One Hundred Years of Gene Balance: How Stoichiometric Issues Affect Gene Expression, Genome Evolution, and Quantitative Traits

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Abstract

A century ago experiments with the flowering plant *Datura stramonium* and the fruit fly *Drosophila melanogaster* revealed that adding an extra chromosome to a karyotype was much more detrimental than adding a whole set of chromosomes. This phenomenon was referred to as gene balance and has been recapitulated across eukaryotic species. Here, we retrace some developments in this field. Molecular studies suggest that the basis of balance involves stoichiometric relationships of multi-component interactions. This concept has implication for the mechanisms controlling gene expression, genome evolution, sex chromosome evolution/dosage compensation, speciation mechanisms, and the underlying genetics of quantitative traits.

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Introduction

One hundred years ago Alfred Blakeslee published a summary of his studies in which he had systematically recovered a trisomic line for each of the 12 chromosomes of the flowering plant, *Datura stramonium* [Blakeslee et al., 1920; Blakeslee, 1921]. He had also found triploids and tetraploids. He noted that each trisomy, which were given names such as “Globe,” “Glossy,” “Poinsettia,” “Cocklebur,” etc., produced a unique morphology, and each trisomy would modulate the quantitative expression of the anthocyanin pigment in the plants that was used as a phenotypic marker. Yet, when all of the chromosome set was changed in dosage in the polyploids, there was much less effect.

The effects on pigment levels in his own words follow: “While there seems to be but a single chromosomal set responsible for the presence or absence of purple pigmentation, probably each chromosome has an influence upon the strength of expression of the pigment since the several mutants {trisomies} appear to differ widely in color when homozygous for the main purple factor.” “Thus, Glossy is darker purple than normal, while Cocklebur is

Fig. 1. Maize plants illustrating the impact of genomic balance. From left to right, maize plants are shown that are a normal haploid containing 10 chromosomes, a haploid that has an additional copy of the short arm of chromosome 9, a normal diploid with 20 chromosomes, and a diploid with an extra copy of the short arm of chromosome 9 all in the same genetic background. Note that the addition of an extra chromosome arm to the haploid has a much more detrimental effect on the plant stature than the addition to the diploid. The greater the stoichiometric difference of chromosomal dosage to the remainder of the genome, the greater the aneuploid effect. Photo by Zhi Gao and Fangpu Han.

distinctly lighter.” “When all of the sets have an extra chromosome, however, as is the case in triploids, no great disturbance of the balance is brought about and the plant is not greatly different from normal.” “Even in tetraploids where all the sets are equally affected, although the total number of chromosomes is doubled, the difference from normal is not so great as in mutants {trisomies} of the Globe and Poinsettia series.” A few years later, Blakeslee and Belling [Blakeslee and Belling, 1924; Blakeslee, 1934] published pictures of this collection and added the result that the addition of a particular chromosome had greater effects on plant morphology with ascending dosage, but the same dosage series in a tetraploid background was less severe at each dosage step, which reflects the lower proportion of the extra chromosome versus the whole genome in tetraploids versus diploids. Blakeslee used the word “balance,” which gained acceptance as similar phenomena were described in other taxa.

One other species that played a major role in the concept of gene balance was *Drosophila*. Calvin Bridges [Bridges, 1921] deduced that genotypes that carried an extra copy of any of the major chromosomes of the X, 2, or 3 were mostly lethal at the adult stage whereas triploid

female flies were viable. Still further, Bridges [1925] found that the ratio of the X chromosome to the number of autosomes was the chromosomal trigger for sex determination. As collections of trisomies were produced in several plant species together with the respective ploidy series, the generalization of these earlier observations was confirmed. In humans, most trisomies are lethal in early development or cause spontaneous abortions [Bond and Chandley, 1983; Schinzel, 2001]. Trisomy 21 or Down syndrome is the least severe and hence was the first discovered [Lejeune et al., 1959]. And because trisomies are the product of nondisjunction, an equal frequency of human monosomies is likely to occur but they have not been observed, leading to the deduction that they are lethal very early in development. Thus, the observation that partial genome changes in dosage are more severe than changing the dosage of the whole genome is a general theme in eukaryotes.

The differential effect of aneuploidy at different ploidy levels noted by Blakeslee and Belling [1924] was extended extensively in hexaploid wheat. Sears [1953] developed a complete set of aneuploids in common wheat. Monosomies were much less affected than in diploid species and indeed, nullisomics that are missing a homologous pair of chromosomes are viable, given the presence of copies of homoeologous chromosomes (the related chromosomes in the separate genomes) being still present (2 each of 2 different genomes). These principles have been repeatedly found in other polyploid species, illustrating the lesser effect of addition or subtraction of a single chromosome in these cases. When new allopolyploids are produced in *Brassica*, compensating aneuploids for a balanced total genome tend to emerge over the early generations [Xiong et al., 2011]. Other classical aspects of balance have previously been noted [Birchler and Veitia, 2012]. The effect of relative dosage on the phenotype of maize is depicted in Figure 1.

Some of these observations were made before the nature of the genetic material was known, let alone aspects of gene expression. Once molecular studies were initiated with aneuploids, the concept arose that the genes on the varied chromosome would produce a proportional dosage effect (i.e., proportionality between copy number and gene expression), and this was the basis of the imbalance. Some investigators cling to this idea and to some degree it is correct but, in this review, we will illustrate how the balance phenomena are a reflection of normal gene regulatory processes that operate across the genome, which are perturbed by aneuploidy.

Molecular Studies of Gene Balance

The beginnings of molecular studies grew from the initial observations that changing the dosage of genes known to encode specific enzymes would change the quantity of that enzyme in cellular extracts. Early examples include the gene for xanthine dehydrogenase in *Drosophila* [Grell, 1962]. With the development of the tools to produce and detect segmental aneuploidy across the chromosomes of *Drosophila* [Lindsley et al., 1972], a popular trend in the 1970s was to screen these materials to define the position of the structural gene for specific enzymes [O'Brien and Gethman, 1973; Rawls and Lucchesi, 1974; Hodgetts, 1975; Hall and Kankel, 1976; Pipkin et al., 1977; Detwiler and MacIntyre, 1978; Moore and Sullivan, 1978; Oliver et al., 1978]. When there was success at finding a small trisomic region that increased the amount of the assayed enzyme, the structural gene was assigned to that location.

In maize, the availability of translocations between the supernumerary B chromosome and various normal chromosome arms allowed the dosage of the latter to be varied in 1–3 doses. The B chromosome is a nonvital chromosome found in some varieties and traces back to teosinte, the wild maize relative. It is maintained in populations by a drive mechanism consisting of nondisjunction at the second pollen mitosis that produces the 2 sperm followed by preferential fertilization of the egg by the B containing sperm in the process of double fertilization. Thus, translocations between the B chromosome and any A chromosomal segment will vary the latter in the sperm and consequently in the zygotes produced. Among functionally diploid organisms, maize is one of the most tolerant of aneuploidy and thus a wider spectrum of aneuploids can be examined. Nevertheless, they exhibit the classical aneuploidy and ploidy syndromes phenotypically [Lee et al., 1996a; b; Kato and Birchler, 2006; Sheridan and Auger, 2008; Yao et al., 2011; Birchler and Veitia, 2012; Brunelle and Sheridan, 2014] and can serve as an excellent model system for the study of aneuploidy.

A study of enzyme levels in a 1–4-doses series of the long arm of chromosome 1 in maize, which was produced by a B-A translocation for that arm, revealed a novel gene expression phenomenon [Birchler, 1979]. Enzymes that were not encoded on 1L were inversely modulated for the level of expression, which came to be called the inverse effect. In other words, in the monosomies, there were increases in expression more or less to a 2-fold range, and in trisomies and tetrasomies there were reductions to a lower range of 2/3 and 1/2, respectively. In that publica-

tion, it was pointed out that in the *Drosophila* segmental trisomies scanning the genome for dosage effects of structural genes for enzymes, the inverse effect was found in virtually all of these studies, and multiple regions could produce this effect on any one enzyme [O'Brien and Gethman, 1973; Rawls and Lucchesi, 1974; Hodgetts, 1975; Hall and Kankel, 1976; Pipkin et al., 1977; Detwiler and MacIntyre, 1978; Moore and Sullivan, 1978; Oliver et al., 1978]. The inverse effect was also observed in *Datura* [Smith and Conklin, 1975] and barley [McDaniel and Ramage, 1970] trisomies. Different regions produced inverse effects for different enzymes. These effects were generally ignored presumably being considered as a secondary consequence of aneuploidy, but the specificity of regions for different enzymes suggests otherwise when all of the studies are viewed collectively. However, the study in maize was able to compare the effects not only in trisomy but monosomy and tetrasomy, which illustrated that the inverse effect tracked with the chromosomal dosage and thus was not just a generic effect of aneuploidy.

In the same study it was found that an enzyme encoded in the long arm of chromosome 1, namely alcohol dehydrogenase 1, was dosage-compensated in the 1–4-dosage series. In other words, its levels were maintained near the diploid level despite the change in the copy number of the known structural gene. In a subsequent study [Birchler, 1981], it was demonstrated that the basis of this compensation was that the structural gene was modulated inversely by a different portion of the long arm of chromosome 1 and that varying the structural gene alone showed a proportional dosage effect. Together the 2 effects cancel each other to produce the dosage compensation.

When profiles of kernel embryo proteins were examined for several dosage series of the maize genome, the inverse effect was found to be quite prevalent [Birchler and Newton, 1981]. The implication from *Drosophila* studies that any one gene product could be inversely affected by multiple regions of the genome was confirmed. For the protein profiles in the dosage series of 1–3 doses of the long arm of chromosome 1, again a protein encoded therein was dosage compensated, while numerous other proteins were inversely affected, fewer were positively affected, and some were not modulated. However, comparison of the protein profiles in haploids and tetraploids compared to diploids showed few relative changes in amounts. The various types of effects are depicted in Figure 2.

Studies in *Drosophila* of whole-arm trisomy (~20% of the genome) at the larval stage revealed several cases of

Fig. 2. Types of dosage effects in aneuploid series. Left, the types of dosage effects seen for genes on the varied chromosome. A structural gene that shows an expression that is proportional to the change of chromosomal dosage is said to show a proportional gene dosage effect. If the change in chromosomal dosage does not produce a change in gene expression of the varied genes, it is said to exhibit gene dosage compensation. A spectrum is seen between gene dosage effects and dosage compensation. Right, the types of *trans*-acting effects on gene expression in the nonvaried portion of

the genome in aneuploids. The inverse effect is an inversely proportional change in expression with the dosage of the varied chromosomal region. A direct effect shows a positive correlation with the varied chromosomal region. In dosage series involving monosomy through trisomy, a decreased effect is often observed in which there is a reduced expression in both monosomy and trisomy relative to the diploid. Also, sometimes observed but not shown is an increased effect in which there is an increase in both monosomy and trisomy relative to the diploid.

autosomal dosage compensation on the enzyme expression level [Devlin et al., 1982]. The compensation was found to be gene-specific [Devlin et al., 1982] and occur on the transcriptional level [Devlin et al., 1984]. Later, related experiments showed the presence of the inverse effect in similar genotypes [Devlin et al., 1988]. In a study of a trisomy encompassing a large portion of the left arm of chromosome 2 surrounding the alcohol dehydrogenase (*Adh*) gene, dosage compensation was documented on the mRNA level [Birchler et al., 1990]. When smaller trisomies were produced surrounding just the *Adh* gene, a proportional dosage effect was found. Shorter-length trisomies within the limits of the larger one, but which did not include the *Adh* locus, exhibited the inverse dosage effect on *Adh*. Thus, the basis of the dosage compensation was again illustrated to be based on the cancellation of a proportional gene dosage effect by an inverse effect produced in larger aneuploids simultaneously. Further, in this study, the trisomies were found to produce an inverse dosage effect on an *Adh* promoter-*white* eye color reporter gene construct. This latter experiment demonstrated that the inverse effect can operate on the promoter and that the effect can be observed phenotypically.

A survey of the genome of short aneuploids demonstrated that many regions of the genome had a dosage effect on the phenotypic expression of the *white* eye color gene [Sabl and Birchler, 1993]. Indeed, in the classical literature, Muller [1950] had shown that small aneuploids along the length of the X chromosome would modulate the expression of the leaky allele, *white-apricot*. In a genome-wide survey, Guo and Birchler [1994] examined several model gene systems whose structural genes were located in a collection of dosage series across the genome in maize and found that the mRNA from any one of these genes could be modulated in *trans* by multiple regions of the genome. The most common effect was an inverse correlation of the mRNA levels with the varied dosage of the various chromosome arms. In diploid embryos versus triploid endosperm, the magnitude of the positive and negative modulations was more at the diploid level than the triploid, reflecting the relative change in dosage analyzed with respect to the remainder of the genome, analogously to how aneuploidy of an additional single chromosome with ascending ploidy has lesser and lesser effect as noted above. An examination of a ploidy series in maize revealed fewer modulations than with aneuploidy [Guo et al., 1996].

Fig. 3. Ratio distribution analysis to reveal global trends of gene expression. **a** Idealized scatterplot of expression data where the mRNA levels of most genes are not changing between 2 conditions, normal and mutant. Only 4 genes (displaced from the diagonal) appear to be differentially expressed (DE) between the 2 conditions (the ratios of mutant/normal depart from 1). This is the typical result obtained when a transcription factor with a few targets is up- or downregulated in the mutant condition. In such cases one can normalize the expression of each gene assuming that most of the genes are not DE. **b** In this case, aneuploidy (trisomy

in this example) introduces large expression departures between the 2 conditions (many ratios will depart from 1). **c** To visualize the changes in a different way, the ratio of the expression for each gene between the trisomy condition over that in the normal condition can be generated and plotted in a distribution. A ratio of 1.00 (black vertical bar) represents dosage compensation in the aneuploid versus the diploid. A ratio of 1.50 (red bar) represents a proportional gene dosage effect. A ratio of 0.67 (green bar) represents the inverse ratio of gene expression in *trans*. The frequency is the number of genes in each ratio bin.

Single-Genes Dosage Modifiers

The fact that the inverse effect could be produced by relatively short aneuploid regions and specific aneuploids have this effect for a subset of genes suggested that single genes were responsible. To investigate this hypothesis, mutageneses were conducted using a leaky allele of the *white* eye color gene (*white-apricot*) in *Drosophila* to find heterozygous mutations that would produce an up- or downregulation of the eye color. The logic was that a heterozygous mutation would act similarly as does a monosomy that is missing one of the 2 copies of a chromosome. This approach produced a multitude of mutations that were analyzed over many years [Birchler et al., 2001].

The first one was *Inverse regulator-a* (*Inr-a*), which is present on the right arm of chromosome 2 and as a heterozygote will elevate the pigment and mRNA levels of *white* structural gene alleles about 2-fold in diploid males and females [Rabinow et al., 1991]. The region where the gene mapped produced an inverse effect on the *white* alleles when the small region was changed in dosage. When the *Inr-a* mutation was combined with lesions in the 5' regulatory region of the *white* gene, the target *white* gene failed to respond in contrast to the *white* alleles with an

intact regulatory region. When *Inr-a* mutations were combined with a *white* promoter-*Adh* reporter, the mutations caused about a 2-fold increase in the expression of ADH in enzyme assays. Thus, in this case the promoter of *white* was found to be necessary and sufficient to mediate the inverse effect onto the *white* gene expression. Examination of other genes found that *Inr-a* also affected the expression of the *scarlet* and *brown* loci, which are also involved in eye pigment expression.

Many other single gene dosage-sensitive modifiers of the *white* eye color gene were recovered and characterized [e.g., Bhadra et al., 1997a, b]. A summary of the 47 *white* eye color modifiers that were eventually identified revealed that they were involved in a heterogeneous collection of molecular functions but all with some relationship to gene regulation, being transcription factors, signal transduction components, or chromatin proteins [Birchler et al., 2001]. The observation of dosage sensitivity was consistent with the emerging findings that many developmental regulators in *Drosophila* are dosage sensitive [Driever and Nüsslein-Volhard, 1988; Struhl et al., 1989; Warrior and Levine, 1990; Sauer and Jäckle, 1991; Schulz and Tautz, 1994; Cribb et al., 1995] and genes modifying position effect variegation are also dosage sensitive [Henikoff, 1996].

Global Studies of Gene Expression

The examples above involved the study of single genes and their implication in genomic imbalance. When the molecular tools became available, it was of interest to examine how the genome as a whole reacted to imbalance. Larvae of *Drosophila* trisomic for the X chromosome (metafemales) were examined via RNA-seq [Sun et al., 2013a], and ratios were produced between the levels in metafemales and normal females for each expressed gene. When ratios were plotted in a distribution (see Fig. 3 for a description of ratio distribution analysis), for the X chromosome, the major peak of the distribution surrounded the ratio of 1.00, which indicates no change or, in other words, dosage compensation. There was also a small peak surrounding 1.50, which indicates that a subset of genes exhibit a proportional gene dosage effect. For autosomal genes, there was a spread of ratios but the major peak was at 0.67 indicating an inverse dosage effect of the triple X on a large fraction of autosomal genes. Ratio distributions were also examined comparing normal males versus females. The X chromosomal distributions showed the major peak approaching the level for dosage compensation between the sexes but a substantial fraction was at 0.50 exhibiting a gene dosage effect in males. The autosomal gene ratio distribution surrounded 1.00. A further discussion of these results is given below.

By way of comparison, RNA-seq was performed for trisomy for the left arm of chromosome 2 (2L) in *Drosophila* larvae compared to the normal diploid for both males and females [Sun et al., 2013c]. The expression of genes on 2L was mostly centered around the value of 1.00 indicating dosage compensation for a major subset, but there was also a subset that exhibited a proportional gene dosage effect. Genes on other autosomes showed a spread of effects with the major peak at the inverse level in the trisomy compared to the normal of the same sex. Interestingly, genes that showed sex-biased expression between males and females showed a greater modulation by trisomy for 2L, suggesting that sex-biased expression is mediated by dosage-sensitive balance interactions. Also of interest, the genes on the X chromosome responded in a significantly different distribution between males and females although both showed a major inverse response. A study of global expression in a collection of small heterozygous deficiencies along 2L revealed examples of proportional gene dosage effects and compensation as well as modulations across the genome [Malone et al., 2012]. The generalized similarity between the X and 2L large trisomies (each ~20% of the genome) illustrates a related

mechanism between sex chromosomal and autosomal dosage compensation.

A study in *Arabidopsis* examined global gene expression for all 5 trisomies compared to the diploid and to a ploidy series consisting of diploid, triploid, and tetraploid plants [Hou et al., 2018]. For each trisomy there was a spread of gene expression for the genes on the varied chromosome from dosage compensation to a proportional dosage effect. For the genes on other chromosomes, not varied in dosage, the major effect was an inverse response for a substantial subset of genes. The range of effects was much greater in the trisomies compared to the ploidy series demonstrating that genomic imbalance upsets gene expression more than a balanced ploidy series.

Other aspects of this study found that DNA methylation could be altered by trisomy, which could have an impact on gene expression by extension, but the modulations of expression were not necessarily based on the status of the gene methylation [Hou et al., 2018]. Also, in example cases of the co-expression data of transcription factors and their target genes, the response between the two in trisomy was discordant in the direction of the effect for about half of the cases, illustrating how genomic imbalance can alter this relationship. Also, genes associated with the Polycomb complex, which typically is involved with gene silencing, could also be modulated by trisomy.

This study [Hou et al., 2018] also re-examined global gene expression in a set of disomic yeast lines compared to the normal haploid from a previous study. The original data normalized the expression of the genes on the varied chromosome to the remainder of the genome [Torres et al., 2007], which, if there is an inverse effect operating, will produce a greater degree of apparent proportional gene dosage effects than in reality and will obscure the presence of the *trans*-acting inverse effect across the genome (see further discussion below). Similar data from mouse trisomic cells [Williams et al., 2008] were reanalyzed without this previous type of normalization. Together it was revealed that the inverse effect is realized in yeast disomies and mouse trisomies as it is in aneuploids of other organisms [Hou et al., 2018], tracing back to the phenotypic studies of anthocyanin pigment by Blakeslee 100 years ago.

The biology and genetic tools available in maize allow the production of a much broader spectrum of aneuploids than in any other multicellular eukaryote [Carlson, 1988]. Most chromosome arms can be examined in 1–4 doses in diploids as well as 1 and 2 doses in haploids. Extensive ploidy series can also be produced. In a study of a

collection of disomic haploids compared to normal haploids for 17 chromosome arms covering about 80% of the genome, an inverse effect was realized in all of them for a subset of the genes across the genome [Yang et al., 2021]. As evidenced in the classical literature, disomic haploids provide a greater imbalance for analysis than trisomic diploids [Satina et al., 1937]. Comparing across all regions, when there was a greater number of genes showing an inverse effect, the genes on the varied chromosome arm had a greater number that trend toward dosage compensation, showing on a global scale the relationship between the inverse dosage effect and dosage compensation for genes on the varied regions. A few disomies also showed a subset of genes that were increased in expression across the genome resulting in both positive and negative modulations being produced by a single disomy.

RNA-seq analysis will only determine the relative gene expression and does not alone produce an absolute measure of the expression per cell [Lovén et al., 2012]. The study of Yang et al. [2021] also determined the transcriptome size in the disomy collection by comparing mRNA levels to genomic DNA of the same genes in total nucleic acid preparations as previously proposed [Coate and Doyle, 2010]. Some chromosome arms showed a slight increase in the transcriptome size but the most common effect, when there was a change, was an inverse effect on the total transcriptome size. The regions with the greatest reduction in transcriptome size were also the disomies that were most detrimentally affected phenotypically [Yang et al., 2021].

In a related paper, 1–3-dose series for 14 regions in diploids showed that the inverse effect is the most common global effect, but reductions in both monosomy and trisomy or increases in both are common [Shi et al., 2021]. In some cases, it was possible to examine a 1–4-dose series for selected chromosome arms. In general, the effects become stronger in the tetrasomies compared to the trisomies [see also, Johnson et al., 2020]. In comparing the haploid disomy and diploid trisomy data, the inverse and positive effects are stronger in the former in which the genomic imbalance is greater. In parallel, a ploidy series of haploid to tetraploid showed much fewer disturbances of gene expression illustrating that gene expression in balanced genomes is much less upset globally.

In the hyperploids of both ploidies the most common response across the genome was an inverse effect, but in the monosomies, many regions also have a positive effect on a subset of genes across the genome [Shi et al., 2021]. In other words, both types of aneuploids reduce the global expression of a subset of genes. In the next section, we

Fig. 4. Heuristic simplified example of how the stoichiometry of multicomponent interactions can be affected by differential quantities of the bridge component relative to its interactors. Imagine a complex required for a cellular function consisting of blue (B), green (G), and red (R) subunits. At the top left, B, G, and R components in a similar 1/1/1 relationship will proceed to completion of the BGR complex. However, at the top right is shown a situation in which the G component is present in a 3/2 relationship (e.g., as might occur in a trisomy). In this case, partial complexes are formed and the complete complex is reduced in quantity to an inverse relationship to the stoichiometry of the components. At the bottom left, the 1/1/1 relationship is repeated. At the bottom right, the effect of reducing the bridge component G to half is shown (e.g., as might occur in a monosomy). In this case, the completion of the complex is limited by the quantity of the bridge component. Thus, for multicomponent interactions, the complete complex can be reduced by both over- and underexpression of bridge components. In analogy, aneuploidy of both increased and decreased chromatin is detrimental to the organism.

will discuss how this finding is consistent with limiting and “trans-dominant negative” effects of bridge molecules in multicomponent interactions.

Haploinsufficiency, Overexpression, and Subunit Stoichiometry

The heterogeneous nature of the dosage-sensitive genes became unified from modeling and experimental work that indicated that contributors to multicomponent interactions would be dosage sensitive based on the kinetics of assembly, the topology of the complexes, and their assembly routes [Veitia, 2002, 2003, 2004; Kondrashov and Koonin, 2004]. Modeling of the assembly of multi-subunit complexes [Bray and Lay, 1997; Veitia, 2002] suggests that peripheral subunits are limiting at low con-

centrations as they prevent completion of the whole complex. Their excess is irrelevant from the point of view of the formation of the complex but might lead to spurious protein-protein interactions (PPI) [Vavouri et al., 2009]. However, a decrease of their quantities when they are represented several times in the complex (i.e., AaBbCc) can lead to more-than-proportional decrease of the whole [Veitia, 2002, 2010]. Bridge subunits that interact with multiple other subunits, on the other hand, will be limiting at low concentration, proceed through a peak and then cause a decline at higher concentrations because partial subunits would be produced that cannot proceed to completion of the whole. Thus, both under- and overexpression of bridging components can be detrimental showing a semidominant effect (overexpression *trans* dominance) (Fig. 4). This parallels typical aneuploid effects at the monosomic and trisomic levels [Lee et al., 1996a, b; Sheridan and Auger, 2008; Birchler and Veitia, 2012; Brunelle and Sheridan, 2014] and at the molecular level [Birchler and Newton, 1981; Guo and Birchler, 1994; Shi et al., 2021].

An experiment in yeast examined the fitness of heterozygous deletions of essential genes and found that when they encoded subunits of multimeric complexes, the subsequent fitness defects were stronger [Papp et al., 2003]. Gene overexpression studies in yeast showed that those with the greatest fitness consequences tend to encode proteins involved in multicomponent complexes and those with many physical interactions [Makanae et al., 2013; Robinson et al., 2021]. Schuster-Bockler et al. [2010] found that there was a negative correlation between the number of PPI domains encoded in genes with their frequency found in copy number variants in human populations, suggesting a negative influence from dosage change for genes with such protein interactions. In yeast and fruit fly populations, Lemos et al. [2004] found a negative correlation between the number of protein interactors of a gene and the variation in expression. Together these studies illustrate that changing the dosage of highly connected genes has detrimental fitness consequences.

While the kinetics of assembly of multisubunit complexes has been modeled and can serve as an explanation of the types of gene expression alterations found with genomic imbalance, it is possible that the same type of phenomenon would apply to any multicomponent interactions in which different components must assemble to regulate various cellular functions. For example, it is conceivable that DNA might serve as one component for the stochastic binding of multiple regulatory molecules for which the stoichiometry of the components impacts the

eventual outcome [Veitia et al., 2008]. The effect of rates of synthesis versus steady state levels of complexes and how that affects the function of the whole is an underexplored area of investigation. Regulatory stoichiometry that has an impact on morphology has been documented in some examples in plants [Chen et al., 2010; Stahl et al., 2013; Clark et al., 2020]. These examples illustrate a connection between regulatory balance and the phenotypic effects of imbalance that trace back to the pioneering studies of Blakeslee and colleagues. This connection is further discussed below with regard to quantitative traits.

An examination of protein complexes in mammalian cells indicated that there is, in general, a correlation between the mRNA and the protein levels of individual subunits suggesting a selection during evolution for co-expression [Ori et al., 2016]. However, posttranscriptional and protein turnover are also involved in determining the stoichiometry of subunits. Further, macromolecular complexes can have a shifting composition of subunits. Together these considerations illustrate the intricate nature of stoichiometry or balance to multicomponent function. The phenomenon of overdosage in yeast [Chen et al., 2019], in which different balanced complexes are out of register with others has a detrimental effect on growth rate, illustrates the layers of balance that operate in a cell.

Whole-Genome Duplication and Balance

As sequenced genomes increased in number, it has become obvious that many eukaryotes have experienced multiple rounds of whole-genome duplication (WGD) [Wolfe and Shields, 1997; Simillon et al., 2002; Maere et al., 2005; Aury et al., 2006; Blomme et al., 2006; Thomas et al., 2006; Freeling, 2009; Jiao et al., 2011]. The species span such examples as yeast, protozoa, vertebrates including human, and especially numerous examples in the plant kingdom. A reoccurring theme in these studies is that following the WGD, genes are deleted back to the singleton state (Fig. 5) with exceptions depending on the type of gene function. The exceptions include those genes that are involved with multicomponent interactions and that would be predicted to be dosage sensitive. These genes are retained for longer periods of evolutionary time than other classes of genes [Blanc and Wolfe, 2004; Maere et al., 2005; Freeling and Thomas, 2006; Chang et al., 2010; Makino and McLysaght, 2010; McGrath et al., 2014; Li et al., 2016]. The implication is that if one member of the duplicate pair becomes mutated or deleted,

Fig. 5. Gene numbers from multiple whole-genome duplications (WGD) over evolutionary time. Starting at a baseline of the blue line, a WGD will double the number of total genes. Over time, genes are deleted to reduce the total number with retention of some classes. A second WGD will double all genes again, which is followed by gene deletion. A third WGD will double the total number of genes, which is followed by gene deletion. Selected gene retention and some cases of sub- or neofunctionalization will increase the total gene number over time.

there is a negative fitness consequence, and the individuals carrying the deletion are selected against. In other words, this circumstance would mimic an aneuploid effect to the detriment of the individual [Birchler et al., 2005]. Thus, the duplicate pair of dosage-sensitive genes would be preserved for longer periods than other classes of genes [Birchler et al., 2005]. It is also the case that dominant-negative mutations in members of macromolecular complexes can have a similar effect on the active amount of the complex as do dosage alterations [Veitia, 2010].

With the example of the model plant species *Arabidopsis*, there are 3 easily recognizable WGDs (but also more ancient ones) with receding time from the present. The number of retained duplicate genes from each WGD continues to decline with increasing time into the past (Fig. 6). This fact indicates that over time new effective balances are achieved among interactors that permit the eventual progression to singleton state. As time elapses from a WGD, mutations can accumulate in the duplicate pair. Even though the expression levels of individual members of the pair might vary, compensatory changes, as modeled [Thompson et al., 2016], might maintain a functional total level from all members.

An example of the expression of the flowering gene, *FLC*, in polyploid *Brassica* illustrates this principle, where the total *FLC* expression is maintained despite the divergence of the individual contributors to that total [Calderwood et al., 2021]. It is important to note that divergence of expression levels between duplicate members of a pair

Fig. 6. Typical decline in the number of retained duplicate genes following multiple whole-genome duplications (WGDs). In genome sequences that show evidence of multiple WGDs, the numbers of duplicate genes retained from a particular WGD become fewer and fewer with increasing time from the present. The retained duplicates are typically those classes of genes that are involved with multicomponent interactions. The fact that the number of retained duplicates from each WGD will become fewer with time suggests that new functional balances allow the interacting genes to become singletons as new functional balances evolve.

is not necessarily an indication of a change in function or deviation from a critical balance [Lan and Pritchard, 2016]. Also, the expression levels that are most critical could be specific to cell type, in plants particularly in the haploid gametophyte generation, while different expression levels of duplicates in other cell types could be essentially neutral [Conant et al., 2014]. Over time, new effective balances can evolve.

In mammals, protozoa, and yeast, the expression levels of duplicated genes can diverge from each other with the total expression being reduced [Qian et al., 2010; Gout and Lynch, 2015]. In these cases of degeneration of the gene copies, there is retention of both to supply sufficient total function. The singleton state can eventually be achieved with the deletion of the more lowly expressed copy [Gout and Lynch, 2015].

As noted above, a gene overexpression study in yeast found the most detrimental effects were for genes encoding members of multicomponent complexes [Robinson et al., 2021]. In that study, the overexpression was conducted in several natural genetic backgrounds with great variation found for the individual gene fitness detriments. Although such variation could be attributed to many factors, it is likely that one contributor is differences to some degree in the level of individual components that would generate subtle changes in balance that could facilitate the

emergence of altered functional stoichiometries over evolutionary time.

When the WGD involves distinct genomes to produce an allopolyploid, one genome is often expressed at a higher overall rate than the other, a phenomenon referred to as genome dominance [Wang et al., 2006; Flagel et al., 2008; Garsmeur et al., 2014; Renny-Byfield et al., 2015]. Analyses of the fractionation (deletion of one copy of the duplicate genes) process in these cases indicates that genes are lost more often from the genome that is expressed at a lower level [Thomas et al., 2006; Woodhouse et al., 2010; Edger et al., 2017]. The deletion of genes from the more lowly expressed genome are less likely to be subjected to purifying selection from an upset of the balance because of their lower overall contribution to the gene expression pool [Thomas et al., 2006; Schnable et al., 2011].

In addition to specific types of protein-encoding genes that are retained in duplicate for longer periods of time than others, microRNA (miRNA) genes are also often retained in duplicate [Zhang et al., 2009; Abrouk et al., 2012; Desvignes et al., 2021]. miRNAs are dosage sensitive [Guo et al., 2010] and target mRNAs as a means of regulation of protein quantities. Because of this connection, it is reasonable to suggest that they are constrained with regard to total expression in balance with their targets and thus would follow a similar type of retention over time. Conserved miRNA targeting in humans is involved with dosage-sensitive genes [Naqvi et al., 2018]. This fact raises the interesting unexplored idea that individual regulation of separate components of multicomponent interactors at multiple levels might also be constrained in a quantitative manner.

Balance, Hypofunctionalization, Subfunctionalization, and Neofunctionalization

The divergence of function of duplicate genes is an important source of new genes, as has long been recognized [Lewis, 1951; Stadler and Emmerling, 1956; Ohno, 1970; Lynch and Conery, 2000]. When duplicate genes split functions, this phenomenon is referred to as subfunctionalization. When one member of the pair takes on a new function, this is referred to as neofunctionalization. Hypofunctionalization is the reduction of the expression level of both members of a duplicate pair as might occur by random mutation, thereby becoming dependent upon each other [Qian et al., 2010; Gout and Lynch, 2015; Veitia, 2017]. Subfunctionalization and neofunctionalization

will hold a duplicate pair as such in perpetuity preventing the progression to the singleton state. Gene balance can also cause duplicate gene retention for dosage-sensitive genes for longer periods of time than other classes of genes. In general, selection for retention of highly networked, dosage-sensitive duplicate genes is expected to be stronger than for genes encoding poorly connected products. The former genes are also the most likely to have a strong effect on the phenotype. Therefore, their longer retention as duplicates provides increased opportunities for evolutionary innovation through mutation of one of the members of the pair. Accordingly, balance principles can explain the longer retention time of genes involved in multicomponent interactions, but eventually functional balances can evolve such that they can return to a singleton state. Retention of duplicates by balance will decay over time but sub- and neofunctionalization is forever. Thus, the decline in the number of retained duplicates in successive WGDs going back in time in a genome is a signature of decaying balance retention, because the duplicates with sub- or neofunctionalization would remain as duplicates. The fact that singletons can vary in quantitative or tissue-specific expression in a population illustrates that when this occurs between members of a duplicate pair of genes, it is not necessarily hypo-, sub-, or neofunctionalization. This fact illustrates the difficulty in deciding the basis of retained duplicates. However, it is important to note the connection among the various possibilities because a substantial fraction of the retained genes is involved with various regulatory and developmental processes. Thus, genomic balance maintains the very genes in duplicate for longer periods of evolutionary time that are critical for evolutionary novelty allowing that novelty more time to emerge [Birchler et al., 2005; Birchler and Veitia, 2012; Gout and Lynch, 2015].

Small-Scale Duplication

The fate of gene pairs from small-scale duplication is the complement to that of WGD with genes involved with multicomponent interactions being underrepresented. In this case, there would also be a mimic of an aneuploid effect in that such interacting genes would have a detrimental effect when present in extra copies. An examination of duplicate genes from WGD versus small-scale duplication fulfills these predictions [Veitia, 2004; Hakes et al., 2007; Freeling et al., 2008; Korbel et al., 2008; Ionita-Laza et al., 2009; Rodgers-Melnick et al., 2012; Tasdighian et al., 2017]. Nevertheless, small-scale duplications that

confer a strong selective advantage or a new function will be retained.

Retained WGD duplicates show dosage balance constraints on divergence at the sequence and expression level compared to small-scale duplicates. In a study of *Arabidopsis*, tomato, and maize [Defoort et al., 2019], the sequence and expression level of duplicates from WGD were more constrained than for duplicates from small-scale duplication. The PPI data from *Arabidopsis* were used to examine the impact on evolutionary constraint. For both WGD and small-scale duplicates, those with PPI tend to evolve more slowly than those without such interactions. In all 3 species studied, duplicates from WGD showed an overrepresentation with PPI but an underrepresentation in tandem duplications. This study illustrates the role of PPI in dosage sensitivity and the differential retention of classes of genes between WGD and small-scale duplication.

A study in *Lotus* examined the fate of duplicate genes following the single WGD present in this species, a fact that simplifies such studies [Shi et al., 2020]. The genes retained from the WGD were enriched for those involved with transcriptional processes and signaling whereas those at the singleton state were enriched in metabolic functions and others that are not dosage sensitive. The retained duplicates were more heavily involved with PPI, followed similar patterns of coexpression, and had a greater level of sequence conservation than small-scale duplicates, illustrating selective constraints. There is, not surprisingly, a gradient of divergence among the retained duplicates with the greater degree of divergence in function corresponding to those with a lesser degree of connectivity (i.e., number of interactors). These results illustrate the importance of PPI and connectivity in retention of duplicate pairs as well as the spectrum involved that will allow divergence.

Speciation

It is possible that gene balance can mediate speciation in some cases [Birchler et al., 2005; Birchler and Veitia, 2019]. Speciation can emerge at many levels including mating behavior and other pre-mating processes [Coyne and Orr, 2004]. However, division of a population will allow divergence, and when the subpopulation members come together again and intermate, sterility or lethality can often occur as a “hybrid” incompatibility [Dobzhansky, 1937; Muller, 1942; Orr and Turelli, 2001]. Specifically, new functional balances can evolve in isolated sub-

populations, whose combination in a hybrid might not operate correctly [Tirosh et al., 2009; Walia et al., 2009]. Different aneuploid configurations can cause sterility or lethality with only a 1/2 or 3/2 dosage differential, so shifts in balance in subpopulations could produce such a level of divergence, which could mimic an aneuploid effect when brought back together in hybrids. Indeed, in the aforementioned overexpression study in yeast [Robinson et al., 2021], there were many differences in various backgrounds, illustrating extensive variation for the degree of fitness consequences that can exist in populations. Numerous examples of postzygotic hybrid incompatibilities [Matute and Gavin-Smyth, 2014; Guerrero et al., 2017] exist, so a fruitful direction of investigation would be to interrogate these examples from the perspective of gene balance.

Endosperm Balance and Speciation

Aneuploidy and dosage effects in general can have an effect on basically any cell type or tissue [Sinnott et al., 1934]. It is reasonable therefore to suggest that the same should be true for the triploid endosperm in plants. However, using the example of the endosperm of maize, some different aspects appear to operate [Birchler and Hart, 1987; Birchler, 1993].

Using B-A translocations as described above, endosperms that are missing certain paternally derived regions of the genome are smaller than normal [Birchler and Hart, 1987]. Lin [1982] found that for a region of chromosome 10, the introduction of extra copies from the female parent would not rescue this phenotype. The interpretation given to this result was that the responsible genes are parentally imprinted such that they did not function from the female parent but were active from the male parent. Thus, when missing from the male parent, a defective phenotype would result. However, in other backgrounds, introduction of an extra copy of this chromosome from the female parent will actually make the lack of a male contribution even worse [Birchler and Hart, 1987; Birchler, 2014]. Similarly, extra copies of one genomic region from the female parent will also make the small kernel effect from paternal absence for other regions an even smaller kernel [Birchler and Hart, 1987]. The full collection of B-A translocations covers the vast majority of the genome but no region is lethal when missing in the endosperm from the male parent. Thus, if there are imprinted genes that are expressed only from the male parent, none of them are vital. There is in fact an interacting dosage ef-

fect between female and male contributions to the endosperm that appears to be of most consequence at the initiation of endosperm development [Birchler, 1980, 2014]. Spontaneous changes in dosage later in development show no autonomous detrimental effect in the sectors missing a chromosomal segment [Birchler, 1980] or when the ploidy is changed during development [Birchler, 2014].

Crosses between diploid and tetraploid lines in angiosperms in most cases will result in endosperm failure [Birchler, 1993], which has also been attributed to parental imprinting [Lin, 1984]. Haig and Westoby [1989] postulated that parental conflict for resources for progeny was the driving evolutionary force for imprinting. Maternal parents were suggested to evenly distribute resources to all their progeny and different paternal parents would seek an advantage for their own progeny against the others. Some ecotypes of *Arabidopsis* will produce viable seeds with interploidy crosses in which diploid females by tetraploid males produce larger seeds in apparent support of this concept [Scott et al., 1998], but there appears to be great variability for this trait [Dilkes and Comai, 2004]. In most angiosperms, reciprocal crosses between diploid and tetraploid lines cause endosperm failure in both directions [Birchler, 1993] rendering resource distribution irrelevant, even though imprinting could be involved in the developmental process.

The available evidence suggests that a stoichiometric interaction between regulatory factors from the female gametophyte with the dosage of the complement resulting from fertilization of the central cell is responsible for the effects [Birchler, 2014]. Imprinting is a non-mutational means to modulate the level of a gene product [Beaudet and Jiang, 2002] and thus such genes can be involved in endosperm development [e.g., Kradolfer et al., 2013; Martinez et al., 2018], but it is likely that dosage-sensitive biparentally expressed regulatory genes also contribute to the maternal/zygotic interaction as occurs in other tissues. The interploidy hybridization barrier is an effective speciation mechanism given that it will block gene flow between tetraploids and their diploid progenitors for many plant species [Birchler, 1993].

Nuclear/Organelle Stoichiometry

The mitochondria and chloroplasts are organelles in eukaryotes that possess their own DNA and carry out cellular functions with a combination of gene products from the organelle itself and from the nucleus. A collection of

chromosome arm dosage series in maize was examined to assess expression of nuclear and mitochondrial genes. Interestingly, the mitochondrial encoded genes were also modulated by various aneuploid regions of the nuclear genome [Auger et al., 2001], illustrating that their regulation can be affected by nuclear gene dosage. In mice and nematodes, the nuclear-mitochondrial imbalance triggers the “unfolded protein response” in mitochondria, which results in lifespan extension [Houtkooper et al., 2013].

In a study of nuclearly encoded plastid genes in soybean, *Medicago*, and *Arabidopsis*, genes encoding photosystem components are preferentially retained following WGD and are underrepresented in small-scale duplications similarly to cases described above [Coate et al., 2011]. In contrast, genes for the Calvin cycle and the light-harvesting complex do not follow this pattern. Given the importance of organelles to metabolism, further exploration of these relationships is an important research direction.

Sex Chromosome Evolution and Balance

Heteromorphic sex chromosomes have evolved multiple times in the plant and animal kingdoms [Bachtrog et al., 2014; Vicoso and Bachtrog, 2015; Furman et al., 2020]. A typical scenario is that one of a pair of homologues becomes restricted for recombination along part of its length, often by inversions, to hold together alleles that foster the sex determination mechanism. Genes then become deleted from one member of the pair of homologues that becomes the sex chromosome that is restricted to one of the 2 sexes. The other member of the chromosomal pair can recombine in the opposite sex and is present in both sexes. With the continued deletion of genes, a genotype similar to an aneuploid would occur. Different mechanisms operate to prevent any potential detrimental effects of this situation. In cases in which the degenerating sex chromosome is found in males, the sex chromosomes are referred to as X and Y, but when it is present in females, the chromosomes are designated as Z and W. Below we consider 2 examples of how the evolution of heteromorphic sex chromosomes has accommodated issues of genomic balance, namely, in eutherian mammals and in *Drosophila melanogaster*. However, in any sex chromosome situation, changes of dosage-sensitive genes must be accommodated in some way.

Mammalian Sex Chromosomes

The situation in most mammals involves X and Y chromosomes, with X inactivation in the female [Disteche, 2012]. The X inactivation system is typically referred to as a dosage compensation mechanism between males and females but not between the X and the autosomes [Pessia et al., 2012]. The latter would be achieved by upregulating the expression of the active X in both sexes. Whether the single X shows any increase of expression of critical genes has been debated but seems likely at least to some degree [Disteche, 2012].

However, 2 recent revelations factor into an understanding of the sex chromosome dosage considerations. Comparison of the genes that are retained between the mammalian X and Y chromosomes indicates that dosage-sensitive regulatory genes are preferentially retained on both chromosomes while other classes of genes are lost from the Y [Bellott et al., 2014, 2017]. The role of miRNA in sex chromosome evolution has also been investigated [Naqvi et al., 2018]. In some respects, the maintenance of regulatory factors is reminiscent of the retention of dosage-sensitive regulatory genes following WGD as repeatedly found in diverse eukaryotes, as described above. Indeed, in a comparison of mammals, birds, and snakes, different homologous pairs of chromosomes have evolved into the sex chromosomes in the respective taxa, and in each case there is preferential retention of dosage-sensitive regulatory genes [Bellott and Page, 2021]. Thus, the balance principle of gene retention also applies to sex chromosome evolution in these cases.

The second revelation is that a dosage series of human sex chromosomes from a single copy (X0, Turner syndrome), to 2 copies in male (XY) and female (XX), to 3 copies (XXY, Klinefelter syndrome) can modulate the expression of genes across the genome [Raznahan et al., 2018; Zhang X et al., 2020]. The X and Y chromosomes share segments in common, namely the pseudoautosomal regions, which will be varied in dosage in these genotypes although some genes outside these regions also exhibit a proportional dosage effect (i.e., those escaping X inactivation). Along the X chromosome, these modulations are diverse owing to X inactivation, but on the autosomes, the most common modulation is a negative or inverse correlation with the dosage of the sex chromosomes. Examination of gene regulatory networks provided candidate genes responsible for these effects. Integrating observations of X inactivation, retention of dosage-sensitive regulatory genes, and the dosage effect across the genome of the sex chromosomes is a valuable direction of investigation.

Dosage Compensation in Drosophila

In *Drosophila*, the sex chromosomes consist of an X and a Y. The Y in this species contains only a few genes required for male fertility [Zhang J et al., 2020]. The X consists of approximately 20% of the genome. Thus, females have 2 X chromosomes and males have one. Muller [1932] found that the expression of genes between the 2 sexes was similar, but when individual genes on the X were varied in dosage, they exhibited a proportional dosage effect. Each copy in a male was expressed at about twice the level as each copy in a female. Females with 3 X chromosomes are highly inviable, but when they do survive to the adult stage, Stern [1960] found that they too exhibited dosage compensation in that the expression in the triple X genotype was similar to normal females and males. Studies in triploid flies found that dosage compensation also occurred among X;AAA, XX;AAA, and XXX;AAA genotypes via upregulation of the Xs, where X indicates the dosage of the X and AAA indicates the triploid nature of the autosomes [see Birchler, 2016 for further discussion]. Diploid and triploid females have a similar per gene expression but the other 5 genotypes each must modulate gene expression by different amounts equivalent to an inverse relationship between the X versus autosomal dosage to achieve a balanced expression between the X and the A [Birchler, 1996].

Some years ago, mutations were sought in *Drosophila* that were lethal to males but viable in females. A number of such mutations were found and termed male-specific lethal (MSL) loci [Belote and Lucchesi, 1980]. Once the products of these genes were identified, they were found to be at high concentration on the male X chromosome [Kuroda et al., 1991]. One of these genes encodes a histone acetyl transferase that acetylates lysine16 of histone H4 [Bone et al., 1994; Hilfiker et al., 1997]. The recognition of the MSL complex and its association with the male X was an important set of contributions to an understanding of sex chromosome evolution in *Drosophila* [Kuroda et al., 2016]. Thus, the hypothesis arose that the MSL complex accumulated this chromatin modifier on the male X to activate the expression about 2-fold to bring about dosage compensation. Other data that contributed to this hypothesis were that when autoradiography of nascent transcription on salivary gland polytene chromosomes was examined in the MSL *maleless* mutation, the ratio of the number of grains over the X versus an autosomal region was reduced compared to the normal ratio [Belote and Lucchesi, 1980].

It has been pointed out [Hiebert and Birchler, 1994; Bhadra et al., 1999; Bhadra et al., 2005; Sun et al., 2013a;

Fig. 7. Illustration of how a change in transcriptome size can affect the interpretation of RNA-seq experiments. **a** The expression of genes on the varied chromosome (*cis*) versus the remainder of the genome (*trans*) in the normal condition is shown in blue. If in a monosomy (shown in red) the expression of genes on the *cis* chromosome shows generalized dosage compensation and the genes in *trans* are in general inversely affected, the transcriptome size would be nearly doubled. **b** If RNA-seq is conducted on the situation shown in **a** without normalization to a per cell value or DNA, an apparent proportional gene dosage effect would be found for the *cis* genes and a slight increase would be found for the remainder of the genome (*trans*). This is the case because sampling the same amount of RNA from an experimental sample with an altered

transcriptome size compared to the normal control will only reveal the relative relationship of transcripts but not the absolute relationship [Lovén et al., 2012]. **c** The expression of genes on the varied chromosome (*cis*) versus the remainder of the genome (*trans*) in the normal condition is shown in blue. If in a disomic haploid (shown in red) the expression of genes on the *cis* chromosome shows a generalized dosage compensation and the genes in *trans* are in general inversely affected, the transcriptome size would be nearly halved. **d** If RNA-seq is conducted on the situation shown in **c** without normalization to a per cell value or DNA, an apparent proportional gene dosage effect would be found for the *cis* genes, and a slight decrease would be found for the remainder of the genome (*trans*).

Birchler, 2016], however, that the grain counts over the X and the autosomes when considered in absolute terms rather than as a ratio show that the X more or less retained dosage compensation and the autosomal numbers are about doubled, i.e., exhibit an inverse dosage effect. The same situation occurs with the *msl3* mutation [Okuno et al., 1984], another MSL gene, in that the autoradiographic grain numbers as an absolute measure show retention of X dosage compensation and an autosomal increase. The data treatment to produce an X-to-A ratio seemed reasonable in the context of the time when all regulation in eukaryotes was considered positive. However, when studies were conducted in such a manner as to assay the absolute effect on gene expression in the *maleless* mutation, in adults [Hiebert and Birchler, 1994], larvae [Hiebert

and Birchler, 1994; Bhadra et al., 1999, 2000; Bhadra et al., 2005], and embryos [Bhadra et al., 2005], the trend was for retention of dosage compensation of X-linked genes and an increase in expression of autosomal genes.

In global studies, if the X-to-autosomal expression does not change between mutant and normal, one can conclude that dosage compensation is operating. However, a lower X-to-A ratio could be a loss of compensation or an increased transcriptome size from the increase of the autosomes, as suggested by the absolute data of Belote and Lucchesi [1980] and Okuno et al. [1984], and that was visualized directly in embryos [Bhadra et al., 2005]. The transcriptome size in aneuploids can change, which cannot be detected in RNA-seq analyses alone [Yang et al., 2021]; so it is an important consideration for studies of

dosage compensation. It was recently acknowledged that in MSL2 knockdowns the relative X expression is decreased and a moderate autosomal increase is found [Valsecchi et al., 2021b]. A similar trend is evident in the microarray data on MSL2 knockdown in SL2 tissue culture cells [Hamada et al., 2005] and in the roX RNA (noncoding RNAs essential for MSL localization on the male X) mutants [Deng and Meller, 2006]. This trend is in fact consistent with the absolute data that suggest an increase in the transcriptome size (Fig. 7). A global change in transcriptome size can cause a generalized dosage compensation of a single chromosome to appear as a proportional dosage effect (loss of compensation) to some degree (Fig. 7).

Examination of expression in triple X metafemales, where there is no MSL complex, found that X-linked genes generally show dosage compensation, but there is a generalized inverse effect on the autosomes which could account for the detrimental effects of metafemales [Birchler et al., 1989; Sun et al., 2013b]. An examination in metafemales of alleles of the *white* eye color gene that do or do not exhibit dosage compensation in males, showed the same response, namely, increased expression if there was no dosage compensation in males, and no difference from normal females for the male dosage-compensated alleles [Birchler, 1992]. This experiment demonstrated a relationship between the mechanism of dosage compensation in males and metafemales and implicated the inverse dosage effect in both. An involvement of the inverse effect in *Drosophila* dosage compensation provides an explanation for how the 5 levels of gene expression can be explained [Birchler, 1996], whereas an all-or-none presence of a complex on the X does not. The stoichiometric relationship of regulatory gene products provides a means to read the relative dosage at multiple doses to produce the appropriate inverse level.

What then is the function of the MSL complex? It was discovered that the MSL complex will actually constrain the effect of H4 lysine16 acetylation to prevent it from causing an overexpression of genes [Bhadra et al., 1999, 2000; Bhadra et al., 2005; Sun and Birchler, 2009; Sun et al., 2013a]. Indeed, in the chromatin remodeling imitation switch (ISWI) mutants, the male X chromosome was specifically overexpressed suggesting that its normal function is needed for the constraint to occur [Bhadra et al., 2005]. Independently, the Mtor nuclear pore component shows a similar specific male X upregulation when Mtor is knocked down, and this effect was shown to operate on the transcriptional level [Aleman et al., 2021]. Interestingly, the increased expression of the X in the Mtor

knockdown is accompanied by an inverse modulation of a substantial subset of autosomal genes, illustrating why the constraining process evolved to counteract male X overexpression. The constraining effect might involve a number of gene products [Birchler, 2016] and is an interesting direction for further study.

Furthermore, when the H4Lys16 acetylase was individually targeted to reporter genes, there was an increase in expression [Sun et al., 2013a]. However, when other members of the MSL complex were present in normal males or ectopically expressed, this increase did not occur [Sun et al., 2013a]. It thus appears that the function of the MSL complex is to sequester the acetylase from the autosomes and constrain it on the X [Bhadra et al., 1999, 2000; Bhadra et al., 2005; Sun and Birchler, 2009; Sun et al., 2013a]. Indeed, in the Mtor knockdowns, the X-linked genes with the greatest upregulation were those with the greatest association with the acetylase and the H4Lys16ac histone mark [Aleman et al., 2021], supporting the idea of a constraining activity that becomes released in the Mtor knockdown. When the MSL complex is disrupted, there is an inverse effect on the autosomes that occurs, but in normal circumstances the sequestration mutes this effect. The MSL complex disruption releases the acetylase to be uniformly distributed across all chromosomes as determined cytologically [Bhadra et al., 1999] or via ChIPseq for H4Lys16ac [Valsecchi et al., 2021a]. Indeed, the quantity of the H4Lys16ac mark only has a proportional effect on gene expression when the constraining activity is disrupted [Aleman et al., 2021] or when the MSL complex is not present [Sun et al., 2013a]. Thus, in normal flies, there is a more or less equalized expression of the X and autosomes despite the monosomy for 20% of the genome.

The component of the complex that is expressed in males and thought to organize the complex to the male X, MSL2, was recently found to also be associated with autosomal dosage-sensitive regulatory genes [Valsecchi et al., 2018]. MSL2 was found to modulate these autosomal dosage-sensitive genes in various directions, which illustrates that MSL2 has a role in dosage balance that goes beyond the X chromosome. This finding is consistent with the evolutionary scenario that the initial attraction of the complex to the X chromosome was to upregulate dosage-sensitive genes in hemizygous regions as the progenitor Y became deleted for them, with the constraining activity evolving to prevent an overexpression, as the amount of acetylase on the male X increased with time [Birchler, 2016].

The MSL hypothesis takes an X-centric view that does not accommodate what is now known about dosage effects across the whole genome. The idea that dissolution of the MSL complex would cause reduction in expression of the male X-linked genes without any consequences for the autosomes is not consistent with current knowledge of gene regulatory networks and their dosage sensitivity. Indeed, the finding of dosage compensation in large autosomal aneuploids that operates on the transcriptional level [Devlin et al., 1982, 1984; Birchler et al., 1990; Sun et al., 2013c] as well as a predominant inverse effect by the same [Devlin et al., 1988; Birchler et al., 1990] argues that one must consider the inverse effect for an understanding of the evolution of dosage compensation in *Drosophila* (and other species). Further discussion of models of *Drosophila* compensation has been published [Birchler, 2016].

What is likely to be the most critical issue with sex chromosome evolution is the consequences for dosage-sensitive regulatory genes. This fact is nicely illustrated by the retention of these types of genes in different independent vertebrate sex chromosome evolutions [Bellott and Page, 2021]. In *Drosophila*, by contrast, dosage compensation is more of a blanket effect on most but not all X-linked genes despite the fact that many genes on the X are unlikely to have dosage-detrimental effects, although dosage-sensitive genes might have played a role as noted above. The particularly large size of the X in *Drosophila* compared to the whole genome is potentially a contributing factor because such large genomic imbalance is likely to modulate most genes across the genome. Also, there is no evidence of a WGD in *Drosophila* as there is in other species for which the inverse effect has been documented (maize, *Arabidopsis*, yeast, mice, human). These other species have various numbers of retained duplicate dosage-sensitive regulatory genes from WGDs. Thus, the relative change of regulatory genes in aneuploids in these species will not be as great compared to some of their interactors in the remainder of the genome, which could result in lesser magnitudes of some *trans*-acting effects, similarly to how aneuploids in ascending ploidies have fewer effects as described above. The absence of duplicate regulatory genes in *Drosophila* would predict a stronger inverse effect in general, and that could have been capitalized upon to produce dosage compensation of most genes on the X chromosome despite many of them not being dosage critical. While sex chromosome evolution must accommodate dosage-sensitive genes for proper development and viability, it could also be capitalized upon to evolve sexual dimorphisms in expression [Sun et al., 2013c].

Commonalities of Gene Imbalance Effects and Quantitative Trait Loci

It is likely that there is an overlap of the genes responsible for aneuploid syndromes and for multigenic quantitative traits. Aneuploidy for multiple chromosomes will modulate the same phenotypic traits as illustrated beginning with the work of Blakeslee [Blakeslee, 1921; Blakeslee and Belling, 1924]. The effects extend to anatomical differences [Sinnott et al., 1934]. Quantitative traits are controlled by multiple genes that, as a general rule, show additive effects in hybrids between true breeding lines with extreme effects [Tanksley, 1993]. This convergence was noted by Guo and Birchler [1994]. When quantitative trait loci (QTL) eventually became identified on the molecular level, many of them are indeed transcription factors and components of signaling cascades [Frery et al., 2000; Cong et al., 2002, 2008; Liu et al., 2002] that fit the criteria of being dosage sensitive as do genes responsible for aneuploidy effects.

The integration of high-throughput DNA and RNA analyses has offered a unique view of the genetic causes of gene expression variation. These developments have allowed the discovery of expression QTL (eQTL), which are genetic differences that affect gene expression (in absolute terms or the ratio of different transcript isoforms) that can act in *cis* and *trans* [Michaelson et al., 2009]. Not surprisingly, genome-wide association studies, which pinpoint genetic variation scattered throughout the genome, show that a proportion of the variants found are indeed responsible for eQTL. In the present context, eQTL associations have been modeled as sequences of transcriptional and protein-protein interactions that link a locus with its affected targets. This approach has allowed prioritization of candidate genes by their proximity within a network to an affected target and also highlights the importance of the underlying regulatory pathways [Tu et al., 2006].

Heterozygous mutations for regulatory genes in mice have phenotypic effects illustrating their dosage effects [Boell et al., 2013]. Tumor suppressor genes are exquisitely dosage sensitive across a spectrum of expression levels [Berger et al., 2011], illustrating a gradient effect. In poplar, a large collection of small heterozygous deficiencies and duplications was assembled from irradiation due to the fact that they could be vegetatively propagated and maintained [Henry et al., 2015; Bastiaane et al., 2019, 2021]. Their characterization for effects on various phenotypic traits illustrates the fine scale and extensive nature of the dosage sensitivity involved with quantitative traits.

Large quantitative variation in regulatory genes would not be tolerated as indicated by the types of genes retained

following WGD and underrepresented in copy number variants. However, a subtle variation for dosage-sensitive genes below a 2-fold range is likely and may be essentially neutral under most conditions. QTL experiments have too little statistical power to detect them and they might account for missing heritability, i.e., the lack of ability to account for the predicted heritability in QTL experiments based on the difference between the parents [Birchler et al., 2016]. Nevertheless, strong selection could utilize this standing variation to evolve new traits rapidly. Because the effects depend not only on the quantity of gene expression of any one regulatory gene affecting its targets but also its quantitative relationship to its interactors, an astronomical array of expression levels could be available for selection. This spectrum is further expanded by the fact that gene regulation often operates in cascades in that one transcription factor affects another one et cetera, leading to an ultimate readout of target genes. A dosage modulation at an early step in a cascade would be transmitted through the system. The dosage sensitivity of regulatory processes allows selection to act on heterozygous alleles that do not need to be homozygous to produce differences in phenotype. The fact that regulatory genes are dosage sensitive in a relative manner compared to the remainder of the genome will maintain the status quo in the absence of selection for change but provides an incredible number of paths for the evolution of quantitative traits in the presence of selection that occurs with new conditions or environments.

C-Value and Gene Dosage Balance

Veitia and Bottani [2009] have applied the principle of balance to explain the observation that genome size (also known as the C-value when referring to the haploid genome) does not correlate with organismal complexity, that is, the “C-value paradox” [Gregory, 2001; Veitia and Bottani, 2009]. They used models and simulations to explore how a WGD accompanied by deletions (fractionation process) might affect dosage balance because extensive genomic deletions would lead to changes in nuclear/cell volumes and gene product stoichiometries. They posit that the potentially negative impact of genomic deletions could be buffered, at least temporarily, if deleted coding DNA were replaced by DNA repeats so that the nuclear/cell volume remains compatible with normal cell function. Thus, either retention of non-functionalized DNA regions and/or their replacement by selfish elements might help avoid dosage imbalances in cycles of poly-

ploidization. More recently, Freeling et al. [2015] have provided a complementary explanation based on the study of the phenomenon of genome dominance, mentioned above. The starting point was the finding that coverage of 24-nt siRNA targeting transposable elements near genes was more important for the “recessive” subgenome in a study involving *Arabidopsis thaliana* and *Arabidopsis lyrata* [Hollister et al., 2011]. These authors argue that TEs might act through epigenetic position effects to downregulate nearby genes. This would require a more or less even dispersion of transposons across the genome. Accordingly, stretches of TEs would act as rheostats of gene expression. If transposons proliferate in a polyploid and get distributed differently than in the ancestor, out-of-balance genomes would be selected against [Freeling et al., 2015].

Future Directions

Despite the fact that the idea of gene balance was introduced 100 years ago, in many respects its study is still in its infancy and deserving of attention. Understanding the nature of multicomponent interactions by examining how each component affects the function of the whole is a worthwhile endeavor [Huang et al., 2018]. Specifically, with regard to gene regulation, these issues would be important to investigate with respect to how target genes are modulated. The role of DNA in multicomponent kinetics is not understood and is by nature stochastic. How the synthesis and degradation of individual subunits of a complex affect the function of the whole complex [Veitia et al., 2008, 2013; Dephoure et al., 2014] is another important avenue for investigation. Genetic variation for aneuploidy tolerance has been found in yeast [Hose et al., 2020; Robinson et al., 2021], which deserves study of the mechanism. Overdosage of separate complexes in the cell has a detrimental effect on growth [Chen et al., 2019] that poses an interesting problem at a higher level than individual interactions. The basis of the inverse effect and, to a lesser degree positive modulations, which have been repeatedly observed but under-appreciated and which trace back those 100 years to Blakeslee’s observations on modulation of pigment levels, is a fertile area for understanding genetic stoichiometric balance. Grasping the molecular basis and the underlying kinetics of dosage effects on gene expression is an important future direction.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

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