A Bird's-Eye View of Sex Chromosome Dosage Compensation

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Abstract

Intensive study of a few genetically tractable species with XX/XY sex chromosomes has produced generalizations about the process of sex chromosome dosage compensation that do not fare well when applied to ZZ/ZW sex chromosome systems, such as those in birds. The inherent sexual imbalance in dose of sex chromosome genes has led to the evolution of sex-chromosome-wide mechanisms for balancing gene dosage between the sexes and relative to autosomal genes. Recent advances in our knowledge of avian genomes have led to a reexamination of sex-specific dosage compensation (SSDC) in birds, which is less effective than in known XX/XY systems. Insights about the mechanisms of SSDC in birds also suggest similarities to and differences from those in XX/XY species. Birds are thus offering new opportunities for studying dosage compensation in a ZZ/ZW system, which should shed light on the evolution of SSDC more broadly.

INTRODUCTION

Sex chromosomes have attracted a great deal of attention for more than a century (70, 92) because, unlike autosomes, they are not a matched pair. One sex chromosome is often much larger and more gene rich than the other, which is often atrophied and gene poor. These differences have enormous ramifications for the organization and evolution of the two chromosomes, and indeed for the rest of the genome (28, 41, 71, 98). From the standpoint of gene dose, the sex chromosomes present two main "difficulties." One issue is that the sex chromosomes, unlike autosomes, are differentially represented in the two sexes, which leads to sexual bias in selection pressures that operate on the sex chromosomes but not on the autosomes. These pressures result in alterations in gene content relative to the autosomes and differences in sex-biased gene expression. The second problem is that a twofold difference in genomic dose of one sex chromosome means that if the gene dosage difference is not mitigated, gene networks in the two sexes cannot function equally, and hence not optimally, in at least one sex. The two main vertebrate heteromorphic sex chromosome systems, XX/XY and ZZ/ZW, are considered here. In XX/XY systems, the male is heterogametic XY and the female is homogametic XX, whereas in ZZ/ZW systems the female is ZW heterogametic. The vast majority of molecular genetic research has been carried out on three genetically tractable XX/XY systems: mammals (mouse and human), *Drosophila*, and *Caenorhabditis elegans*. Accordingly, the theories concerning sex chromosome evolution and dosage compensation have disproportionately arisen from work on XX/XY systems, and have been tested almost exclusively in those systems.

The recent emergence of genome resources for birds, including the sequencing of two avian genomes (19, 102), now allows for the first time a global approach to the study of sex chromosome dosage compensation and evolution in ZZ/ZW systems. The early returns from global studies of dosage compensation challenge existing ideas that arose from the study of XX/XY systems. The novel avian perspective is likely to continue to offer fresh insights that might not previously have been possible if we were limited to the study of XX/XY systems.

THEORIES OF SEX CHROMOSOME EVOLUTION

Why Are Sex Chromosomes Heteromorphic?

XX/XY and ZZ/ZW systems have dramatic similarities. Both often have one large (homogametic) sex chromosome (X or Z, in mammals and birds with ~1000 genes) and one small (heterogametic) sex chromosome (Y or W, typically containing only a few dozen genes). The repeated, independent, and convergent evolution of the mismatched sex chromosome pair in totally unrelated animal taxa likely reflects a common series of evolutionary events. The initiating event is thought to have been the emergence of a dominant sex-determining allele on an autosome in a species lacking well-differentiated sex chromosomes (16, 17). For example, in the therian ancestors of marsupial and eutherian (placental) mammals, the *Sox3* gene probably sustained a series of mutations that transformed it into the *Sry* gene, the dominant testis-determining gene on the Y chromosome of marsupial and eutherian mammals (41, 78). Accumulation of other male-advantage genes near the male-determining mutation would have differentiated one region of the proto-Y from the proto-X, and led to a loss of X-Y recombination of that segment. The subsequent inexorable march to degeneration of the Y would have been the result of accumulation of deleterious mutations, caused by Muller's ratchet, genetic hitchhiking, population size effect, and background selection (98, 106). As genes were lost from the Y, corresponding regions of the X would have been stranded as single-copy segments in the heterogametic sex, whereas the homogametic sex would have had two copies of these segments. Moreover, the dosage of the stranded X genes relative to their interacting partners on the

autosomes [X to autosomal (A) ratio] would have been different in the two sexes, so that reciprocal regulatory effects of X and A genes would have been disrupted in one or both sexes. The loss of Y genes would therefore have set up a male-specific selection pressure to increase expression of X genes to a level comparable to that of females (or at least to a level at which it no longer had deleterious functional consequences), and/or a female-specific selection pressure to reduce X gene expression until deleterious functional consequences were reduced.

These ideas are attractive because they represent a unified theory to explain the evolution of diverse dosage compensation systems among XX/XY species. In mammals, a female-specific mechanism evolved to reduce transcription of one entire X chromosome (X-inactivation) in each female cell, leaving one active X chromosome. However, evidence suggests that the expression of genes across the single active X chromosome of both sexes in mammals is also increased by an unknown mechanism so that X gene expression occurs at a level comparable to autosomal expression (9, 46, 68). Thus, balance is achieved between the sexes in X gene expression, and between X and A expression within each sex. In *Drosophila*, both types of balance are achieved because of the evolution of a mechanism in males to increase X gene expression to the level of females. In *C. elegans*, the XX hermaphrodite reduces expression of X genes on both X chromosomes to the level of expression of the XO male, but both sexes upregulate the expression of X genes to a level comparable to the expression of A genes. The strongest argument for the critical importance of sex chromosome dosage compensation is that these three unrelated groups have each evolved a different but elaborate set of genetic tricks to solve the same problem.

Although most authors seem to suggest that the degeneration of the proto-Y or proto-W chromosome is an almost unavoidable consequence of a dominant sex-determining mutation on that chromosome, we note that some sex chromosomes are not well differentiated in species with genetic sex determination. Among birds, the ancient ratites (flightless birds such as the ostrich, emu, and rhea) have poorly differentiated sex chromosomes (87, 96), even though these chromosomes have had as much chance to differentiate as those of other birds. Although the Z and W chromosomes in these species are different, the wholesale degeneration of the W has not occurred and remains unexplained.

Gene Networks and Dosage Compensation

Genes operate in complex networks in which tens of thousands of genes have positive and negative regulatory influences on one another. Gene networks show scale-free properties (5), meaning that a minority of genes (hub genes) have widespread connections or reciprocal regulatory interactions with many other genes, but progressively larger sets of genes have progressively smaller numbers of connections. Most genes influence the expression of only a small number of other genes. Therefore, removing a random gene abruptly from a stable gene network can have an enormous deleterious influence if the gene is a hub gene on which many other genes rely for proper regulation, but typically will have relatively little effect when one of the more common non-hub genes is removed (48, 104). Many individual genes play roles that are redundant with others, or are functionally important in restricted environments, so there is no obvious phenotypic disadvantage if their copy number is reduced. Accordingly, variability in the copy number of individual genes or small segments of the genome is maintained with significant frequency in wild and laboratory populations (22, 32) and is often compatible with survival.

In contrast, monosomy or trisomy for a whole large chromosome usually presents a major problem; in humans, for example, either is usually lethal. This is probably because differences in dose over a large genomic segment are more likely to involve differences in dose of critical hub genes. The more hub genes involved, the larger the problem. Thus, one can imagine that as the Y chromosome proceeded along

its inexorable degenerative path, the reduction in copy number of many or most X genes in males had relatively small phenotypic effects with minor effects on fitness. This view suggests that the reduction in dose of a minority of X genes drove the selection of complex molecular mechanisms to adjust X dosage in one sex or the other.

Specialization of Gene Content on the Sex Chromosomes

Other forces have also contributed to altering the gene content of the sex chromosomes because they are differentially represented in the two sexes. For example, genes involved in late stages of spermatogenesis are absent from the X chromosome of *C. elegans* and mammals because the X chromosome is silenced at later stages of spermatogenesis (41, 77).

More significant in the context of avian dosage compensation is the effect of sexually antagonistic mutations, which increase the fitness of one sex more than the other (28, 34, 79, 98). Sexually antagonistic alleles on autosomes have a net fitness that is the average fitness between males and females. Because the alleles are passed equally to males and females, if the loss of fitness to one sex substantially decreases the gain to the other sex, the allele will not become fixed. However, this balance is upset in the sex chromosomes because they are unequally represented in the two sexes. Recessive X mutations that benefit males produce a phenotype in the hemizygous male that is selected for even if the mutations are deleterious to females, because the phenotype would not be expressed in females until the allele's frequency in the population reaches the point at which homozygous females become more abundant (79). The allele will therefore likely be fixed as a polymorphism at frequencies that promote its survival in males but are not yet sufficient to produce significant loss of fitness in females (34). The disadvantage to females may set up selection pressure to downregulate the gene specifically in females, for example in vertebrate species if ovarian hormones or other female-specific factors evolve the ability to reduce the gene's expression. Thus, sex differences in gene expression can be the result of sex-specific selection pressures resulting from sexual antagonism and the differential representation of X genes in the two sexes. The selection of sexually antagonistic alleles can lead to some masculinization of the X chromosome, defined as an increase in frequency of male-benefit genes on the X relative to autosomes, which may be observed as an increase in male-biased genes (defined as genes with higher expression in males). Perhaps more significantly, the X chromosome is also subject to opposing pressures to be feminized (accumulation of female-benefit alleles) or demasculinized (loss of male-benefit alleles). The X chromosome spends twice as much of its evolutionary history in XX females than in XY males, so X genes (especially those controlling dominant traits) are subject to greater selection pressure to benefit females even if they lower fitness for males. Again, the accumulation of female-benefit male-detriment alleles on the X chromosome would set up selection pressure to reduce expression of the genes specifically in males (for example, if the gene evolves sensitivity to male-specific factors that lower expression). In ZZ/ZW systems, similar selection pressures are predicted to masculinize the Z chromosome, enriching it in male-biased genes, or lead to polymorphisms involving recessive Z alleles of female-biased genes.

In addition, the X chromosome seems to be subject to unusually high rates of retrotransposition in *Drosophila* and mammals (7, 29, 52), in which copies of genes are moved both from the X chromosome to autosomes and in the reverse direction. The retroposition of genes might in part mitigate the effects of sex-specific selection pressures. For example, as the Y chromosome diverged from the X chromosome, the sex-specific selection pressures on newly hemizygous X genes would be reduced if an autosomal copy of the gene were present at a functional dose. Sexual antagonism on the sex chromosomes can also set up selection pressures on autosomal genes that are redundant with or influence the function of sex chromosome genes

(91, 107) For example, if a male-benefit male-biased allele arises on the Z chromosome of birds, changes in regulation of a redundant autosomal gene could compensate for the deleterious effect of the male-benefit Z allele in females.

Evidence suggests that these processes have had a significant influence on the X chromosome gene content and bias in gene expression. For example, the X chromosome of *Drosophila* has an overrepresentation of female-biased genes in gonads and fewer genes with male-biased expression in all tissues (41, 72, 91, 98). In *C. elegans*, the X chromosome contains significantly fewer germline-intrinsic and sperm-enriched genes (98). The mammalian X chromosome is enriched for genes involved in reproductive, brain, and muscle function and those involved in the early stages of spermatogenesis, all of which have been explained as the result of selection for male-benefit alleles driven by natural or sexual selection (41, 51).

Types of Dosage Compensation

Here we define dosage compensation as any mechanism that reduces the disparity in the expression of genes that differ in copy number between groups of animals. Thus, if the male to female (M:F) ratio of expression of an X-linked gene is significantly higher than 0.5 in a mammal, we conclude that some process has acted to bring the ratio above the simple 1:2 ratio expected on the basis of genomic dose. One type of dosage compensation is sex-specific dosage compensation (SSDC), which we define as an evolved molecular mechanism, present in only one sex, that reduces the sexual disparity in the expression of sex chromosome genes. Examples of SSDC include female-specific X-inactivation in mammals, male-specific upregulation of X genes in *Drosophila*, and hermaphrodite-specific downregulation of X chromosome expression in *C. elegans*. In each of these cases, SSDC is chromosome wide, although it appears to have evolved gradually, perhaps on a gene-by-gene basis as the X chromosome became differentiated from the degenerating Y (30, 38, 90). Importantly, SSDC is triggered by a mechanism originating on the sex chromosomes that measures the number of X (or Z) chromosomes directly or indirectly, so that a sex-specific regulation of sex chromosome genes can be initiated.

Another mechanism of dosage compensation is sometimes called autosomal dosage compensation (10, 24, 25), which we call network dosage compensation (NDC) here because it operates on all chromosomes. Because genes are embedded in networks that involve complex regulatory interactions, their expression is influenced by processes such as negative feedback, autoregulation, and competition for limited regulatory factors (8–11, 106). These forces, which are not chromosome specific, usually buffer the effect of a change in genomic dose (10, 49, 99) and therefore reduce the expression ratio to a level below the ratio of genomic dose. In mouse models of Down syndrome, for example, in which the mice have three copies of genes homologous to segments of human chromosome 21, the average ratio of expression of genes (relative to control mice) is often less than 3:2 as expected from a simplistic model in which expression is proportional to genomic dose (33, 97). Similarly, *Drosophila* strains differing in copy number of a specific autosomal segment show highly variable ratios of expression among genes in the segment, which does not track copy number. For example, flies with three copies of a chromosomal segment have approximately 1.5 times higher expression of genes in that segment than flies with one copy of the segment (42, 106). These results strongly support a quantitatively significant NDC mechanism that applies throughout the genome. How big is the NDC effect? How much NDC can one expect in the complete absence of an evolved SSDC mechanism? Unfortunately, relatively few studies have been conducted that bear on this question, and the results are highly variable. We define the percentage NDC effect as 100 (1 - Pe/Pd), where Pe is the percentage difference in expression and Pd is the percentage difference in gene copy number. For example, in the case of trisomy versus disomy, genes compensated 60%

would be expressed at a 20% higher level in the trisomic state relative to disomic state, instead of the expected 50% higher level based on a 3:2 gene dose. In a variety of papers measuring gene expression in mice with known differences in copy number, the percent NDC ranged from 0% to 80%, a rather wide range (4, 31, 33, 58, 74, 83, 97). In *Drosophila* the percent NDC was roughly 50%–60% (42, 106), indicating that if species exist that completely lack SSDC, then their sex ratio of X or Z gene expression is expected to be considerably less than 2:1. However, the NDC effect appears not to be large enough (100%) on average to completely compensate for the 2:1 sex difference in genomic dose of all genes on a large chromosome such as the eutherian X chromosome; otherwise there would have been insufficient evolutionary pressure for SSDC mechanisms to evolve.

Although SSDC mechanisms in eutherian mammals, *Drosophila*, and *C. elegans* all effectively balance X and autosomal expression in the two sexes and balance X gene expression in males and females, the molecular mechanisms are different in each case. When multiple X chromosomes are present in mammals, a counting mechanism (57) triggers the expression of the noncoding Xist RNA, which accumulates on the X chromosome that is to be inactivated. Xist expression triggers a cascade of epigenetic mechanisms that silences the majority of genes in one X chromosome in each female cell (14, 21, 45, 75, 82). Some (15%–25%) genes escape inactivation, at least in humans (15), suggesting that there is no strong selection pressure to apply the SSDC mechanism perfectly across the entire chromosome. In *Drosophila*, dosage compensation results from mechanisms that measure the expression ratio of X to A genes and trigger the expression of *sex lethal* (*Sxl*) in females (23, 35, 63, 86). If the X:A ratio is 0.5, as in males, the *Sxl* transcript, normally expressed in females, is not expressed. In the absence of SXL protein, a dosage compensation complex (DCC) of proteins accumulates on the X chromosome. Among other epigenetic modifications, the DCC causes acetylation of histone 4 at lysine 16, resulting in increased transcription of X genes to the level of females (13). Two noncoding RNAs (ncRNAs), roX1 and roX2, are associated with the DCC and are essential for dosage compensation. In *C. elegans* (XX hermaphrodite versus XO male), X genes are expressed from both X chromosomes but at a reduced level (30). Downregulation of genes on both X chromosomes in XX cells is initiated by SDC-2 (sex determination and dosage compensation defect-2), which assembles the DCC. The DCC is recruited to multiple cis-acting regions of the X and spreads out along the chromosome from its initial binding sites (30). This results in suppression of transcription on the X. Although the SSDC mechanisms of mammals and *C. elegans* both involve reduction of X expression in the homogametic sex in addition to bisexual increase in X expression so that it reaches the level of autosomal expression, the mechanisms for balancing X and autosomal expression are not known (9, 42, 68).

DOSAGE COMPENSATION IN BIRDS

Dosage Compensation is Less Effective in Birds Than in XX/XY Systems

By 1967, only a few years after the identification of the avian W chromosome, and prior to the identification of any specific protein product encoded by the avian Z chromosome, Ohno (70) had already concluded that dosage compensation does not occur in birds. The evidence was that both Z chromosomes of males appear euchromatic along their entire length, a Barr body is absent, late replication of the Z chromosome does not occur (84), and some Z-linked mutant phenotypes are expressed more in ZZ males than ZW females. For example, the Z-linked white bar mutation in chickens produces bars that are twice as wide in ZZ males than ZW females, and ZZ males heterozygous for the mutation have the same phenotype as hemizygous ZW females (20, 70). The first study of sex differences in Z gene expression (6) showed that the aconitase protein was expressed at twice the level in males than in females, supporting

the conclusion that no dosage compensation occurred. That view was left unchallenged for two decades, when studies of mRNA expression of approximately a dozen Z genes found that although some Z genes escape compensation and are expressed at M:F ratios near 2, others have ratios near 1 and hence show evidence of some compensation (55, 64). Several other findings fueled the idea that dosage compensation is weak. For example, studies of sexual dimorphism in gene expression found a disproportionate number of Z genes among male-biased genes (1, 2, 18, 85, 100), and genes with male-specific expression were disproportionately Z-linked rather than autosomal in gene expression databases (50, 89). Moreover, studies using RNA FISH (fluorescent in situ hybridization) that measured the site of transcription for six Z genes in ZZ male chickens showed biallelic expression, leading to the conclusion that one entire Z chromosome is not inactivated in the same manner as occurs in mammals (54, 55).

The first global assessments of Z chromosome dosage compensation in birds (27, 47) were enabled by the sequencing of the chicken genome (102), which provided unprecedented information on chromosomal linkage of genes and led to the availability of high-quality gene expression microarrays. We measured mRNA levels from three somatic tissues of male and female chick embryos at 14 days of incubation, comparing expression of approximately 1000 Z genes with approximately 17,000 A genes (47). The M:F ratios of expression of A genes varied around a mean of 1. In contrast, M:F ratios for Z genes were strongly shifted toward higher expression in males, with means between 1.24 in liver and 1.4 in brain (**Figure 1**). Similar results were found in measurements of smaller numbers of genes in the zebra finch.

The methods used to estimate M:F ratios affect the ratios that are observed. Microarray measurements have some nonlinearities and background hybridization (44) and less dynamic range than more labor-intensive methods such as quantitative RT-PCR. These nonlinearities likely reduce M:F ratios. For example, microarray measurements of Y gene expression in mammals show M:F ratios far below the true ratio of infinity, probably because of background hybridization in females (26, 80, 105). In general, M:F ratios in chicken measured by quantitative RT-PCR are approximately 10%–20% higher than those obtained from microarrays (27, 47, 85). Thus, the mean M:F ratios for chicken Z genes in somatic tissues could be as much as 50%–60% higher than for autosomes. Quantitative estimation of the amount of dosage compensation is best done in tissues in which males and females have a similar distribution of cell types in which the genes are presumably functioning in the same gene networks; an example

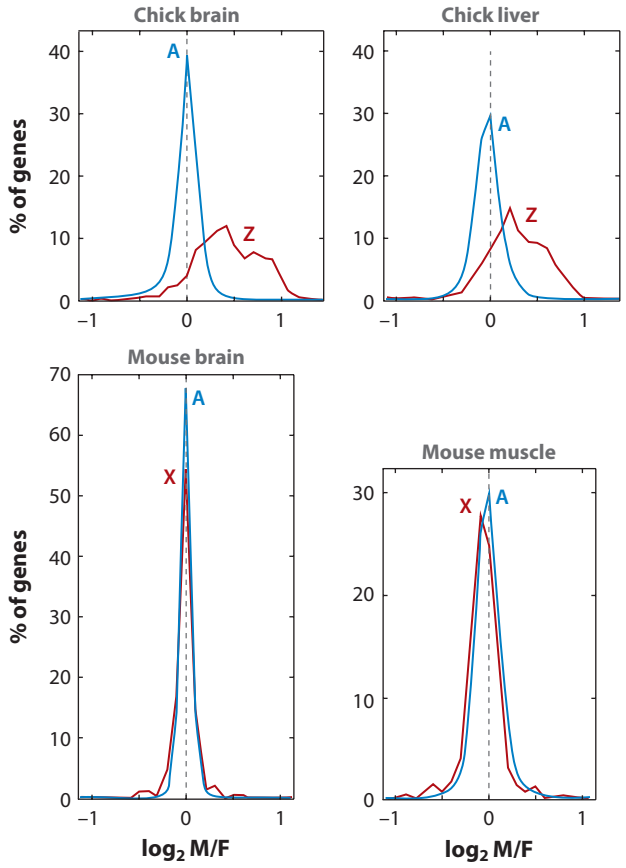


Figure 1

Distributions of male:female (M:F) ratios of mRNA expression (measured by microarrays) are shown for two chick and two mouse tissues, comparing autosomal (A) and Z genes for chick, and A and X genes for mouse. In mouse, both X and A genes show M:F ratios centered around 1 ($\log_2$ ratio 0), whereas in the chick Z genes show much higher ratios. From Reference 47 with permission.

is somatic but not gonadal tissues. In this manner differences in gene expression influenced by cell type do not confound comparison of the two sexes. However, because evolutionary pressures leading to dosage compensation and sexual bias in expression can operate most importantly in individual tissues (often in gonads) or under specific developmental or environmental conditions, measurement of sexual bias in gene expression needs to be performed in many tissues, environments, and developmental stages. Global analyses of dosage compensation so far have been performed in only a few tissues of chick embyros (27, 47).

The contrast between global assessments of dosage compensation for birds and mammals is striking (47). Comparable global measurements of M:F ratios of gene expression in adult mouse (**Figure 1**) and adult human somatic tissues (47) show only small discrepancies in the distribution of M:F ratios for X and A genes. In some cases the X genes are expressed at a slightly lower ratio than A genes. In brain the X genes show a greater amount of sexual dimorphism in both directions than A genes, confirming a previous report (105) indicating that the X chromosome of mammals contains more sexually dimorphic brain genes than the average autosome. The X versus A difference in amount of sexual dimorphism can be explained by the processes, reviewed above, that favor sex bias in expression. However, the percentage of X genes showing unusual amounts of sex bias is small. Clearly, mammalian dosage compensation mechanisms (SSDC plus NDC plus perhaps other unknown mechanisms) are globally effective in bringing M:F ratios of X gene expression in line with autosomal expression. It is interesting that the M:F ratios show different variability across mouse somatic tissues (adipose and liver are more sexually dimorphic in gene expression than whole brain), but that the variance in M:F ratios among X genes closely matches that for A genes in each tissue (**Figure 1**). The explanation probably is not that X and A genes evolved virtually identical percentages of genes that respond in a precisely similar graded fashion to sex-specific regulatory signals (for example, equal variance in regulation by gonadal hormones, which are the predominant signals controlling sexually dimorphic gene expression in mammals). Rather, the variance of X and A M:F ratios more likely match each other because of network interactions, i.e., the reciprocal regulation of X and A genes that underlies NDC in each tissue. The same NDC mechanisms can be invoked to explain the close matching of X to A gene dose in various XX/XY tissues (9), rather than evolution of many precise matching mechanisms for each transcript (90).

In chicks the mean Z:A ratios of mRNA expression are near 1 for ZZ males (range across tissues is 0.92 to 1.03) and near 0.8 for ZW females (range is 0.77 to 0.88) (47). These ratios are comparable to those in mammals, in which male and female X:A ratios are near 1 (range approximately 0.8 to 1.2) across many tissues, developmental stages, species, and sexes (68). *Drosophila* and *C. elegans* also achieve X:A ratios near 1 (42). If the need to balance Z:A (or X:A) gene dosage is the primary force that drives the evolution of dosage compensation mechanisms, as has been argued (46), then it would appear that some mechanism achieves compensation in birds, almost as well as in mammals. Alternatively, the striking bird-mammal difference in M:F ratio curves (**Figure 1**) and the unusual sex difference in Z:A ratios suggest that birds should be considered to be qualitatively different from the three well-known XX/XY systems because the dosage compensation mechanisms are relatively ineffective.

Is the amount of dosage compensation found in birds within the range that can be explained entirely by NDC? As reviewed above, there are relatively few papers that estimate the magnitude of compensation produced by NDC. Moreover, the NDC effect has been estimated most often in models of trisomy rather than monosomy, where the percentage compensation caused by NDC may be greater. Nevertheless, the amount of dosage compensation found for Z genes (roughly 50% compensation) is within the range of the size of the NDC effect previously measured. Also, it is important

to ask whether the amount of compensation of proteins encoded by Z genes mirrors that found for Z transcripts. To our knowledge, only two Z proteins have been measured, and each shows higher expression in males (6, 18).

The contrast of the widespread concentration of sex-biased genes on the avian Z chromosome but not on the X chromosomes of mammals and other XX/XY species may lead to two conclusions. Either SSDC is poorly developed or the pattern reflects gene specialization caused by masculinization of the Z chromosome due to selection of male-benefit male-biased genes. Some evidence suggests that the latter idea, based on sexual antagonism, probably does not provide the entire explanation. First, the degree of concentration of sex-biased genes on the Z chromosome is much greater than that observed to date in any XX/XY system. Those systems show statistically significantly different frequencies of sex-biased genes on the X chromosome versus autosomes, but the differences are on the order of a few percent of genes and are not as dramatic as on the avian Z (72). To our knowledge no theories predict that birds should have a dramatically greater effect of sexual antagonism on the Z chromosome, resulting in greater sex bias on the Z than on the X chromosome. Second, the male-biased expression of Z genes extends even to housekeeping genes that are needed in all cells and are thus unlikely to show sex-specific functional specialization or sexual antagonism. Thus, mitochondrial and ribosomal genes have distinctly higher M:F ratios on the Z than on autosomes (47) (**Figure 2**). Nevertheless, sexually biased expression as a response to sexual antagonism cannot be ruled out and probably contributes to high Z M:F ratios along with the lack of SSDC. No matter which of these factors is responsible, the large difference between M:F ratios on Z versus A is unexpected on the basis of the study of XX/XY systems. SSDC is reduced or absent rather than critical and ubiquitous as previously thought (36, 42, 68), and/or there is an unexpected degree of selection for male-benefit alleles on the Z chromosome. Either conclusion is remarkable.

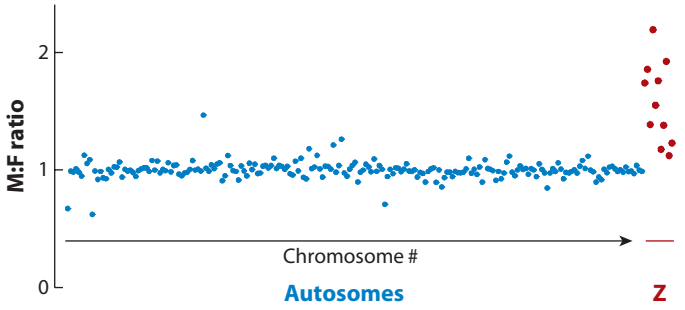
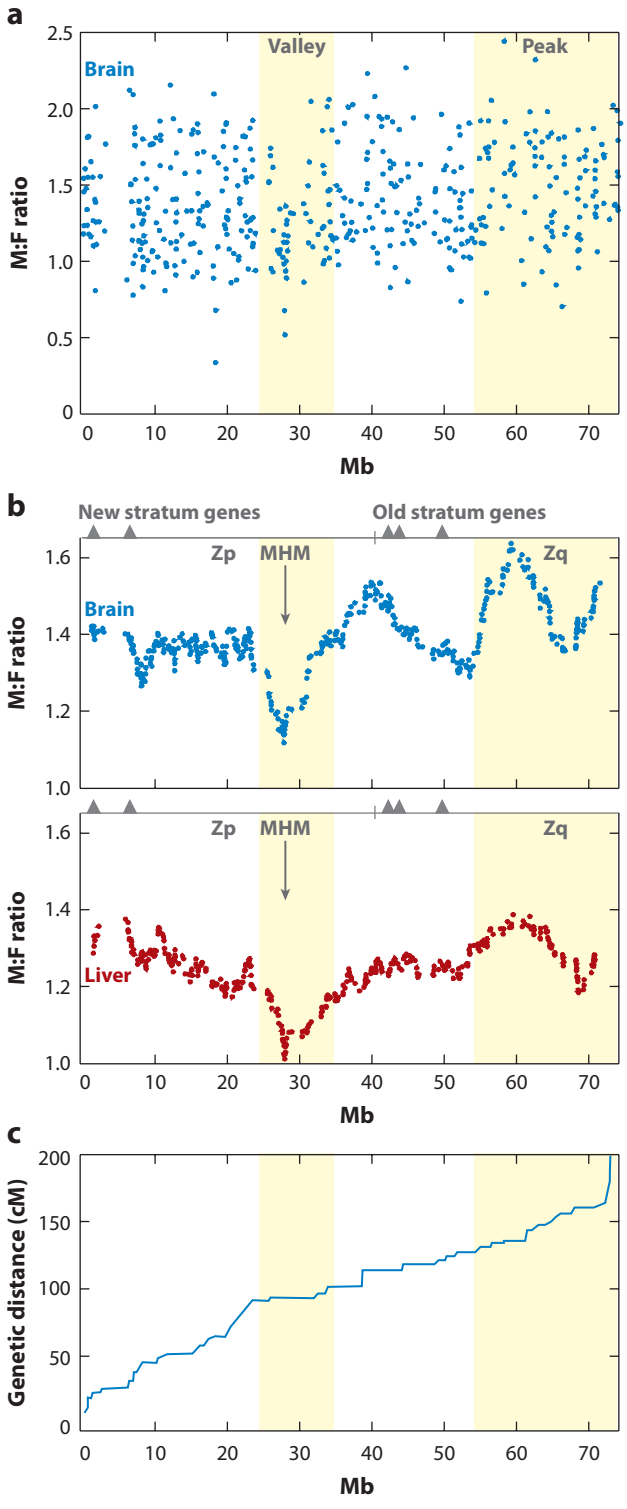


Figure 2

Male:female (M:F) ratios of expression of housekeeping (mitochondrial and ribosomal) genes in chick embryo, mapped by position in the genome. The autosomal positions are mapped by chromosome number (chromosome 1 to the left), and contrasted with ratios on the Z chromosome. Housekeeping genes may be unlikely to include male-benefit genes, but these genes have higher M:F ratios if they reside on the Z chromosome, suggesting that lack of dosage compensation rather than clustering of male-benefit genes better explains the high M:F ratio of Z genes. Data from Reference 47.

Regional Differences in Dosage Compensation on the Z Chromosome

When M:F ratios are mapped by gene position along the Z chromosome, intriguing patterns emerge (65) (**Figure 3**). Genes with high and low M:F ratios are found all along the chromosome, but Zq shows significantly higher M:F ratios than Zp. When the map is smoothed by averaging M:F ratios in a sliding window of genes, a region of low average M:F ratios (valley) is found 25–35 Mb from the telomere on Zp. In addition, one or two broad peaks of high M:F ratios are found toward the distal part of the Zq map. These variations are far from random and are unexpected by chance (65). The valley is not the result of a small number of genes with especially low M:F ratios, but rather reflects the absence of genes with high M:F ratios. For example, the valley includes a statistically unusual, uninterrupted string of genes with lower M:F ratios: 27 genes below 1.5 in brain, 25 genes below 1.2 in liver, and 23 genes below 1.4 in heart (data from Reference 65). The valleys in the curves for the three tissues occur at the same location on the Z chromosome, suggesting that regulation of M:F ratios of Z genes may be controlled in part by factors that are not tissue specific and may influence clusters of genes.



What is special about valley genes, and why do they have unusually low average M:F ratios? Importantly, the valley occurs precisely at a region of female-specific modification of Z chromatin and female-specific expression of a Z ncRNA (12, 93, 94). The ncRNA is expressed only in females from the MHM (male hypermethylated) locus, probably because the DNA at the MHM locus is hypermethylated and transcriptionally silenced in ZZ males. The MHM RNA is approximately 9.5 kb in length and is composed of a 2.2-kb repeat. Treatment of males with the DNA methyltransferase inhibitor 5-azacytidine reduces the methylation at the MHM locus, and the MHM ncRNA is then expressed in male cells (94). MHM ncRNA accumulates along the female Z chromosome near its site of transcription. At the same region of the female but not male Z chromosome, histone 4 is acetylated at lysine 16 (12). The expression of the MHM ncRNA appears to be from the sense but not antisense strand, and occurs in ZZW autosomal triploid and ZW diploid chickens, but not in ZZZ triploids and ZZ diploids. Thus, Teranishi and coworkers (94) suggested that a W chromosome factor is necessary to prevent DNA methylation of the MHM locus, and by extension to trigger the

Figure 3

Regional specialization of the chicken Z chromosome. (*a*) Male:female (M:F) ratios of expression are plotted according to Z gene position for all brain genes. (*b*) The running average of 30 M:F ratios (plotted at the position of the fifteenth gene) is mapped across the Z chromosome for brain and liver. The curve dips at the MHM (male hypermethylated) locus (*valley*) and is higher at the end of Zq (*peak*). Valleys and peaks are similar in diverse somatic tissues such as brain and liver. Data taken from Reference 65. Strata on the Z chromosome have been estimated on the basis of degree of divergence of five Z-W genes, the positions of which are shown as triangles. The old and new stratum genes are mapped. Data taken from Reference 43. (*c*) The graph of genetic (in centimorgans, cM) versus physical distance (in megabases, Mb) based on linkage studies shows that a recombination cold spot, or flat portion of the curve, occurs at the MHM valley. Reprinted from Reference 101 with the permission of S. Karger AG, Basel.

acetylation of H4K16 in this region (12; see also 40). However, in those experiments the presence of the W chromosome is confounded by Z:A ratio, so it is equally feasible that a Z:A ratio below 1 in ZW diploid or ZZW triploid females leads to expression of the MHM RNA, and/or a Z:A ratio of 1 causes methylation of the MHM locus in ZZZ triploid or ZZ diploid males.

Because acetylation of H4K16 is associated with transcriptional activation in a variety of systems, including upregulation of the male's single X chromosome to compensate dosage in *Drosophila* (13), Bisoni and colleagues (12) postulated that the female-specific histone modifications in the MHM region control SSDC. This idea is supported by the clustering of relatively compensated genes in this specific region of the Z chromosome (65) (**Figure 3**). Moreover, the involvement of a female-specific ncRNA at the same locus is reminiscent of the critical involvement of other sex-specific ncRNAs in SSDC, such as *Xist* in mammals and *roX* genes in *Drosophila*. These findings together lead to the theory that MHM ncRNA attracts proteins that modify the Z chromatin in the MHM valley, causing acetylation of H4K16 and probably other modifications in ZW females. Alternatively, the MHM ncRNA may directly modify the chromatin structure in the MHM valley (81). If either mechanism is confirmed, then birds may have evolved an SSDC mechanism that is flylike in that it involves upregulation of the X/Z chromosome in the heterogametic sex. However, numerous other questions remain. What is the molecular signal that initiates the male-specific hypermethylation of the MHM locus? Is the DNA methylation of the MHM locus primary in controlling its expression (suggesting the existence of a male-specific molecular SSDC mechanism as in mammals or *C. elegans*), or is hypermethylation of MHM a secondary consequence of the absence of H4K16 acetylation of the MHM valley in males? Is SSDC triggered by a W chromosome factor, a Z chromosome counting mechanism (Z:A ratio), or both? Who is doing the compensating? Are Z genes upregulated in females, downregulated in males, or both?

The results reviewed so far imply that a mechanism of SSDC has evolved in birds, but the mechanism is not chromosome wide as in mammals, *Drosophila*, and *C. elegans*. Although dosage-compensated genes are concentrated in the region of the MHM locus, they also occur at other regions of the Z chromosome. If the MHM ncRNA is central to SSDC in birds, are compensated genes outside of the MHM valley also regulated at a distance by factors encoded within the MHM valley?

A priori one expects that dosage differences are critical for only a minority of genes, those for which a sexual imbalance in expression is strongly disadvantageous. These would presumably be the hub genes, for which sex differences in expression would have widespread secondary effects on expression in diverse molecular pathways. Evidence in favor of this idea is that Z genes that are dosage compensated show different gene ontology categories from those that are not dosage compensated (27, 65). Compensated genes are enriched for genes involved in development and physiological processes, whereas uncompensated genes are enriched in other categories, including catalytic activity.

Intriguingly, the MHM valley rather closely corresponds to a Z chromosome recombination cold spot, a region of infrequent crossing over during meiosis (101) (**Figure 3**). Similarly, the Xist locus in humans is at the end of a recombination cold spot (67). Recombination cold spots are thought to occur at regions containing specialized DNA sequences, methylated DNA, transcriptional inactivity, and/or specific chromatin status (69, 73). DNA methylation, a feature of the male MHM locus in ZZ male chickens and the Xist locus in female humans, decreases recombination (59). The methylation of repetitive DNA in the MHM region of male chickens may function to inhibit inappropriate heterologous recombination of dispersed DNA repeats in and near the MHM locus to protect the stability of the genome in this region.

Many genes in the MHM valley are found in a syntenic region and in the same gene order in platypus, human, and cow (**Figure 4**). Most

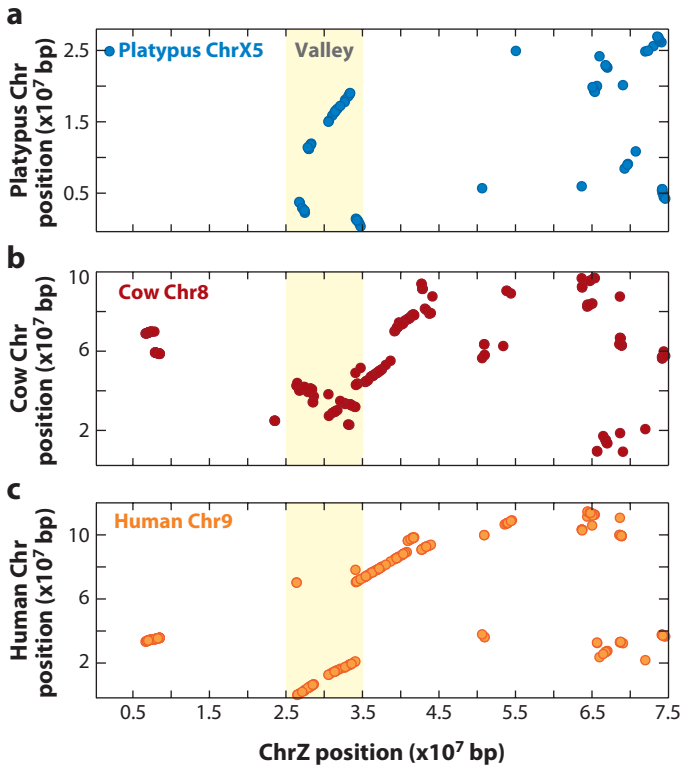


Figure 4

Conserved synteny between the chicken MHM (male hypermethylated) valley and mammalian chromosomes. The position of chicken Z chromosome genes are plotted against the positions of orthologous genes on the chromosome with maximal homology to the chicken MHM valley. In general, MHM valley genes are syntenic with similar gene order on one chromosome in these species. Valley genes are primarily located on sex chromosome X5 of platypus, a monotreme mammal that diverged from therian mammals 210 million years ago, and thus may represent a segment of chromosome from an ancestral precursor to avian ZZ/ZW and monotreme XX/XY sex chromosomes. Data from Reference 65.

interesting is that 65% of MHM valley genes are found on one of the five X chromosomes of the platypus (65, 78). Is it possible that the MHM valley genes represent a remnant of an ancestral sex chromosome that gave rise to both avian and monotreme sex chromosomes? If so, could monotremes share some of the dosage compensation mechanisms that are seen in birds? Approximately 80% of MHM valley genes are also still syntenic in humans and cows, although not as much in other mammalian species, giving rise to the speculation that some factors might have inhibited the breakup of these genes in the 300 million years since the split between human and avian lineages.

Strata on the Sex Chromosomes

The mammalian X and Y are stratified, reflecting an evolutionary history in which segments were added to the sex chromosomes in successive waves, followed by attrition of those genes on the Y (39, 41). Thus, the X and Y chromosome ceased recombination in discrete steps (56). The oldest segment of the human X chromosome contains the primary genes responsible for sex determination and SSDC, possibly because the SSDC mechanism had the longest time to evolve on that segment. The oldest stratum probably arose after the mutation of *Sox 3*, which produced the male-determining gene *Sry* on the proto-Y chromosome. Also in the oldest stratum is *Xist*, responsible for SSDC among eutherians. Newer strata contain genes added to the eutherian X before or after the split from marsupials. The different degrees of divergence of X-Y paralogous genes in each stratum indicate the time since that layer ceased to recombine in X and Y (56). Moreover, the newest X strata in humans contain genes that most frequently escape X inactivation (15), whereas the oldest stratum is more completely dosage compensated. The gradient in dosage compensation suggests that applying the Xist-mediated SSDC mechanism to all new X genes has taken tens of millions of years in each stratum (38). Therefore, the selection pressure to compensate every last X gene seems not to be strong.

The chicken Z chromosome also shows signs of stratification, although much less information is available. On the basis of the degree of divergence of five Z-W paralogous genes, Handley and coworkers (43) found that three genes on Zq near the centromere seem to represent an older stratum that ceased recombination ~102–170 Mya (million years ago), whereas two genes within 7 Mb of the Zp telomere represent a newer stratum with a divergence time of ~58–85 Mya (**Figure 3**). Unfortunately the relative time since loss of recombination of

the MHM valley region has not yet been estimated. The MHM valley includes the putative Z sex-determining gene DMRT1, a testis-determining gene that is present in two copies in males but in one copy in females (88, 94). Moreover, because SSDC evolves slowly, older segments of the Z chromosome might harbor the best-compensated genes. It would make sense if the MHM valley represents the oldest stratum containing the primary SSDC mechanism, clusters of compensated genes, and the origin of the sex-determining mutation, just as occurs in the mammalian X chromosome (65). An alternative view suggests the opposite relation, that older strata contain more poorly compensated genes (27). In animals with poor SSDC, the higher expression of Z genes in males is the default condition, which would promote fixation of male-benefit alleles on the Z chromosome. That process would have had more time to occur on the older strata. Thus, the higher M:F ratios on Zq, relative to Zp, might be related to the presumed older age of Zq. Resolution of this issue requires more information, including a more fine-grained mapping of strata, clarification of the role of the MHM ncRNA in SSDC, and discovery of the sex-determining mechanism of birds.

Our current hypothesis is that SSDC has evolved in birds, but is predominantly restricted to the MHM region and has not spread across the Z chromosome as has happened in mammalian, *Drosophila*, and *C. elegans* XX/XY systems. Thus, the MHM valley can be seen as a possible stratum, differentiated from other Z regions on the basis of the amount of dosage compensation. Why is there no effective SSDC mechanism beyond the MHM valley? One answer may be that major portions of the avian Z-W sex chromosomes have ceased recombination only relatively recently (58–85 Mya) (43) compared with segments of the eutherian X chromosome (41). However, although the eutherian X-Y divergence may have begun prior to the avian Z-W divergence, the most recently diverged segments of the human X-Y chromosome appear to have separated 40 and 100 Mya (103). Thus, there may not have been a large bird-human difference in divergence time of the most recently added segments. Nevertheless, the highly dimorphic Z and W found among most birds diverged approximately 80 Mya from those of the ratites, which have poorly differentiated sex chromosomes, so that most of the genes on the W probably were lost only in that period. Thus, it is possible that differences in the history of avian and eutherian sex chromosomes can account for some of the bird-mammal differences. Further work on bird dosage compensation mechanisms promises interesting insights, especially because of the availability for study of poorly differentiated Z and W chromosomes of the ratites (61, 96).

Who Is Doing the Compensating?

If there is a regional or even chromosome-wide but selective mechanism of SSDC in birds, is the heterogametic female upregulating Z gene expression to match that of males (a fly-like mechanism) or do males downregulate genes (a mammalian or wormlike mechanism)? So far there are only hints at an answer. The existence of female-specific acetylation of H4K16 in the MHM valley strongly suggests that there is transcriptional activation in this region in females but not in males. Conversely, Z genes that are unbiased are expressed at levels in males and females that are below mean autosomal expression, suggesting that the male may be compensating (27). Z gene expression is positively correlated with M:F ratio in males but uncorrelated in females (lower in compensated or unbiased genes than in uncompensated or male-biased genes), a pattern that is compatible with a male-specific mechanism of regulation (65). However, resolution of this question requires better understanding of the molecular mechanisms of compensation.

What Is Special About ZZ/ZW Systems?

Limited evidence suggests that the relative lack of SSDC may also occur in other ZZ/ZW

systems. Most Lepidoptera (butterflies and moths), like birds, are ZZ/ZW (37, 95). Quantitative RT-PCR was used to assess the M:F ratios of expression of 14 contiguous genes from a 340-kb segment of the Z chromosome of the silkworm moth *Bombyx mori* (53). All but one gene showed higher expression in males. However, the median M:F ratio was 2.6 (range 0.5–9.7), which is too high to reflect simply a failure of dosage compensation. Rather, the genes measured may be exceptionally male biased, perhaps clustered in a specialized region of the Z. Still, there is little evidence so far suggesting that dosage compensation occurs in Lepidoptera. More information is needed.

Although numerous theories point to differences between XX/XY and ZZ/ZW systems in the degree and nature of sexual selection or sexual conflict (3, 60, 62, 66, 76), it is far from clear that the nature of sexual antagonism is so fundamentally different in female heterogametic systems that it should favor a Z chromosome that is loaded with male-benefit genes, in contrast to more modest loading in XX/XY systems. On the basis of present information, we suggest that SSDC in birds has evolved in the MHM region, but for unknown reasons did not spread across the entire Z chromosome, as happened in the three major XX/XY systems that have been studied. In the absence of a chromosome-wide SSDC mechanism, the deleterious effects of dosage imbalance are therefore likely to have been mitigated in non-MHM regions by several factors, including the following: (*a*) Some Z genes have individually evolved sensitivity to sex-specific regulatory factors that cause dosage compensation of particularly dosage-critical Z genes such as hub genes. The regulatory factors may include the Z-specific SSDC mechanism (e.g., MHM-mediated), gonadal hormones present in one sex, or uncompensated Z genes that are constitutively higher in male cells. These factors have evolved control over Z genes to downregulate them in males and/or upregulate them in females. The effects of this sex-specific regulation are seen in the low M:F ratios of a minority of Z genes dispersed along the Z chromosome (**Figure 3**). These local, gene-specific regulatory interactions evolved gene by gene as the W chromosome degenerated, leaving Z genes stranded in the hemizygous state in females. (*b*) Adjustments in autosomal genes also occurred to offset the sexual imbalance in the functional effect of the Z genes expressed at different levels in the two sexes (91). For example, mutations would have been favored to replace a dosage-critical Z gene by a related autosomal gene in a functional pathway. In this context it is interesting that female-biased genes have evolved more rapidly during avian evolution, which could reflect in part alterations in female genetic networks that offset the male bias of some Z genes (62). (*c*) The higher expression of some Z genes in males probably reflects some masculinization of the Z chromosome (increase in content of male-benefit genes), because the same pressure accounts for the femininization or demasculinization of the X chromosome in XX/XY systems. However, in XX/XY systems the specialization effect is quantitative, a statistical shift, not a qualitative change in the gene content of the X chromosome (72). Therefore, it seems unlikely that sexual antagonism alone would have produced a Z chromosome with such striking male bias as is observed. Conversely, in species lacking chromosome-wide SSDC, the selection for male-benefit Z alleles might be more efficient. The deleterious effects for females of male-benefit Z alleles would be naturally reduced because of their hemizygous state in females (27), so some Z genes may have rapidly evolved functions that favor males.

SUMMARY AND CONCLUSIONS

Z genes in birds show average M:F ratios that are roughly 50% higher than those for autosomal genes. These ratios are less than the 2:1 M:F ratio of Z gene copy number, suggesting that some sort of dosage compensation occurs. One likely source of dosage compensation is the process of NDC, which is probably quantitatively sufficient to explain 50% compensation. However, the regional concentration of

compensated genes near the MHM locus, together with the sex-specific DNA methylation and histone modifications in that region, strongly suggest that some SSDC also occurs. A minority of Z genes located all along the Z chromosomes shows dosage compensation (near sexual equality), suggesting that the deleterious effects of the genomic dosage difference for hub genes were eliminated by sex-specific adjustments in gene expression [either controlled by MHM, an undiscovered SSDC mechanism, or by gene-specific (and probably tissue-specific) compensatory adjustments]. For other non-sexually antagonistic genes that retain a male bias in expression, the deleterious effects of the sex difference in gene dose may have been reduced by the evolution of autosomal adjustments that reduce the functional sex difference, for example by female-specific upregulation of autosomal genes with functions redundant with the male-biased Z gene (107), or changes in the gene network to make it insensitive to the sex difference. Solving the dosage problem for the most dosage-critical genes might have been enough for birds to forego the evolution of a more effective chromosome-wide mechanism of dosage compensation. Selection for male-benefit alleles may also have contributed to the frequency of male-biased genes on the Z.

Although one can find ways to explain away the greater sex bias of sex chromosome expression in ZZ/ZW birds than in known XX/XY systems, the essential problem is that no theoretical framework exists that explains when SSDC evolves and when it does not. What differences between birds and mammals, or between ZZ/ZW and XX/XY systems, account for the different path of evolution of SSDC mechanisms?

It is remarkable that forty years ago Ohno (70) was more right than wrong in his conclusion that birds do not show dosage compensation. He summed up as follows: "One may conclude that the dosage compensation mechanism for sex-linked genes is a luxury which is desirable to have, but it is definitely not a *sine qua non* of successful speciation. Conversely, one may take the stand that the failure to evolve an effective dosage compensation mechanism for numerous Z-linked genes is one major reason why birds have not escaped the status of feathered reptiles" (70, p. 147). The status of these magnificent "feathered reptiles" is exalted in our view, because birds (both sexes) are amazingly adaptable across extremes of environments, and have highly evolved functions (e.g., flight, spatial orientation and memory, vocal learning) that are truly impressive and often superior to our own. Thus, we are more attracted to Ohno's former rather than latter option, that dosage compensation, at least at the transcriptional level, is not a prerequisite for success for all species with heteromorphic sex chromosomes. The study of dosage compensation and evolution of the sex chromosomes in ZZ/ZW systems has barely begun, and there are more questions than answers. Thankfully, the genomic resources available to address these questions in birds have increased significantly with the sequencing of the chicken and zebra finch genomes, which have catalyzed further advances in other molecular and cytological resources. Thus, the study of birds will likely provide some of the answers. It's going to be exciting.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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Annual Review of Genomics and Human Genetics

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Contents

Indexes

Errata

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