the GAL4 DNA-binding domain [Akhtar and Becker, 2000]. When this targeting was conducted, the expression of the reporter was in fact increased about 10-fold. This yeast experiment was widely taken as evidence that targeting of MOF by the MSL complex to the X chromosome in flies was the basis of dosage compensation, but it assays MOF in isolation of other *Drosophila* cellular components.

By removing the binding sites for SXL from the MSL2 message, the protein can be expressed in females [Kelley et al., 1995] and the MSL complex is organized and localized to the X chromosomes. MOF is brought to the 2 X chromosomes and there is indeed an increase in H4K16Ac that can be readily visualized. However, when genes on the X are assayed for expression in these females, there are none that show any increase in expression [Bhadra et al., 1999, 2000; Sun et al., 2013a]. Many autosomal genes in contrast are reduced in their expression [Bhadra et al., 1999; Sun and Birchler, 2009]. As would be expected from the action of *Sxl*, mutations for this gene will allow the expression of MSL2 in females and localization to the X chromosomes. When endogenous genes were assayed for expression in an Sxl^{-} genotype, again there was no evidence for an increase in expression of any gene studied [Bhadra et al., 2000]. Thus, a key prediction of the MSL hypothesis fails.

When MOF targeting to reporters was conducted in flies, there was a large increase in expression when females were examined but not in males where the MSL complex can be organized [Prestel et al., 2010; Sun et al., 2013a]. In both males and females, there was an increase of acetylation at the reporter, so an absence of histone modification cannot be attributed to the difference in gene expression. Thus, there appears to be a constraining activity in the MSL complex, or associated with the complex, that counteracts the effect of histone acetylation on gene expression. Further, when GAL4-MSL2 was targeted to the same reporters in males and females [Sun et al., 2013a], there was no increase in expression as predicted by the MSL hypothesis but often a slight reduction in expression despite the fact that all assayed components of the MSL complex were associated with the reporter and histone acetylation was increased across the reporter. Ectopic expression of MSL2 in females with MOF targeting to reporters switched their response from upregulation to down, consistent with the idea of a constraining activity accompanying the MSL complex [Sun et al., 2013a].

Indeed, when the MSL complex was dissociated from the X chromosome in *roX* mutants, the complex tended to become associated with heterochromatic regions of the chromosomes including the small 4th chromosome [Meller and Rattner, 2002; Figueiredo et al., 2014]. The new association of the MSL complex with 4th chromosome genes was documented by ChIP-Seq and found to be active in increasing the acetylation of H4K16. However, when the transcriptional output of these genes was compared to normal, there was no evidence for an increased expression. This result, in which there is no change in expression when genes transition from no association to being associated with the MSL complex, is similar to the deliberate targeting of reporters and ectopic expression in females [Sun et al., 2013a].

An RNA-Seq experiment comparing females ectopically expressing MSL2 to normal females shows no evidence for increased expression of any X-linked endogenous genes despite an increased accumulation of the H4K16Ac on the X chromosomes [Sun et al., 2013a]. Thus, all of the multiple lines of evidence (in embryos, larvae and adults using transgene reporters and endogenous genes with assays on the RNA and phenotypic levels using GAL4-specific targeting or nonspecific MSL2 ectopic expression) suggest that the MSL complex does not confer an increased expression on genes when it becomes ectopically associated with them.

A question that arises is whether the lack of an effect of MSL targeting in females is due to a missing component present only in males. This possibility seems unlikely on multiple counts. Targeting of MSL2 to autosomal reporters in males fails to cause an increased expression [Sun et al., 2013a]. Further, the upregulation of reporters in females by targeting MOF alone can be reversed by the ectopic expression of MSL2 [Sun et al., 2013a]. Thus, all

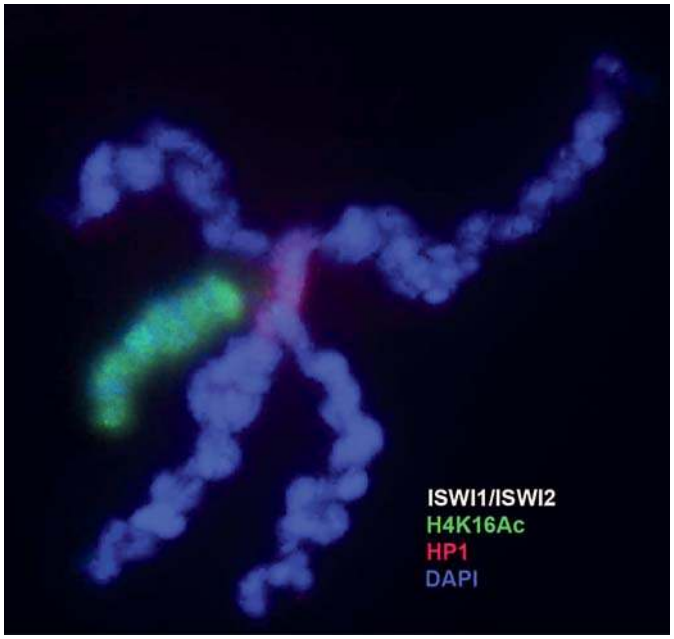

Fig. 5. Chromosomal morphology in the *ISWI* mutant male. Polytene chromosome spread of an *ISWI* mutant male larva. Chromosomes are stained with DAPI (blue). Immunolocalization of H4K16Ac (green) is illustrated on the X chromosome. Heterochromatin protein 1 (HP1, red) is concentrated in the chromocenter. Note the enlarged morphology of the X chromosome in this *ISWI* mutant male. Photo by Lin Sun.

of the components to counteract the effect of histone acetylation on gene expression appear to be present in females.

As noted above, disruption of the MSL complex does not eliminate X chromosome dosage compensation. Together with the targeting results, the combined evidence suggests the need for a revised hypothesis for the role of the MSL complex.

Components of the Constraining Activity

An interesting phenotype of the male X chromosome occurs in mutations of various chromatin components. The male X chromosome is shorter and wider in mutants for the chromatin remodeling components *ISWI* [Brehm et al., 2000; Deuring et al., 2000] (fig. 5), *nurf* [Badenhorst et al., 2005], *JIL-1 kinase* [Wang et al., 2001], *Topo2* [Hohl et al., 2012], overexpression of DNA supercoiling factor [Furuhashi et al., 2006] and for the heterochromatin components, *HP1* and *Su(var)3-7* [Delattre et al., 2004; Spierer et al., 2005, 2008] among other conditions [Pal Bhadra et al., 2005].

A role in gene repression was suggested for ISWI given that it localizes on polytene chromosomes independently of RNA polymerase II [Deuring et al., 2000]. In a subsequent study, the magnitude of the enlarged X phenotype was found to correlate with the amount of H4K16 acetylation [Corona et al., 2002]. The enlarged X phenotype is suppressed by the *mof1* mutation, which strongly reduces the histone modification. It is potentially the case that *ISWI* and other genes with similar effects are involved in the override of histone acetylation or constraining activity by the MSL complex. One speculation is that these mutations release the MSL override, which would produce an extreme overexpression of the X as well as its bloated appearance. The results of Corona et al. [2002] with regards to the impact of levels of histone acetylation on the *ISWI* mutant phenotype are consistent with this idea. An examination of selected X and autosomal gene expression in the *ISWI* mutant embryos showed an overexpression of the X chromosome in males, which suggests that this protein is required for the process involved with overriding histone acetylation [Pal Bhadra et al., 2005]. Heterochromatin protein 1 (HP1) is enriched on the male X [de Wit et al., 2005, 2008] and might play a role, but this possibility has not been explored.

A Model for the Function of the MSL Complex

The narrative above describes evidence that the MSL hypothesis for dosage compensation does not explain the various levels of gene expression that occur in compensating genotypes, as well as the results of ectopic expression and targeting. How then might one account for its presence on the X chromosome in males? Why is the MSL complex attracting an activator of gene expression to the X chromosome? A unifying explanation might be that the imbalance of the X chromosome relative to the remainder of the genome produces a widespread inverse effect on gene expression [Hiebert and Birchler, 1994; Birchler, 1996; Bhadra et al., 1999]. Such an effect can account for the different levels of compensation in both diploid and triploid flies including both reductions and increases in the dosage of the X chromosome that have been documented in *Drosophila*. The inverse dosage effect is likely a reflection of the stoichiometric interactions of multisubunit macromolecular complexes, and thus can be manifested in most aneuploids to some degree because any regulatory complex will exhibit the same kinetic

properties [Veitia et al., 2008, 2013; Birchler, 2010; Birchler and Veitia, 2012]. The MSL complex might have evolved to localize to the X chromosome so as to sequester the histone modifiers MOF and JIL1 kinase away from the autosomes to mute the inverse dosage effect that would otherwise occur there [Bhadra et al., 1999; Pal Bhadra et al., 2005; Sun et al., 2013a, b]. With the increase in histone modifiers on the X, a constraining activity evolved to prevent overexpression. In essence, the sequestration process would be a reaction to genomic imbalance that occurs in normal males, and it would assure that the X is compensated but that the autosomes are not overexpressed.

With the degeneration of the Y chromosome to produce the heteromorphic sex chromosome situation, hemizygous regions of the X might be selected to acquire MOF for increased expression to survive. As the hemizygous region expands, further X-accumulated MOF would be coincident with autosomally depleted MOF, which would mute autosomal upregulation from an expanding inverse effect of the enlarging size of the hemizygous X region in males. As this process accelerates, an activity of the MSL complex might be selected to prevent overexpression due to the higher levels of acetylation now accumulating on the X but still allow dosage compensation to occur. The consequence of this scenario would be that the X chromosome in males is expressed near the level as the 2 X chromosomes in females, and the autosomes in the 2 sexes would be expressed at similar levels. However, when the MSL complex is dissociated in mutants, there is no constraining activity present and MOF becomes distributed uniformly across the genome [Bhadra et al., 1999]. The autosomes would be expected to be increased in expression to some degree, for which experimental evidence exists. This evolutionary hypothesis is consistent with the normal and mutant results of MSL targeting and gene expression.

Resolution

The model that the MSL complex causes a 2-fold increase in the expression of the male X chromosome as the basis of dosage compensation is a simple concept. However, there are many aspects of the biology of dosage compensation that it does not explain. Dosage compensation in triple X metafemales [Stern, 1960] and the concomitant autosomal reductions in expression cannot be accounted for. Likewise, the tendency for male autosomal expression dimorphism is not explained. Other genotypes such as triploid intersexes and metamales have different levels of 'per gene' expression that association with the MSL complex does not readily accommodate. As noted above, triploid intersexes are mosaic for male and female tissues. It might be argued therefore that the intermediate expression results from an average expression from male and female cells. However, the phenotype of *white-apricot* in triploid intersexes (observed in the work of Rabinow et al. [1991]) is not mosaic but uniformly similar to that of triploid females illustrating the true intermediate level of expression per gene. Furthermore, the targeting of the MSL complex to reporter transgenes or to the whole X chromosomes in females shows no evidence for an upregulation of the genes newly associated with the complex. Autosomal dosage compensation has been reported for several different aneuploids by multiple laboratories suggesting that compensation will inherently occur with a change of chromosomal dosage to some degree, and the MSL concept does not fit into this framework. In this vein, it lies outside the eukaryote-wide phenomenon of genomic balance effects on gene expression. The inverse effect model, however, is consistent with these findings in a general sense.

The unresolved issue seems to be whether there is a loss of dosage compensation of the X chromosome when the MSL complex is dissociated. As noted above, studies conducted in fly tissues suggest that a generalized loss of compensation does not occur and many increases in expression operate for the autosomes. If this is in fact the reality, then the method of normalization becomes an issue of whether it obscures the results. If the autosomes are inversely affected and they are used as the normalization standard, as is often the case, then the X chromosome values would be artificially reduced and would suggest a loss of compensation. In concert, the change in autosomal expression would be confounded by normalization to the same. Transcriptome size changes have been shown to be very difficult to decipher in microarray and RNA-Seq studies [Coate and Doyle, 2010, 2015; Lovén et al., 2012; Birchler, 2014]. The situation is further complicated by the possibility that autosomal changes might alter the stoichiometry of regulatory machineries and indeed produce a spectrum of increases and decreases on all chromosomes under different circumstances as occurs in global studies of aneuploidy [Sun et al., 2013c]. If part of this spectrum included reductions of some X-linked genes, a focus on them might lead to the conclusion that there is loss of compensation when a more global change is indeed occurring.

One study purports to have documented a loss of compensation when correcting for 'per cell' expression [Straub et al., 2005]. This study, however, was conducted in S2 tissue culture cells in which MSL2 had been knocked down by RNA interference. S2 cells are highly aneuploid, and there is evidence that there are other global changes in gene expression occurring in these cells [Zhang et al., 2010]. Another consideration is that the level of MOF might be low in S2 cells and consequently, when the MSL complex is disrupted, there may in fact be a generalized loss of compensation as occurs in the *mof[1];mle* double mutant genotype in embryos [Pal Bhadra et al., 2005]. Similar to the fact that targeting of MOF to a reporter in yeast [Akhtar and Becker, 2000] does not give the same result as targeting the MSL complex in flies [Bhadra et al., 1999, 2000; Sun and Birchler, 2009; Sun et al., 2013a], because it is out of the context of the fly cell, testing for loss of dosage compensation in S2 cells might not reflect the situation within the organism.

Indeed, there are distinctions between how autosomal monosomics comparable in genetic content to the X chromosome (~20% of the genome) behave compared to the *msl* mutant males. The former are lethal at embryonic stages [Devlin et al., 1982], but the msl^- mutant males survive to the late larval stages and into the pupal stage. If there were in fact a loss of compensation in the msl^- mutant males, the biology is different than an autosomal monosomic of the same size. Indeed, the *mof[1];mle* mutants are lethal in the embryonic stage. In this case, the loss of compensation is visually apparent in RNA-FISH experiments that do not make intragenomic normalizations but rather provide an absolute measurement of gene expression 'per cell'.

A resolution would come from conducting gene expression studies in fly tissues to insure that the cellular context is maintained. Normalization of global expression patterns should not correct to autosomal levels, given that one model to be distinguished suggests they are modulated, but rather to the DNA or via per cell methods. Procedures to estimate the transcriptome size are available [Coate and Doyle, 2010, 2015], but visualization methods per cell could also be used [Lovén et al., 2012; Birchler, 2014]. However, most RNA-Seq studies have an unwitting normalization built into them that obscures the transcriptome size [Lovén et al., 2012; Birchler, 2014; Coate and Doyle, 2015]. Thus, careful reflection on this matter is needed to move the field forward.

Acknowledgements

Research was supported by NIH grant RO1GM068042. The author is grateful to colleagues who have contributed to the cited work.

Disclosure Statement

The author has no conflicts of interest to declare.

References

Akhtar A, Becker PB: Activation of transcription through histone H4 acetylation by MOF, an acetyltransferase essential for dosage compensation in *Drosophila*. Mol Cell 5:367–375 (2000).

Aury JM, Jaillon O, Duret L, Noel B, Jubin C, et al: Global trends of whole-genome duplications revealed by the ciliate *Paramecium tetraurelia*. Nature 444:171–178 (2006).

Badenhorst P, Xiao H, Cherbas L, Kwon SY, Voas M, et al: The *Drosophila* nucleosome remodeling factor NURF is required for ecdysteroid signaling and metamorphosis. Genes Dev 19: 2540–2545 (2005).

Bellott DW, Hughes JF, Skaletsky H, Brown LG, Pyntikova T, et al: Mammalian Y chromosomes retain widely expressed dosage-sensitive regulators. Nature 508:494–499 (2014).

Belote JM, Lucchesi JC: Control of X chromosome transcription by the *maleless* gene in *Drosophila*. Nature 285:573–575 (1980).

Bhadra U, Pal-Bhadra M, Birchler JA: Role of the *male specific lethal (msl)* genes in modifying the effects of sex chromosomal dosage in *Drosophila*. Genetics 152:249–268 (1999).

Bhadra U, Pal-Bhadra M, Birchler JA: Histone acetylation and gene expression analysis of *Sex lethal* mutants in *Drosophila*. Genetics 155:753–763 (2000).

Birchler JA: A study of enzyme activities in a dosage series of the long arm of chromosome one in maize. Genetics 92:1211–1229 (1979).

Birchler JA: The genetic basis of dosage compensation of *Alcohol dehydrogenase-1* in maize. Genetics 97:625–637 (1981).

Birchler JA: Genetic analysis of a modifier of the sexual dimorphism of *glass* in *Drosophila melanogaster*. Genet Res 44:125–132 (1984).

Birchler JA: Expression of *cis*-regulatory mutations of the *white* locus in metafemales of *Drosophila melanogaster*. Genet Res 59:11–18 (1992).

Birchler JA: X chromosome dosage compensation in *Drosophila*. Science 272:1190 (1996).

Birchler JA: Reflections on studies of gene expression in aneuploids. Biochem J 426:119–123 (2010).

Birchler JA: Facts and artifacts in studies of gene expression in aneuploids and sex chromosomes. Chromosoma 123:459–469 (2014).

Birchler JA, Newton KJ: Modulation of protein levels in chromosomal dosage series of maize: the biochemical basis of aneuploid syndromes. Genetics 99:247–266 (1981).

Birchler JA, Veitia RA: Gene balance hypothesis: connecting issues of dosage sensitivity across biological disciplines. Proc Natl Acad Sci USA 109:14746–14753 (2012).

Birchler JA, Hiebert JC, Krietzman M: Gene expression in adult metafemales of *Drosophila melanogaster*. Genetics 122:869–879 (1989).

Birchler JA, Hiebert JC, Paigen K: Analysis of autosomal dosage compensation involving the *alcohol dehydrogenase* locus in *Drosophila melanogaster*. Genetics 124:679–686 (1990).

Birchler JA, Bhadra U, Pal-Bhadra M, Auger DL: Dosage-dependent gene regulation in multicellular eukaryotes: implications for dosage compensation, aneuploid syndromes and quantitative traits. Dev Biol 234:275–288 (2001).
Birchler JA, Riddle NC, Auger DL, Veitia RA: Dosage balance in gene regulation: biological implications. Trends Genet 21:219–226 (2005).
Blakeslee AF: New Jimson weeds from old chromosomes. J Hered 25:81–108 (1934).
Blakeslee AF, Belling J, Farnham ME: Chromosomal duplication and Mendelian phenomena in *Datura* mutants. Science 52:388–390 (1920).
Blanc G, KH Wolfe: Functional divergence of duplicated genes formed by polyploidy during *Arabidopsis* evolution. Plant Cell 16:1679–1691 (2004).
Blomme T, Vandepoele K, De Bodt S, Simillion C, Maere S, Van de Peer Y: The gain and loss of genes during 600 million years of vertebrate evolution. Genome Biol 7:R43 (2006).
Boell L, Pallares LF, Brodski C, Chen Y, Christian JL, et al: Exploring the effects of gene dosage on mandible shape in mice as a model for studying the genetic basis of natural variation. Dev Genes Evol 223:279–287 (2013).
Bone JR, Lavender J, Richman R, Palmer MJ, Turner BM, Kuroda MI: Acetylated histone H4 on the male X chromosome is associated with dosage compensation in *Drosophila*. Genes Dev 8:96–104 (1994).
Bowers JE, Chapman BA, Rong J, Paterson AH: Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. Nature 422:433–438 (2003).
Bray D, Lay S: Computer-based analysis of the binding steps in protein complex formation. Proc Natl Acad Sci USA 94:13493–13498 (1997).
Brehm A, Langst G, Kehle J, Clapier CR, Imhof A, et al: dMi-2 and ISWI chromatin remodeling factors have distinct nucleosome binding and mobilization properties. EMBO J 19:4332–4341 (2000).
Bridge CB: Sex in relation to chromosomes and genes. Am Nat 59:127–37 (1925).
Cline TW, Meyer BJ: Vive la différence: males vs females in flies vs worms. Annu Rev Genet 30:637–702 (1996).
Coate JE, Doyle JJ: Quantifying whole transcriptome size, a prerequisite for understanding transcriptome evolution across species: an example from a plant allopolyploid. Genome Biol Evol 2:534–546 (2010).
Coate JE, Doyle JJ: Variation in transcriptome size: are we getting the message? Chromosoma 124:27–43 (2015).
Conant GC, Birchler JA, Pires JC: Dosage, duplication, and diploidization: clarifying the interplay of multiple models for duplicate gene evolution over time. Curr Opin Plant Biol 19:91–98 (2014).
Conrad T, Cavalli FM, Vasquerizas JM, Luscombe NM, Akhtar A: *Drosophila* dosage compensation involves enhanced Pol II recruitment to male X-linked promoters. Science 337:742–746 (2012).
Corona DF, Clapier CR, Becker PB, Tamkun JW: Modulation of ISWI function by site-specific histone acetylation. EMBO Rep 3:242–247 (2002).
da Cunha PR, Granadino B, Perondini ALP, Sánchez L: Dosage compensation in sciarids is achieved by hypertranscription of the single X chromosome in males. Genetics 138:787–790 (1994).
de Clare M, Pir P, Oliver SG: Haploinsufficiency and the sex chromosomes from yeasts to humans. BMC Biol 9:15 (2011).
Delattre M, Spierer A, Jaquet Y, Spierer P: Increased expression of *Drosophila* Su(var)3-7 triggers Su(var)3-9-dependent heterochromatin formation. J Cell Sci 117:6239–6247 (2004).
Deng X, Meller VH: *roX* RNAs are required for increased expression of X-linked genes in *Drosophila melanogaster* males. Genetics 174:1859–1866 (2006).
Deuring R, Fanti L, Armstrong JA, Sarte M, Papoulos O, et al: The ISWI chromatin-remodeling protein is required for gene expression and the maintenance of higher order chromatin structure in vivo. Mol Cell 5:355–365 (2000).
Devlin RH, Holm DG, Grigliatti TA: Autosomal dosage compensation in *Drosophila melanogaster* strains trisomic for the left arm of chromosome 2. Proc Natl Acad Sci USA 79:1200–1204 (1982).
Devlin RH, Holm DG, Grigliatti TA: The influence of whole-arm trisomy on gene expression in *Drosophila*. Genetics 118:87–101 (1988).
de Wit E, Greil F, van Steenstel B: Genome-wide HP1 binding in *Drosophila*: developmental plasticity and genomic targeting signals. Genome Res 15:1265–1273 (2005).
de Wit E, Braunschweig U, Greil F, Bussemaker HJ, van Steensel B: Global chromatin domain organization of the *Drosophila* genome. PLoS Genet 4:e1000045 (2008).
Edger PP, Pires JC: Gene and genome duplications: the impact of dosage sensitivity on the fate of nuclear genes. Chromosome Res 17:699–717 (2009).
Figueiredo ML, Kim M, Philip P, Allgardsson A, Stenberg P, Larsson J: Non-coding *roX* RNAs prevent the binding of the MSL-complex to heterochromatic regions. PLoS Genet 10:e1004865 (2014).
Freeling M: Bias in plant gene content following different sorts of duplication: tandem, whole-genome, segmental, or by transposition. Annu Rev Plant Biol 60:433–453 (2009).
Freeling M, Thomas BC: Gene-balanced duplications, like tetraploidy, provide predictable drive to increase morphological complexity. Genome Res 16:805–814 (2006).
Freeling M, Lyons E, Pedersen B, Alam M, Ming R, Lisch D: Many or most genes in *Arabidopsis* transposed after the origin of the order Brassicales. Genome Res 18:1924–1937 (2008).
Furuhashi H, Nakajima M, Hirose S: DNA supercoiling factor contributes to dosage compensation in *Drosophila*. Development 133:4475–4483 (2006).
Gergen JP: Dosage compensation in *Drosophila*: evidence that *daughterless* and *Sex-lethal* control X chromosome activity at the blastoderm stage of embryogenesis. Genetics 117:477–485 (1987).
Goldschmidt RB: Different philosophies of genetics. Science 119:703–710 (1954).
Guo M, Birchler JA: Trans-acting dosage effects on the expression of model gene systems in maize aneuploids. Science 266:1999–2002 (1994).
Guo M, Davis D, Birchler JA: Dosage effects on gene expression in a maize ploidy series. Genetics 142:1349–1355 (1996).
Hakes L, Pinney JW, Lovell SC, Oliver SG, Robertson DL: All duplicates are not equal: the difference between small-scale and genome duplication. Genome Biol 8:R209 (2007).
Hamada FN, Park PJ, Gordadze PR, Kuroda MI: Global regulation of X chromosomal genes by the MSL complex in *Drosophila melanogaster*. Genes Dev 19:2289–2294 (2005).
Hazelrigg T, Levis R, Rubin GM: Transformation of *white* locus DNA in *Drosophila*: dosage compensation, zeste interaction and position effects. Cell 36:469–481 (1984).
Henikoff S: Dosage-dependent modification of position-effect variegation in *Drosophila*. Bioesssays 18:401–409 (1996).
Hiebert JC, Birchler JA: Effects of the *maleless* mutation on X and autosomal gene expression in *Drosophila melanogaster*. Genetics 136:913–926 (1994).
Hilfiker A, Hilfiker-Kleiner D, Pannuti A, Lucchesi JC: *mof*, a putative acetyl transferase gene related to the Tip60 and MOZ human genes and to the SAS genes of yeast, is required for dosage compensation in *Drosophila*. EMBO J 16:2054–2060 (1997).
Hohl AM, Thompson M, Soshnev AA, Wu J, Morris J, et al: Restoration of topoisomerase 2 function by complementation of defective monomers in *Drosophila*. Genetics 192:843–856 (2012).
Jin Y, Wang Y, Walker DL, Dong H, Conley C, et al: JIL-1: a novel chromosomal tandem kinase implicated in transcriptional regulation in *Drosophila*. Mol Cell 4:129–135 (1999).
Jin Y, Wang Y, Johansen J, Johansen KM: JIL-1, a chromosomal kinase implicated in regulation of chromatin structure, associates with the male specific lethal (MSL) dosage compensation complex. J Cell Biol 149:1005–1010 (2000).
Karess RE, Rubin GM: A small tandem duplication is responsible for the unstable *white-ivory* mutation in *Drosophila*. Cell 30:63–69 (1982).

Kelley RL, Kuroda MI: The *Drosophila roX1* RNA gene can overcome silent chromatin by recruiting the male-specific lethal dosage compensation complex. Genetics 164:565–574 (2003).

Kelley RL, Solovyeva I, Lyman LM, Richman R, Solovyev V, Kuroda MI: Expression of *msl-2* causes assembly of dosage compensation regulators on the X chromosomes and female lethality in *Drosophila*. Cell 81:867–877 (1995).

Kelley RL, Wang J, Bell L, Kuroda MI: *Sex lethal* controls dosage compensation in *Drosophila* by a non-splicing mechanism. Nature 387: 195–199 (1997).

Kind J, Vaquerizas JM, Gebhardt P, Gentzel M, Luscombe NM, et al: Genome-wide analysis reveals MOF as a key regulator of dosage compensation and gene expression in *Drosophila*. Cell 133:813–828 (2008).

Kondrashov FA, Koonin EV: A common framework for understanding the origin of genetic dominance and evolutionary fates of gene duplications. Trends Genet 20:287–290 (2004).

Kruesi WS, Core LJ, Waters CT, Lis JT, Meyer BJ: Condensin controls recruitment of RNA polymerase II to achieve nematode X-chromosome dosage compensation. eLife 2:e00808 (2013).

Kuroda MI, Kernan MJ, Kreber R, Ganetsky B, Baker BS: The *maleless* protein associates with the X chromosome to regulate dosage compensation in *Drosophila*. Cell 66:935–947 (1991).

Larschan E, Bishop EP, Kharchenko PV, Core L, Lis JT, et al: X chromosome dosage compensation via enhanced transcriptional elongation in *Drosophila*. Nature 471:115–118 (2011).

Levis R, Hazelrigg T, Rubin GM: Separable *cis*-acting control elements for expression of the *white* gene of *Drosophila*. EMBO J 4:3489–3499 (1985).

Lim CK, Kelley RL: The *Drosophila over compensating males* gene genetically inhibits dosage compensation in males. PLoS One 8:e60450 (2013).

Lovén J, Orlando DA, Sigova AA, Lin CY, Rahl PB, et al: Revisiting global gene expression analysis. Cell 151:476–482 (2012).

Lucchesi JC, Kuroda MI: Dosage compensation in *Drosophila*. Cold Spring Harb Perspect Biol 7:a019398 (2015).

Lucchesi JC, Rawls JM Jr: Regulation of gene function: a comparison of enzyme activity levels in relation to gene dosage in diploids and triploids of *Drosophila melanogaster*. Biochem Genet 9:41–51 (1973a).

Lucchesi JC, Rawls JM Jr: Regulation of gene function: a comparison of X-linked enzyme activity levels in normal and intersexual triploids of *Drosophila melanogaster*. Genetics 73: 459–464 (1973b).

Lucchesi JC, Rawls JM Jr, Maroni G: Gene dosage compensation in metafemales (3X;2A) of *Drosophila*. Nature 248:564–567 (1974).

Lucchesi JC, Belote JM, Maroni G: X-linked gene activity in metamales (XY;3A) of *Drosophila*. Chromosoma 65:1–7 (1977).

Lucchesi JC, Kelly WC, Panning B: Chromatin remodeling in dosage compensation. Ann Rev Genet 39:615–651 (2005).

Maere S, De Bodt S, Raes J, Casneuf T, Van Montagu M, et al: Modeling gene and genome duplications in eukaryotes. Proc Natl Acad Sci USA 102:5454–5459 (2005).

Makino T, McLysaght A: Ohnologs in the human genome are dosage balanced and frequently associated with disease. Proc Natl Acad Sci USA 107:9270–9274 (2010).

Margolis OS: The effect of a supernumerary X chromosome on members of the Bar series of *Drosophila*. Genetics 19:18–84 (1934).

Maroni G, Plaut W: Dosage compensation in *Drosophila melanogaster* triploids. I. Autoradiography study. Chromosoma 40:361–377 (1973).

Meller VH, Rattner BP: The *roX* genes encode redundant *male-specific lethal* transcripts required for targeting the MSL complex. EMBO J 21:1084–1091 (2002).

Meller VH, Wu KH, Roman G, Kuroda MI, Davis RL: *roX1* RNA paints the X chromosome of male *Drosophila* and is regulated by the dosage compensation system. Cell 88:445–457 (1997).

Muller HJ: Further studies on the nature and causes of gene mutations. Proc 6th Int Congr Genet 1:213–255 (1932).

Muller HJ: Evidence of the precision of genetic adaptation. Harvey Lect 43:165–229 (1950).

Nawata H, Kashino G, Tano K, Daino K, Shimada Y, et al: Dysregulation of gene expression in the artificial human trisomy cells of chromosome 8 associated with transformed cell phenotypes. PLoS One 6:e25319 (2011).

Okuno T, Satou T, Oishi K: Studies on the sex-specific lethals of *Drosophila melanogaster*. VII. Sex-specific lethals that do not affect dosage compensation. Jpn J Genet 59:237–247 (1984).

Pal Bhadra M, Bhadra U, Birchler JA: Cosupression of nonhomologous transgenes in *Drosophila* involves mutually related endogenous sequences. Cell 99:35–46 (1999).

Pal Bhadra M, Bhadra U, Kundu J, Birchler JA: Gene expression analysis of the function of the male-specific lethal complex in *Drosophila*. Genetics 169:2061–2074 (2005).

Papp B, Pal C, Hurst LD: Dosage sensitivity and the evolution of gene families in yeast. Nature 424:194–197 (2003).

Park Y, Kelley RL, Oh H, Kuroda MI, Meller VH: Extent of chromatin spreading determined by *roX* RNA recruitment of MSL proteins. Science 298:1620–1623 (2002).

Pessia E, Makino T, Bailly-Bechet M, McLysaght A, Marais GAB: Mammalian X chromosome inactivation evolved as a dosage-compensation mechanism for dosage-sensitive genes on the X chromosome. Proc Natl Acad Sci USA 109:5346–5351 (2012).

Philip P, Stenberg P: Male X-linked genes in *Drosophila melanogaster* are compensated independently of the male-specific lethal complex. Epigenetics Chromatin 6:35 (2013).

Prestel M, Feller C, Straub T, Mitlohner H, Becker PB: The activation potential of MOF is constrained for dosage compensation. Mol Cell 38:815–826 (2010).

Rabinow L, Nguyen-Huynh AT, Birchler JA: A *trans*-acting regulatory gene that inversely affects the expression of the *white*, *brown* and *scarlet* loci in *Drosophila*. Genetics 129:463–480 (1991).

Ruiz MF, Esteban MR, Donoro C, Goday C, Sanchez L: Evolution of dosage compensation in Diptera: the gene *maleless* implements dosage compensation in *Drosophila* (Brachycera Suborder) but its homolog in *Sciara* (Nematocera Suborder) appears to play no role in dosage compensation. Genetics 156:1853–1865 (2000).

Sabl JF, Birchler JA: Dosage dependent modifiers of *white* alleles in *Drosophila melanogaster*. Genet Res 62:15–22 (1993).

Schuster-Böckler B, Conrad D, Bateman A: Dosage sensitivity shapes the evolution of copy-number varied regions. PLoS One 5:e9474 (2010).

Seidman JG, Seidman C: Transcription factor haploinsufficiency: when half a loaf is not enough. J Clin Invest 109:451–455 (2002).

Simillion C, Vandepoele K, Montagu MC, Zabeau M, Van de Peer Y: The hidden duplication past of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 99:13627–13632 (2002).

Smith PD, Lucchesi JC: The role of sexuality in dosage compensation in *Drosophila*. Genetics 61:607–618 (1969).

Spierer A, Seum C, Delattre M, Spierer P: Loss of the modifiers of variegation Su(var)3-7 or HP1 impacts male X polytene chromosome morphology and dosage compensation. J Cell Sci 118:5047–5057 (2005).

Spierer A, Begeot F, Spierer P, Delattre M: SU(VAR)3-7 links heterochromatin and dosage compensation in *Drosophila*. PLoS Genet 4:e1000066 (2008).

Stern C: Dosage compensation – development of a concept and new facts. Can J Genet Cytol 2: 105–118 (1960).

Straub T, Gilfillan GD, Maier VK, Becker PB: The *Drosophila* MSL complex activates the transcription of target genes. Genes Dev 19:2284–2288 (2005).

Sun L, Fernandez HR, Donohue RC, Li J, Cheng J, Birchler JA: Male-specific lethal complex in *Drosophila* counteracts histone acetylation and does not mediate dosage compensation. Proc Natl Acad Sci USA 110:E808–E817 (2013a).

Sun L, Johnson AF, Donohue RC, Li J, Cheng J, Birchler JA: Dosage compensation and inverse effects in triple X metafemales of *Drosophila*. Proc Natl Acad Sci USA 110:7383–7388 (2013b).

Sun L, Johnson AF, Li J, Lambdin AS, Cheng J, Birchler JA: Differential effect of aneuploidy on the X chromosome and genes with sex-biased expression in *Drosophila*. Proc Natl Acad Sci USA 110:16514–16519 (2013c).
Sun X, Birchler J: Interaction study of the male specific lethal (MSL) complex and trans-acting dosage effects in metafemales of *Drosophila melanogaster*. Cytogenet Genome Res 124: 298–311 (2009).
Turner BM, Birley AJ, Lavender J: Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in *Drosophila* polytene nuclei. Cell 69:375–384 (1992).
Veitia RA: Exploring the etiology of haploinsufficiency. Bioessays 24:175–184 (2002).
Veitia RA, Bottani S, Birchler JA: Cellular reactions to gene dosage imbalance: genomic, transcriptomic and proteomic effects. Trends Genet 24:390–397 (2008).
Veitia RA, Bottani S, Birchler JA: Gene dosage effects: nonlinearities, genetic interactions and dosage compensation. Trends Genet 29:385–393 (2013).
Veitia RA, Veyrunes F, Bottani S, Birchler JA: X chromosome inactivation and active X upregulation in therian mammals: facts, questions and hypotheses. J Mol Cell Biol 7:2–11 (2015).
Vicoso B, Bachtrog D: Numerous transitions of sex chromosomes in Diptera. PLoS Biol 13:e1002078 (2015).
Wang Y, Zhang W, Jin Y, Johansen J, Johansen KM: The JIL-1 tandem kinase mediates histone H3 phosphorylation and is required for maintenance of chromatin structure in *Drosophila*. Cell 105:433–443 (2001).
Wolfe KH, Shields DC: Molecular evidence for an ancient duplication of the entire yeast genome. Nature 387:708–713 (1997).
Xie W, Birchler J: Identification of *Inverse regulator-a (Inr-a)* as synonymous with pre-mRNA cleavage complex II protein *(Pcf11)* in *Drosophila*. G3 (Bethesda) 2:701–706 (2012).
Yang J, Lusk R, Li WH: Organismal complexity, protein complexity, and gene duplicability. Proc Natl Acad Sci USA 100:15661–15665 (2003).
Zachar Z, Bingham PM: Regulation of *white* locus expression: the structure of mutant alleles at the *white* locus of *Drosophila melanogaster*. Cell 30:529–541 (1982).
Zhang Y, Malone JH, Powell SK, Periwal V, Spana E, et al: Expression in aneuploid *Drosophila* S2 cells. PLoS Biol 8:e1000320 (2010).