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Dosage compensation, the origin and the afterlife of sex chromosomes

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Abstract

Over the past 100 years *Drosophila* has developed into an outstanding model system for the study of evolutionary processes. A fascinating aspect of evolution is the differentiation of sex chromosomes. Organisms with highly differentiated sex chromosomes, such as the mammalian X and Y, must compensate for the imbalance in gene dosage that this creates. The need to adjust the expression of sex-linked genes is a potent force driving the rise of regulatory mechanisms that act on an entire chromosome. This review will contrast the process of dosage compensation in *Drosophila* with the divergent strategies adopted by other model organisms. While the machinery of sex chromosome compensation is different in each instance, all share the ability to direct chromatin modifications to an entire chromosome. This review will also explore the idea that chromosome-targeting systems are sometimes adapted for other purposes. This appears the likely source of a chromosome-wide targeting system displayed by the *Drosophila* fourth chromosome.

The problem with sex

Many higher eukaryotes, such as *Drosophila melanogaster*, have strikingly different sex chromosomes. While the homogametic sex carries a pair of identical X or Z chromosomes, the heterogametic sex has an XY or ZW pair. The X and Z chromosomes can carry many protein-coding genes, but the Y and W chromosomes are often small and carry few genes. *Caenorhabditis elegans* represents the extreme situation in which the Y has been lost entirely. Males of this species carry a single X chromosome but no Y chromosome (XO). While hemizygosity for a single gene is usually tolerable, the degeneration of an entire chromosome requires that the organism take action to ensure adequate expression of sex-linked genes. This process involves gene regulation based on chromosomal linkage, and so it is strikingly different from transcriptional control relying on closely situated enhancers that direct individual genes. Many organisms have developed mechanisms that regulate an entire sex chromosome to compensate for unequal gene dosage between the sexes. The importance of these systems can be illustrated by the sex-specific lethality of mutations that disable them.

The impact of evolutionary forces on sex chromosome differentiation is particularly apparent in the mammalian and *Drosophila* lineages. Identical principles drive the differentiation of sex chromosomes in many organisms, but for convenience we will limit this discussion to a few organisms with well-characterized XY chromosome pairs. A fascinating, but less frequently addressed aspect of dosage compensation is the afterlife of these powerful regulatory systems when they are no longer required to compensate an X chromosome. The *Drosophila* fourth chromosome is an autosome that appears to be derived from a fragment of a dosage compensated X chromosome.

The *Drosophila* fourth chromosome provides an opportunity to explore the fate of chromosome-wide targeting systems after sex chromosome reversion to autosomal status.

The Y chromosome in a bad situation

When a gene acquires the ability to determine sex, the chromosome carrying this gene is henceforth limited to passage through a single sex. This results in selective forces quite distinct from those that shape the autosomes. The most potent of these is a bar to recombination (reviewed by Rice 1996). The mechanism of suppression of recombination is not always known. In the mammalian lineage a series of Y-chromosome inversions has protected segments of the Y from recombination (Lahn & Page 1999). The situation in *Drosophila* is more straightforward as males lack recombination entirely. Thus, the male-limited inheritance of a Y chromosome ensures the end of recombination. In the absence of recombination, mutations and mobile elements accumulate without the option of repair or removal. A slow but inexorable erosion of coding potential on the Y chromosome is the result (Figure 1A). Loss of genes from the Y chromosome creates the requirement for a system that increases expression of the remaining X-linked homologues to restore normal gene dosage in males. This process is beautifully illustrated in *D. miranda*, where translocation of an autosomal element onto the Y about 1 Mya created neo-Y and neo-X chromosomes (Bachtrog 2003b). The neo-Y, forced to segregate with the Y and consequently blocked from recombination, has begun to degenerate. While the neo-Y is of similar size to the neo-X and clearly retains homology with it, genes along the neo-Y have acquired point mutations and transposable element insertions, and many are no longer expressed (Steinmann & Steinmann 1998, Bachtrog 2003a, 2005). Meanwhile, numerous sites on the neo-X recruit a protein complex that serves to boost expression of X-linked genes in males (Bone & Kuroda 1996, Marin *et al.* 1996).

In spite of abundant evidence that lack of recombination drives degeneration of the Y chromosome, the origin of the Y chromosome now found in several insects, including *D. melanogaster*, is in doubt. While mammalian Y chromosomes bear remnants of their origin from a homolog of the X, the Y chromosome of *D. melanogaster* does not. The *D. melanogaster* X and Y chromosomes lack sequence similarity in repetitive regions, as well as between functional genes (reviewed by Carvalho 2002). An intriguing idea is that the modern Y of these males is derived from a B element, an optional 'selfish chromosome' that has assumed the position of the Y in meiotic segregation. This is likely to have occurred after degradation and loss of the original Y chromosome. If a B element were to segregate away from the X and pass only through males, it would be a refuge for genes with male-limited benefits. Indeed, functional genes on the *Drosophila* Y chromosome appear to be derived from retrotransposed autosomal genes whose only function is in male fertility. The discovery that the Y carries several dyneins expressed in sperm flagella suggests that this chromosome supports post-mating sperm competition between males (Carvalho *et al.* 2000).

Many strategies for solving one problem

Mechanisms to accomplish coordinated regulation of an entire X chromosome, and thus accommodate a degenerated Y chromosome, have arisen independently several times. This follows the establishment of a sex-determining locus. As the primary signals for sex determination are surprisingly fluid, new sex-determining genes arise with some regularity (Bull 1983). A change in the sex-determining gene nominates a pair of former autosomes to differentiate into sex chromosomes. As might be expected from a process that has had multiple independent origins, the mechanics of dosage compensation differ markedly between animal lineages. However, in each well-studied case it involves the recruitment of a pre-existing chromatin regulatory system to modulate the expression of an entire X chromosome.

The initial event in the acquisition of dosage compensation is thought to be an increase in the expression of X-linked genes that have recently lost their homolog on the Y chromosome (Figure 1A). Examination of X-linked and autosomal expression in several mammalian species reveals that expression from X-linked genes is, on average, about twice that from autosomal genes (Gupta *et al.* 2006, Nguyen & Disteche 2006). This trend can be observed in the autosomal *Clc4* gene, which has been translocated to the X chromosome in one mouse species. *Clc4* displays two-fold

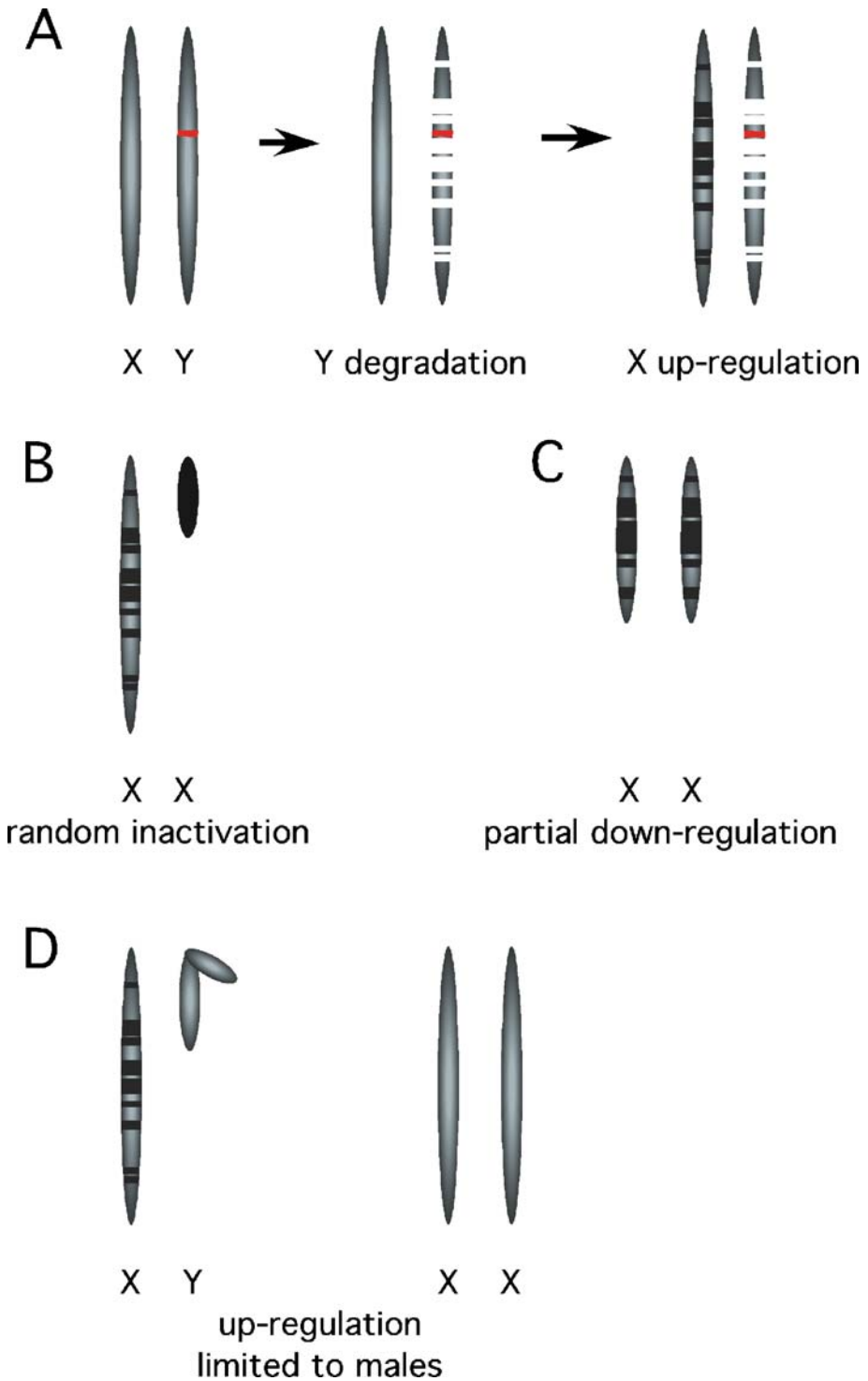

Figure 1. The origin and dosage compensation of differentiated sex chromosomes. **A:** Establishment of a sex-determining locus (red) is followed by degradation of the Y chromosome bearing it. This is represented by gaps in the Y chromosome. X-linked genes then increase expression to compensate for reduction of gene dosage in males (dark bars on X). **B:** Mammalian females randomly inactivate a single X (black oval). The active X is expressed at the same level as the male X chromosome. **C:** *C. elegans* hermaphrodites partially repress both X-chromosomes. **D:** *Drosophila* males produce a complex that activates transcription, but is limited to males. They consequently escape the need to reduce expression of all X-linked genes in females.

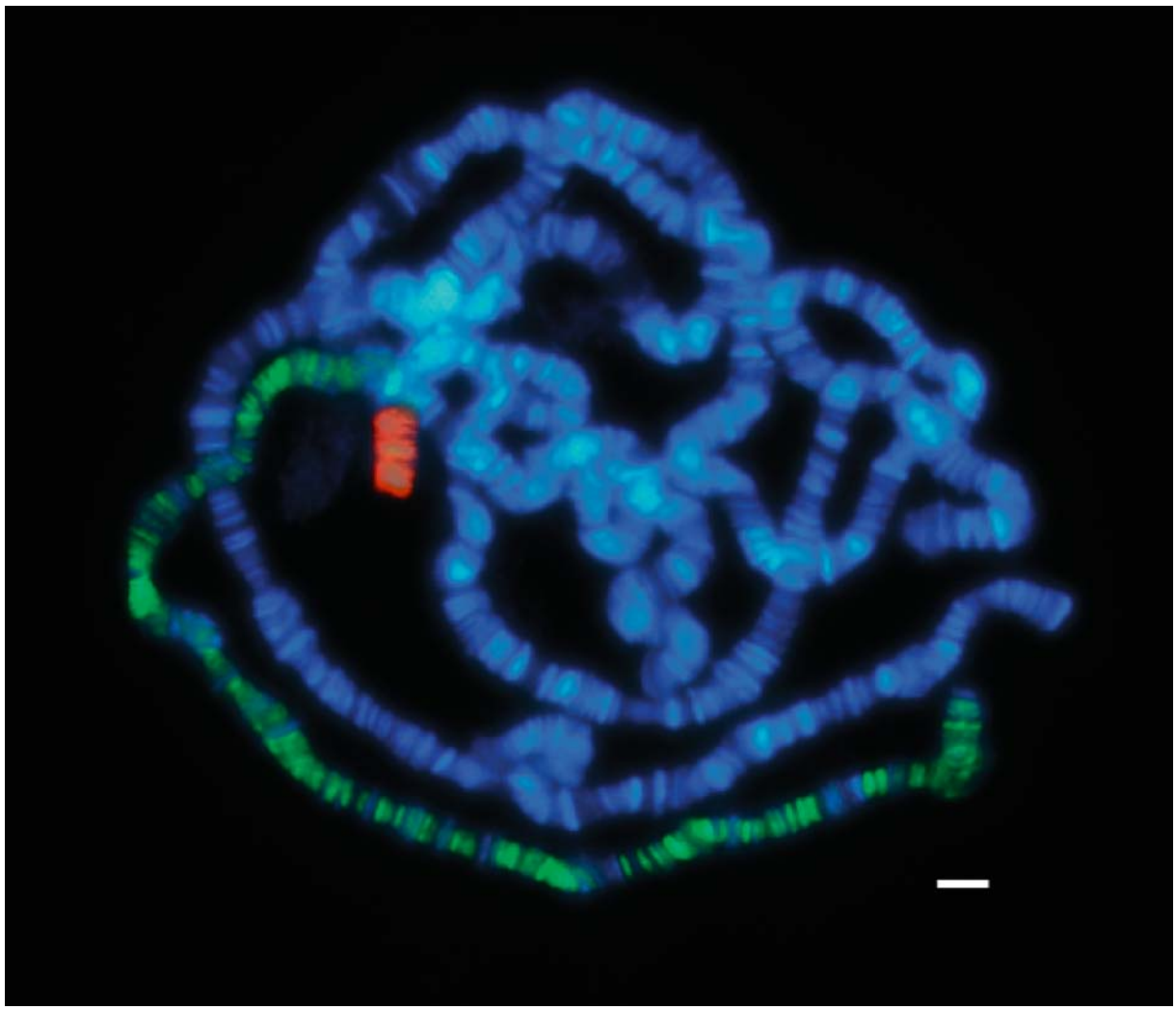

Figure 2. Two chromosome-wide targeting systems exist in *Drosophila melanogaster*. The dosage compensation complex localizes to hundreds of sites along the male X chromosome. The distribution of one protein of this complex, MSL3, is detected in green on a male polytene chromosome preparation. The POF protein, detected in red, paints the fourth chromosome of both sexes. DNA is counterstained with DAPI (blue). The scale bar is 5 μm.

increased expression from the X chromosome site (Adler *et al*. 1997). These findings support the contention that X-linked genes adopt elevated expression to restore normal transcript levels in the hemizygous male. Females, with two X chromosomes, then must undertake a compensatory down-regulation of X-linked genes. This is accomplished in female mammals by silencing one X chromosome (Figure 1B). However, X-linked genes that retain a functional homolog on the Y chromosome escape silencing (Jegalian & Page 1998). This suggests that compensation, accomplished by increased expression in both sexes followed by silencing of one allele in females, occurs on a gene-by-gene basis as genes are lost from the Y chromosome.

A related compensation strategy is used by *C. elegans*. Hermaphrodites with two X chromosomes must reduce X-linked gene expression to avoid lethality, and they do so by down-regulation of both X chromosomes (reviewed in Lucchesi *et al*. 2005). This reduction in X-linked expression in the homogametic sex suggests that *C. elegans* hermaphrodites,

like mammals, must counter elevated expression of X-linked genes (Figure 1C). A comparison of expression intensity between X-linked and autosomal genes in *C. elegans* reveals that expression from the hemizygous X chromosome of males is similar to expression from two paired homozygous autosomes, supporting the idea that worms also increase X chromosome expression (Gupta *et al.* 2006). Reduction of X-linked gene expression in hermaphrodites requires the action of a protein complex that is targeted to both X chromosomes.

Although increased expression of X-linked genes appears to be the initial response to the loss of coding potential from the Y chromosome, the mechanism underlying this increase is unknown. It is possible that X-linked genes are selected for promoter mutations that increase activity. However, examination of expression in mouse germ cells and early zygotes suggests that up-regulation is absent from haploid germ cells but rapidly appears upon fertilization, suggesting a more elaborate mechanism (Nguyen & Disteche 2006).

In contrast to mammals and worms, flies compensate their sex chromosomes using a mechanism limited to males (Figure 1D). A complex of proteins and RNA that forms only in males binds along the length of the X chromosome. This is illustrated by immunolocalization of one of these proteins, detected in green, on the polytene chromosome preparation presented in Figure 2. Proteins in this complex modify chromatin to equalize expression between the single X chromosome and the autosomes. This appears a straightforward solution to the problem, but there are indications that the machinery of compensation may contain additional complexities. Female flies have a mechanism that may act to reduce the expression of some X-linked genes. The Sex lethal (SXL) protein regulates all aspects of sexual differentiation and is present only in females (Cline 1984). SXL is an RNA-binding protein that directs sexual differentiation through its influence on *tra* mRNA splicing (Baker 1989). However, SXL can also reduce the translation of messages containing SXL-binding sites (Beckmann *et al.* 2005). A search for mRNAs with multiple SXL-binding sites revealed a small number of almost exclusively X-linked genes (Kelley *et al.* 1995). One of these, *runt* (*run*), is known to be compensated by a mechanism independent from that used by most X-linked genes (Gergen 1987). The *run* gene also escapes a chromatin modification associated with the increased expression of X-linked genes in males (Smith *et al.* 2001). Insertion of *run* SXL-binding sites into the 3′ untranslated region of a reporter gene was shown to decrease expression in females relative to that in males (Fitzsimons *et al.* 1999). These observations support the idea that some X-linked genes may have increased expression in both sexes. A compensating down-regulation in females is then necessary. Down-regulation of the *run* gene is likely to be mediated by SXL binding directly to mRNA.

Pre-existing regulatory systems are recruited to compensate X chromosomes

In all cases where the molecular basis of compensation is known, it involves the recruitment of chromatin-modifying proteins to the X chromosome. In each instance these proteins participate in a chromatin-binding complex. The members of these complexes have been found to have an ancient association that predates their function in sex chromosome compensation. This suggests that new systems of dosage compensation do not require the emergence of novel regulatory proteins, but the development of a recruiting mechanism that targets pre-existing regulatory factors to an entire chromosome.

Silencing of one of the two X chromosomes of mammalian females is directed by a single locus on the X chromosome, the *Xic* (*X inactivation center*). The large, non-coding *Xist* RNA is transcribed from the *Xic* and directs silencing to flanking chromatin (Andersen & Panning 2003). *Xist* action is limited to the chromosome of its origin and is essential for silencing. It acts by directing repressive complexes to a single X chromosome. Two Polycomb repressive complexes, PRC1 and PRC2, are sequentially recruited to the future inactive X chromosome shortly after *Xist* begins to accumulate on this chromosome (Plath *et al.* 2003, 2004, Silva *et al.* 2003). These complexes modify histones, an early step in an ordered series of chromatin changes leading to a stably inactivated chromosome (Chadwick & Willard 2003). Methylation of CpG islands on the inactive X chromosome occurs subsequently, and may be necessary for stable, long-term maintenance of the silent X chromosome (Mohandas *et al.* 1981, Pfeifer *et al.* 1990).

Using an alternative strategy, *C. elegans* hermaphrodites down-regulate expression of both copies of X-linked genes, thereby equalizing X chromosome expression between XX hermaphrodites and XO males. A complex of proteins that includes members of the mitotic condensin complex, as well as paralogs of condensin subunits, covers both X chromosomes (reviewed by Hagstrom & Meyer 2003). The molecular mechanism by which this complex reduces gene expression is unknown, but the involvement of condensin-like molecules suggests that changes in chromatin architecture are involved.

The fly MSL complex is the prototype of a widely distributed chromatin regulator

The genetic basis of dosage compensation in flies was first revealed by screens for mutations with male-specific lethal phenotypes (Belote 1983). This produced a group of five genes termed the *male-specific lethals* (*msls; maleless* (*mle*), the *male specific lethals1*, *-2* and *-3* (*msl1*, *-2* and *-3*), and *males absent on first* (*mof*)). These genes encode proteins that form a complex which binds selectively to the male X chromosome (reviewed by Meller & Kuroda 2002). Mutation of an *msl* gene causes male lethality as third-instar larvae or pupae, but no *msl* is essential in females. In spite of this, all of the MSL proteins, with the exception of MSL2, are expressed in females. As all members of the complex must be present for dosage compensation, the absence of MSL2 limits this process to males (Kelley *et al*. 1995, Zhou *et al*. 1995). The MSL complex equalizes expression of X-linked and autosomal genes, but precisely how this occurs remains unresolved. The inverse regulator model posits that expression of the entire genome is elevated by aneuploidy, or by hemizygosity of the X chromosome in males (Birchler *et al*. 2003, Pal Bhadra *et al*. 2005). Sequestration of the MSL proteins, MOF in particular, to the X chromosome prevents inappropriate increases in autosomal expression. An alternative model proposes that the MSL complex acts on chromatin to increase X-linked gene expression two-fold in males (Hamada *et al*. 2005, Straub *et al*. 2005).

The *mof gene* encodes an H4 acetyltransferase (Hilfiker *et al*. 1997). MOF acetylates H4 on lysine 16 (H4Ac16), a modification associated with increased transcription (Akhtar & Becker 2000, Smith *et al*. 2000). In *Drosophila*, H4Ac16 is exclusive to the male X chromosome (Turner *et al*. 1992, Bone *et al*. 1994). The MSL complex and H4Ac16 are more highly enriched in the coding regions of compensated genes than on their promoters, supporting the notion that the rate of elongation, rather than promoter activation, accounts for elevated expression (Smith *et al*. 2001, Alekseyenko *et al*. 2006, Gilfillan *et al*. 2006).

While the *msl*s appear to be the only protein-coding genes that are uniquely essential for male survival, several other genes are known to either participate in dosage compensation, or interact genetically with mutations in dosage compensation, but have additional essential functions in both sexes. The most prominent of these is JIL-1, an H3 kinase that is enriched on the male X chromosome (Jin *et al*. 1999, 2000). The male X chromosome is also enriched for H3 phosphorylated on serine 10 (H3pS10), a modification attributable to the JIL-1 kinase and associated with increased expression (Wang *et al*. 2001). In spite of being enriched on the male X chromosome, JIL-1 is not restricted to this chromosome and binds throughout the genome of both sexes. JIL-1 acts to antagonize the spread of heterochromatin, possibly due to an incompatibility between H3pS10 and the heterochromatic histone modification dimethyl H3K9 (Zhang *et al*. 2005). It is possible that the essential role of JIL-1 in females is related to regulation of the heterochromatin/euchromatin balance (Ebert *et al*. 2004).

Two non-coding RNAs, *roX1* and *roX2* (*RNA on the X*), play a central role in dosage compensation. The *roX* transcripts are large and polyadenylated, but dissimilar in sequence. Both *roX* genes have strikingly male-preferential expression that is regulated by one or more members of the MSL complex (Bai *et al*. 2004, Lee *et al*. 2004, Rattner & Meller 2004). Both are X-linked, and their transcripts 'paint' the male X chromosome (Meller *et al*. 1997, 2000). While males with a single *roX* gene are completely normal, survival is sharply reduced in $roX1^{-}roX2^{-}$ males. By contrast, $roX1^{-}roX2^{-}$ females appear normal and are fully viable (Meller & Rattner 2002). Thus, *roX1* and *roX2* are redundant *male-specific lethal* genes. MSL3 and MOF have RNA binding activity *in vitro*, and MLE and MOF may be removed from the X chromosome by RNase A digestion (Richter *et al*. 1996, Akhtar *et al*. 2000,

Buscaino *et al*. 2003). This suggests a central role for RNA in assembly or organization of the MSL complex. In accordance with this idea, the MSL proteins can be coimmunoprecipitated with one another, the *roX* RNAs, and JIL-1 kinase (Copps *et al*. 1998, Jin *et al*. 2000, Meller *et al*. 2000, Smith *et al*. 2000).

The discovery that the yeast NuA4 transcriptional regulator contains subunits with similarity to MSL3 and MOF (Eaf3p and Esa1p) suggests that some members of the complex have an ancient association (Eisen *et al*. 2001). Homologs of all of the protein-coding *msl* genes have also been identified in mammals (Lee & Hurwitz 1993, Marin 2003). A complex containing MSL homologs, with the exception of MLE, has been isolated from human cells (Smith *et al*. 2005, Taipale *et al*. 2005). MLE, an RNA/DNA helicase, appears to have a more peripheral association with the complex in flies, and it is possible that its primary role in dosage compensation is to integrate the *roX* transcripts into the complex (see Meller 2003). However, MLE may also have a general role in transcription of some X-linked and autosomal genes (Kotlikova *et al*. 2006). Human MOF (hMOF) participates in multiple protein assemblies and is responsible for the majority of H4Ac16 acetylation in the cell. Interestingly, hMOF is required for normal function of human ATM (ataxia–telangiectasia-mutated) protein, reinforcing the idea that homologs of proteins necessary for dosage compensation in one species may have quite different roles in other organisms (Gupta *et al*. 2005).

Recognition of an entire chromosome

The RNA and proteins that mediate X chromosome compensation in flies can be presumed to perform several functions. One of the most critical of these is selective recognition of the X. The molecular basis of targeting systems that direct compensation to an entire chromosome remains the most mysterious aspect of dosage compensation. Exploration of the basis of X chromosome binding by the MSL complex suggests that multiple factors influence the striking selectivity of localization. Flies provide an additional twist: the *D. melanogaster* fourth chromosome is specifically decorated in both sexes by Painting of Fourth (POF) protein (Larsson *et al*. 2001). Immunolocalization of POF (detected in red) is illustrated on the chromosome preparation presented in Figure 2. This is particularly intriguing, as it appears likely that the fourth chromosome was once part of a dosage-compensated X chromosome. It is therefore possible that global recognition of the fourth chromosome is derived from a system for X chromosome recognition. This, combined with the extensive analysis of evolutionary processes that is being done in *Drosophila,* make flies an outstanding model for exploration of chromosome targeting and sex chromosome evolution.

Clues emerging from studies of dosage compensation in mammals, *C. elegans* and flies point to mechanisms that combine X-linked DNA sequence elements with spreading of modifications from *cis*-acting sites. Together these mechanisms direct modification to an entire chromosome. However, the relative importance of these elements is strikingly different in each system. The most straightforward situation appears to be mammals, where the *Xic* provides a single strong, *cis*-acting element that directs silencing to flanking chromatin. Placement of an *Xic* on an autosome by translocation is sufficient for at least partial silencing of that autosome (White *et al*. 1998). *Xist* is necessary for inactivation, and coats autosomes silenced by ectopic *Xist* expression (reviewed by Nusinow & Panning 2005). This demonstrates the profound ability of the *Xic* to direct silencing in *cis*, as well as the absence of local DNA elements on the X that are essential for silencing. But while silencing of autosomal chromatin will occur, silencing does not spread as far or repress as stably as when it occurs on the X chromosome (White *et al*. 1998). Thus, X-linked sequence elements that promote the spread and maintenance of silencing have been proposed. Intriguingly, a class of LINES that is enriched on the X chromosome does appear to facilitate the spread of silencing into X:A translocations (Lyon 1998, Bailey *et al*. 2000).

X to autosome translocations have also been used to probe the process of X chromosome recognition in *C. elegans*. These studies reveal that worms target modification to their X chromosomes by a combination of *cis*-acting elements and spreading from these elements to coat the entire X chromosome. Translocations have identified several regions of the X chromosome that are capable of attracting the dosage compensation complex, and some regions that do not (Csankovszki *et al*. 2004, Lieb *et al*. 2000). Interest-

ingly, a large region that was incapable of recruiting the compensation machinery when detached from the X was coated by these proteins if located on the X chromosome. This suggests a plausible model for X chromosome recognition in *C. elegans* involving widely spaced *cis*-acting sites and spreading from these to coat intervening chromatin that lack recruiting elements.

Flies have a system that shares aspects of mammalian compensation, but includes a strong helping of X-linked 'identity' elements. As the driving force for dosage compensation is a progressive degeneration of the Y chromosome, a gene-by-gene mechanism of recognition and modification seems likely. An early view was that *cis*-acting elements close to individual genes served as targets for the MSL complex (reviewed by Baker *et al.* 1994). Analyses of transgenes carrying the dosage compensated *white* (*w*) gene support the idea that sequences close to genes contribute to compensation (Qian & Pirrotta 1995). So far, no sequence determinants for this targeting have been identified.

An alternative model based on a limited number (35–40) of chromatin entry sites (CES) followed by spreading of the MSL complex has been proposed (Kelley *et al.* 1999). This model relies in part on the observation that in males mutant for *mle, msl3* or *mof*, MSL1 and MSL2 are bound to this limited group of sites (Lyman *et al.* 1997). On the other hand, mutations in *msl1* or *msl2* release the entire complex from the X chromosome. Spreading of the MSL complex from the CES into the surrounding chromatin is supported by the fact that the *roX* genes overlap the two strongest CES. Spreading in *cis* from autosomal insertions of the *roX* genes is well documented and is dependent on the levels of MSL proteins that are available (Kelley *et al.* 1999, Park *et al.* 2002, Kelley & Kuroda 2003). Recently it has been shown that the X chromosome harbors a larger number of binding sites of different strengths. The ability of these sites to recruit the MSL complex depends on the presence and concentration of protein subunits available (Demakova *et al.* 2003, Dahlsveen *et al.* 2006). Sufficiently large X chromosome regions lacking strong recruitment sites can still recruit the MSL complex when transposed to an autosome or inserted into autosomal sites (Fagegaltier & Baker 2004, Oh *et al.* 2004). In addition, no spreading into autosomal regions flanking these insertions could be observed, nor was there spreading into autosomal regions transposed onto the X chromosome. This led Fagegaltier & Baker (2004) to propose a model based not on linear spreading, but on a progressive binding of the MSL complex from high-affinity sites to sites of lower affinity. However, the lack of spreading into autosomal regions transposed to the X chromosome, and X regions transposed to autosomes, may also be a consequence of selection against spreading in these stocks (Lucchesi *et al.* 2005). The question remains whether spreading is limited to *roX*-containing transgenes, or can occur from other fragments that attract the MSL complex. Nine non-*roX* sites that attract the MSL complex have so far been analyzed as transgenes inserted on autosomes (Oh *et al.* 2004, Dahlsveen *et al.* 2006). Only in rare cases was spreading or binding to additional nearby sites observed. This additional binding depends on concentration of MSL complex components and on the surrounding chromatin (Dahlsveen *et al.* 2006). If binding to additional sites should be called spreading or not is to some extent a matter of definition.

Recent evidence points to a role for active transcription in attraction of the MSL complex to individual genes. MSL is attracted to a site of Gal4-induced expression on the X chromosome (Sass *et al.* 2003). MSL enrichment is also much more likely at transcribed genes than at those with undetectable levels of transcript (Alekseyenko *et al.* 2006). These observations, and H4Ac16 enrichment in the body of compensated genes, rather than at the promoters, suggests that attraction of the MSL complex and chromatin modification may be co-transcriptional (Smith *et al.* 2001).

Not only do the *roX* genes provide *cis*-acting sites that can attract the MSL complex and direct its spread into surrounding chromatin, but they are also the source of RNAs that are essential for X recognition. Although the importance of *roX* action *in cis* to its site of synthesis remains unclear, the requirement for at least one of the *roX* transcripts for correct localization of the MSL complex is well established (Meller & Rattner 2002). Unlike mammalian *Xist*, *roX* RNA originating from an autosome can rescue $roX1^{-}roX2^{-}$ males and direct MSL localization to the X chromosome.

Flies are unique in having a second chromatin-targeting system that may be contrasted to X-chromosome recognition. The small fourth chromosome is coated with Painting of Fourth (POF) in a

manner that appears superficially similar to MSL coating of the X chromosome (Larsson *et al*. 2001). But while POF paints the fourth chromosome, it is not attracted to large segments of the fourth chromosome that are translocated to other chromosomes. This has prevented the identification of entry sites by mapping of translocations. The fourth chromosome is enriched for several mobile elements and is heavily heterochromatic, giving it a unique composition (Miklos *et al*. 1988, Sun *et al*. 2000). It is possible that sites of differential POF affinity exist on the fourth chromosome, but depend on nearby heterochromatin to be functional.

While it is expected that recognition of the *Drosophila* X and fourth chromosomes will involve the contribution of local sequence determinants, none has been identified at this time. Both the *roX* high-affinity sites and the nine additional high-affinity sites contain GAGA-like elements, suggesting a role for these sequence elements in recognition (Park *et al*. 2003, Dahlsveen *et al*. 2006). To identify sequence determinants for targeting is an important future task, and advances have been made using multivariate analysis of genome sequences from three *Drosophila* species to identify fourth chromosome-specific sequences, and sequences correlating to POF binding (Stenberg *et al*. 2005). One element, a nonamer pair found in the *Drosophila* DINE-1 element, was shown to be significantly enriched at cytologically determined POF binding locations. Although these elements are by themselves not sufficient to recruit POF, they may be involved in the targeting of POF binding. Furthermore, both exon and non-exon fragments of the X chromosome can be distinguished from autosomal fragments using this methodology (Stenberg *et al*. 2005). An important test of this bioinfomatic approach will be to see if elements that distinguish the X chromosome are involved in targeting of the MSL complex. However, mapping of MSL binding along the length of the X chromosome has revealed enrichment in the body of most transcribed genes (Alekseyenko *et al*. 2006, Gilfillan *et al*. 2006, Legube *et al*. 2006). This suggests that the final distribution of MSL proteins is determined in part by RNA polymerase activity. If the mature pattern of MSL binding to the X chromosome is created by the interaction between chromosome-specific sequence elements and the propensity of the MSL complex to bind and modify transcribed regions, it may prove difficult to pinpoint the sequence determinants of chromosome identity from analysis of the regions that are coated by the MSL complex.

The end of compensation

The establishment of a new mechanism for sex determination will, in most cases, nominate a pair of autosomes to become the new sex chromosomes. In addition to triggering differentiation of the new XY pair, the organism acquires an autosome that brings with it a global targeting mechanism. Can these mechanisms be adapted for coordinated regulation of groups of autosomal genes? The regulation of contiguous groups of genes is a common feature of the genomes of higher eukaryotes, and may be necessitated by large genome size and complexity. Regulation may be quite complex, for example, at the imprinted gene clusters in mammals (reviewed by Verona *et al*. 2003). Imprinted clusters are groups of genes with mono-allelic expression patterns established in the parental germ lines. Imprinted loci of mammals share several similarities with the *Xic*, and an argument has been made for their derivation from a duplication of an *Xic* (Huynh & Lee 2001). Controlling elements that direct the expression of surrounding genes regulate the X chromosome as well as imprinted gene clusters. In the case of the X chromosome, non-coding *Xist* RNA transcribed from the *Xic* is essential for silencing of genes in flanking chromatin (reviewed by Chadwick & Willard 2003). The enormous range of *Xist*, which silences an entire chromosome, is striking. However, other aspects of *Xic* influence on gene regulation are echoed on a reduced scale at other locations of the mammalian genome. Large non-coding RNAs that are transcribed in a mono-allelic fashion from imprinted loci are pervasive (recently reviewed by O'Neill 2005). Although the function of these transcripts is often elusive, in some instances they have been found essential for imprinted expression of genes within the cluster (Jones *et al*. 1998, Luikenhuis *et al*. 2001, Sleutels *et al*. 2002, Thakur *et al*. 2004). These similarities between X inactivation and imprinting have inspired the suggestion that *Xic* duplications gave rise to imprinted loci, but 'retirement' of an X

chromosome upon designation of a new sex chromosome pair would also free the targeting mechanism to assume a new role, perhaps to direct imprinting.

A related situation could occur if an X chromosome breaks to form an X and a new autosome. This is proposed to have occurred > 50 mya in an ancestor of the modern *D. melanogaster* lineage, giving rise to the modern X and fourth chromosomes (Tamura *et al.* 2004). This is a particularly intriguing situation as the fourth chromosome of *D. melanogaster* is the only autosome known to bind a chromosome-specific protein (Larsson *et al.* 2001).

Did an ancestral X chromosome produce the modern X and fourth chromosomes?

Different naming principles have been used to identify the *D. melanogaster* chromosomes. For an evolutionary discussion it is most convenient to use the element names, where A corresponds to the *D. melanogaster* X chromosome, B, C, D and E to the *D. melanogaster* 2L, 2R, 3L and 3R chromosome arms, respectively. The F-element corresponds to the *D. melanogaster* fourth, or dot, chromosome (Muller 1940). These elements are well conserved in evolution and the major differences between species are paracentric inversions and whole chromosome arm fusion and fissions. The elements A through E are roughly the same size, while the F element is typically a small, dot-like chromosome. Despite this size difference, the F element is remarkably well conserved as a unique chromosome in most species (summarized by Ashburner *et al.* 2005). In only a few instances is the F element fused to other chromosomes. In *D. busckii* and *Scaptodrosophila lebanonensis* the F element is fused with the X chromosome (element A), while in *D. willistoni* the F element is fused to element E (Krivshenko 1955, Papaceit & Juan 1998).

Substantial evidence points to a relationship between the F element and the X chromosome. In *D. busckii* the F element is located at the base of the X and the Y chromosomes. The F element portion of the *D. busckii* X chromosome is separated from the A element by the nucleolus organizer (NO). The F element on the Y chromosome is seen in polytene chromosome preparations as a euchromatic banded region (Krivshenko 1952, 1955, 1959). Krivshenko also characterized three loci in the F-element portion of the *D. busckii* X chromosome whose mutant phenotypes mimic the *D. melanogaster* mutants *Cell*, *shaven* and *cubitus interuptus*. These genes are situated on the fourth chromosome of *D. melanogaster* (Krivshenko 1955, 1959). The fourth chromosome of *D. melanogaster* has also been ascribed a minor role in sex determination. The primary signal for sex determination in *Drosophila* is the X to autosome ratio. Flies with two X chromosomes become female, and those with only one become male. Manipulation of the X:A ratio can produce mosaics of male and female tissue (reviewed by Cline & Meyer 1996). In contrast to the other autosomes, the fourth chromosome has 'female tendencies', shifting 2X:3A intersexes towards female development when its dosage is increased, and towards male development when decreased (Bridges 1925, Fung & Gowen 1960). The fourth chromosome therefore behaves more like an X chromosome than a typical autosome in promoting sex determination. A triplo-4 condition causes an increased frequency of X chromosome nondisjunction, suggesting a tendency of chromosome 4 to pair with the X in meiosis (Sandler & Novitski 1956). In contrast to other autosomes, but like the X chromosome, flies carrying a single chromosome 4 are viable and fertile. This and other observations prompted the suggestion of a dosage compensation mechanism for the fourth chromosome (Hochman 1976). An alternative explanation for the haplosufficient nature of the fourth chromosome is that this chromosome is very small. In general, deletions spanning more than one of Bridges' numbered divisions, i.e., ~800–1500 kb, are lethal (Lindsley *et al.* 1972). The banded and sequenced region of the fourth chromosome is 1280 kb, but its total length is estimated at 4.5–5.2 Mb (Locke & McDermid 1993). It should also be stressed that long haplosufficient deletions have been actively screened for, while the fourth chromosome is a natural-occurring haplosufficient chromosome. Thus, the fourth chromosome is the only haplosufficient autosome and the longest haplosufficient autosomal region in *D. melanogaster*. We can conclude that the F element shows a strong relationship to the X chromosome and it is tempting to argue that the fourth chromosome originates from the X. It should be noted though that this issue is not resolved and the

conclusion drawn by Krivshenko (1959) is still valid: 'the present condition of the microchromosome of *D. melanogaster* is not primary, nor is that of the short euchromatic elements of the X and Y of *D. busckii*'.

Retention of a global targeting mechanism by the fourth chromosome

The relation between the fourth and X chromosomes extends beyond cytological and genetic data. The *D. melanogaster* fourth chromosome, like the X chromosome, has a unique, chromosome-wide targeting mechanism that is revealed by POF decoration of the fourth chromosome in both sexes. POF binds throughout the entire euchromatic portion of the fourth chromosome (Larsson *et al.* 2001). POF binding is lost in translocations that attach euchromatic regions of the fourth to other chromosomes. However, in instances where the translocated distal 4 (d4) is able to pair with an intact fourth chromosome, POF binding is restored to the translocated fragment, suggesting spreading *in trans*. Material from other chromosomes, when translocated to the tip of the fourth chromosome, is not labeled by POF. POF association with the fourth chromosome thus appears to nucleate in the basal region of this chromosome and spread *in cis* or *in trans* to coat the length of the fourth chromosome (Larsson *et al.* 2001). Inability to spread into chromatin transposed onto the fourth chromosome suggests a requirement for chromosome 4-specific sequence or chromatin structure.

POF represents the first example of a protein specifically targeted to a single autosome, and the question arises whether its association with chromatin is functional. The high degree of conservation of POF binding to the F element would suggest that it is. Several species within the genus *Drosophila*, e.g., *D. virilis* and *D. pseudoobscura*, also display POF localization to the F element (Larsson *et al.* 2004). In several species the banded region of the F element has been inverted relative to *D. melanogaster*, e.g., in *D. virilis* and *D. simulans*, but POF binding is unaffected by these inversions, indicating that gene order does not influence targeting (Podemski *et al.* 2001, Larsson *et al.* 2004). In *D. busckii*, where the fourth chromosome counterpart is located at the base of the X chromosome, POF decorates the entire X chromosome, but is limited to males. In this species POF co-localizes with H4Ac16, a marker for dosage compensation (Larsson *et al.* 2001, 2004). In *D. ananassae* and *D. malerkotliana* POF binds to the highly heterochromatic F element in both sexes, but also paints the male X chromosome, where it co-localizes perfectly with MSL3. These findings support the idea of a shared ancestry between the F element and the X chromosome, but also suggest that POF may have been part of an ancient dosage compensation mechanism. When expressed in *D. melanogaster*, *D. ananassae* POF stains only the fourth chromosome, not the male X. This indicates that the chromosome specificity is not determined by variations in the POF protein, but by some other factor. POF has a predicted RNA-binding domain (RRM1) in the central part of the protein. It is tempting to speculate that a non-coding RNA is involved in targeting of POF to the fourth chromosome. However, the existence of such RNA, as well as the function of the POF protein, remains to be discovered. The fourth chromosome is unusual in being highly heterochromatic. If the fourth chromosome indeed originated from the X chromosome, heterochromatinization may have evolved as a way to repress hypertranscription. The retention of POF might be necessitated by this repressive influence (Larsson *et* al. 2004). In the event that a regulatory function is assigned to POF, it will represent the first instance of gene regulation directed to an entire autosome.

The power of sex

The differentiation of sex chromosome pairs has given rise to powerful chromosome-wide regulatory mechanisms that govern the sex chromosomes, and may have also contributed to systems that regulate small or large clusters of autosomal genes. The discovery that the *D. melanogaster* fourth chromosome retains a global targeting mechanism, possibly originating from its former status as part of a dosage-compensated X chromosome, suggests that these targeting mechanisms have an unusual longevity after their role in compensating sex chromosomes is over. The identification of two chromosome-wide targeting mechanisms in *D. melanogaster* emphasizes the value of this system for the study of

chromosome recognition. It further supports the importance of comparative evolutionary studies using the *Drosophila* lineage.

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