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Molecular Features and Functional Constraints in the Evolution of the Mammalian X Chromosome

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ABSTRACT Recent advances in genomic sequencing of multiple organisms have fostered significant advances in our understanding of the evolution of the sex chromosomes. The integration of this newly available sequence information with functional data has facilitated a considerable refinement of our conceptual framework of the forces driving this evolution. Here we address multiple functional constraints that were encountered in the evolution of the X chromosome and the impact that this evolutionary history has had on its modern behavior.

KEYWORDS retrotransposition, sex determination, meiotic sex chomosome inactivation, MSCI, epigenetics, sexual antagonism, sex-biased genes

INTRODUCTION

The modern X chromosome of mammals is very different from autosomes. First, due to its special role in sex determination, the X chromosome is present in one copy in males and in two copies in females. One consequence of this unequal representation of the X chromosome between sexes is the evolution of X inactivation. To compensate for the unequal dose, one of the copies of the X is inactivated in females. As a result, as in males, only one copy of the X is transcriptionally active in females. These profound differences between the autosomes and the modern X chromosome did not emerged instantaneously. They are the result of evolutionary selection over a long period of time. Currently these differences themselves are acting as an evolutionary force and are driving the evolution of the X chromosome even further. Here we address the multiple evolutionary implications of the specific functions of the sex chromosomes. The evolution of the X chromosome is intricately linked to the evolution of its male counterpart, the Y chromosome. Therefore, to understand the evolution of the X chromosome, we also must consider the history of the Y chromosome. Many extensive reviews detail various aspects of X chromosome evolution (Ayling & Griffin, 2002; Bull, 1983; Charlesworth, 2002; Charlesworth *et al.*, 2005). In our review, we will focus on several recent developments related to the evolutionary history of the mammalian chromosome X and inevitably will omit some important topics covered elsewhere.

As previously mentioned, the most obvious and, perhaps, the most important difference between sex chromosomes and autosomes is the unequal dose of the

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sex chromosomes in males and females. This discrepancy in the copy number of the X chromosome in the two sexes results in multiple characteristic features distinguishing the X chromosome from autosomes. One major consequence of the unequal representation is the evolution of such unique chromosome-wide regulatory mechanisms as dosage compensation (Heard, 2004). The need for dosage compensation arises from the twofold excess of X-linked genes in females over males. Because there is only one copy of the X in males, there are fewer X chromosome copies than for autosomes in the population. Lower effective population size results in a lower population diversity for the X (Sachidanandam *et al.*, 2001). Another consequence of the unequal representation is the accumulation of sex-specific genes on the X and Y chromosomes. The autosomes and X chromosome in females have a homologous chromosome with which to pair and recombine, but the lone X chromosome in males does not recombine. Many of the special features of the X chromosome have evolved as a result of the lack of meiotic recombination for the X in males. For example, the lower mutation rate observed on the X is partly explained by its lower average recombination rate (Gaffney & Keightley, 2005). In addition, there are some unusual and unexpected differences, such as an excess of inverted repeats (Warburton *et al.*, 2004), long interspersed nuclear element (LINE) retrotransposons (Ross *et al.*, 2005), and a higher density of genes involved in cognition (Zechner *et al.*, 2001).

An important milestone in the study of the X chromosome is the recent publication of the complete sequence of the human chromosome X (Harsha *et al.*, 2005; Ross *et al.*, 2005). The sequence of the Y chromosome is also known (Skaletsky *et al.*, 2003). In addition, while not yet finished, the mouse X chromosome is also close to completion and is at a stage suitable for comparative analysis (Waterston *et al.*, 2002). The recently available completed genomic sequences are a very rich source of evolutionary information. Therefore it is not surprising that some of the details of the evolution of the sex chromosomes have only become evident after a careful analysis of the completed sequences of the X chromosome in multiple species (Ross *et al.*, 2005). The sequencing of the chicken genome has also been an important landmark in our understanding of the evolutionary history of the sex chromosomes (Hillier *et al.*, 2004). Birds have sex chromosomes, but their sex determination system is different from that of mammals (discussed below). Sequence comparison has clearly established the independent origin of mammalian and bird sex chromosomes. Several other recent important advances relate to the inactivation and reactivation of the X chromosome in early embryonic devlopment (Huynh & Lee, 2003; Mak *et al.*, 2004; Okamoto *et al.*, 2004) and the gene content of the X (Reinke, 2004). These newly available data permit testing evolutionary models that until recently were subject exclusively to theoretical analysis and modeling. As a result, we now understand better than ever the forces driving the evolution of sex chromosomes and the various stages of this process.

WHY SEX CHROMOSOMES?

It is widely believed that sexual reproduction offers significant benefits in adaptability and viability (Charlesworth, 1996, 2002; Zarkower, 2001). Although the exact nature of the evolutionary advantages is still debated and may appear somewhat counterintuitive, an overwhelming majority of contemporary species have a sexual mode of reproduction (Otto & Lenormand, 2002). This widespread utilization of a sexual reproduction strategy strongly supports the notion that sex is indeed beneficial from an evolutionary point of view. Once sexual reproduction is the chosen method, sex determination mechanisms evolve, and dimorphic sex chromosomes are its typical byproduct.

However, not all of the consequences of sex are beneficial from an evolutionary standpoint. Sexual reproduction requires a higher energy investment in reproduction and decreases the degree of genetic relatedness between parents and their offspring. The higher energetic efficiency of asexual reproduction is, perhaps, reflected in the bias of parthenogenic (that is, multiplying by simple division) species for higher latitudes, higher altitudes, and arid environments ("geographical parthenogenesis"). Impressive examples of geographical parthenogenesis are found in the Australian desert, where several species have independently reverted to an asexual mode of reproduction (Kearney, 2003). Moreover, while the majority of asexual species have been short-lived and have become extinct, sexual reproduction is not a prerequisite for the prolonged survival of a species. Ancient asexual animal groups, such as bdelloid rotifers and *Darwinulidae*, have been propagating without sexual reproduction for tens of millions of years (Butlin, 2002; Judson & Normark, 2000; Mark Welch *et al.*, 2004; Martens *et al.*, 2003; Welch & Meselson, 2000).

The key distinction of sexual reproduction over simple division is genetic recombination and exchange of genetic material among individuals. As a rule, recombination is not necessary for the completion of cellular division in mitosis, but recombination between homologous chromosomes is required to complete meiosis in mammals. Moreover, even if recombination occurs in a clonal organism, there will be no exchange of genetic material between individuals. Recombination allows for a much faster spreading and fixation of beneficial mutations in the population (Rice, 2002). The price for this flexibility is the need to maintain a substantially more complicated system of cell division, that of meiosis I. While it has been a very challenging task, considerable progress in experimentally testing the evolutionary advantages of sexual recombination was achieved recently, and the results strongly support the adaptive advantage of recombination (Goddard *et al.*, 2005; Hoekstra, 2005; Rice, 2002). It can be argued that simple models that support the advantages of recombination and sexual reproduction are too artificial to adequately reflect evolutionary forces. However, the strongest evidence for the advantages of sex comes from its widespread prevalence among modern species.

Currently, a huge variety of mechanisms of sex determination exists in different organisms (Ayling & Griffin, 2002; Charlesworth, 2002; Charlesworth & Charlesworth, 2005). Most likely, the starting point for the evolution of modern mammals was a cosexual organism (Charlesworth, 1991). In the cosexual state, male and female functions are expressed in the same individual. Later these different developmental pathways have been separated between individuals, leading to the formation of different sexes and sex determination systems. The ancestral state of sex determination systems was most likely environmental sex determination (ESD). ESD is currently found in a variety of cold-blooded vertebrates (where the environmental stimulus is temperature) and in some other species, such as mermithid nematodes or marine echiurid worms (where sex may be determined by the nutritional status or the presence of females) (Charlesworth, 1991, 2002). The next stage in the evolution of sex determination systems was most likely genic (sex-determining gene[s] promoting the development of males or females independent of the surrounding environment) and, eventually, chromosomal sex determination (sex determined by the set of chromosomes present in an organism). The simplest and most often used explanation for the development of chromosomal sex determination is via the appearance of male- and female-sterility mutations (Charlesworth, 1991) (Figure 1). The fixation of antagonistic mutations resulted in the formation of primitive sex chromosomes (Ayling & Griffin, 2002; Charlesworth *et al.*, 2005). Then, additional sexually antagonistic (beneficial for one sex but harmful for the opposite sex) mutations were acquired and finally resulted in the formation of heteromorphic sex chromosomes.

Sex determination systems are very diverse and are ever evolving (Bull, 1983; Charlesworth & Charlesworth, 2005). The diversity is, perhaps, explained by the fact that sex determination is a hierarchical regulatory network in which a signal is transmitted downstream and eventually results in one sex or the other. This developmental pathway has multiple upstream regulators. Any of these upstream regulators can be used for sex determination, and even more than one mechanism can be utilized in one species (Zarkower, 2001). Chromosomal sex determination has evolved independently upon multiple occasions as, for example, in birds and mammals. The most commonly used and, perhaps, the simplest and most stable system is that of a pair of heteromorphic sex chromosomes such as the X and Y chromosomes used by most mammals (XY males and XX females) or that of the Z and W chromosomes common in birds (ZZ males and ZW females). More complex examples include systems with an increasing number of sex chromosomes (X_1Y_1 X_2Y_2 and many other variations) or sometimes with a single X chromosome (XX/XO), like in *C. elegans* and some mole voles (Zarkower, 2001). Perhaps one of the most extreme examples is the sex determination system of the platypus, with 5 X and 5 Y chromosomes (Rens *et al.*, 2004). Even two sexes are apparently not the absolute limit. It has been reported that some species of ants have three or even four different sexes, all of which are necessary for reproduction (Parker, 2004). Despite this great variability of sex determination systems a number of common themes have emerged. These themes include an overall similarity in the design of sex chromosomes and a conservation of downstream regulators. Such conservation is consistent with deep evolutionary roots for sex determination systems. Common elements that arose in the ancestral sex-determination system are still discernable at the bottom of the regulatory hierarchy, and top-level regulators have been recruited independently in different species (Zarkower, 2001).

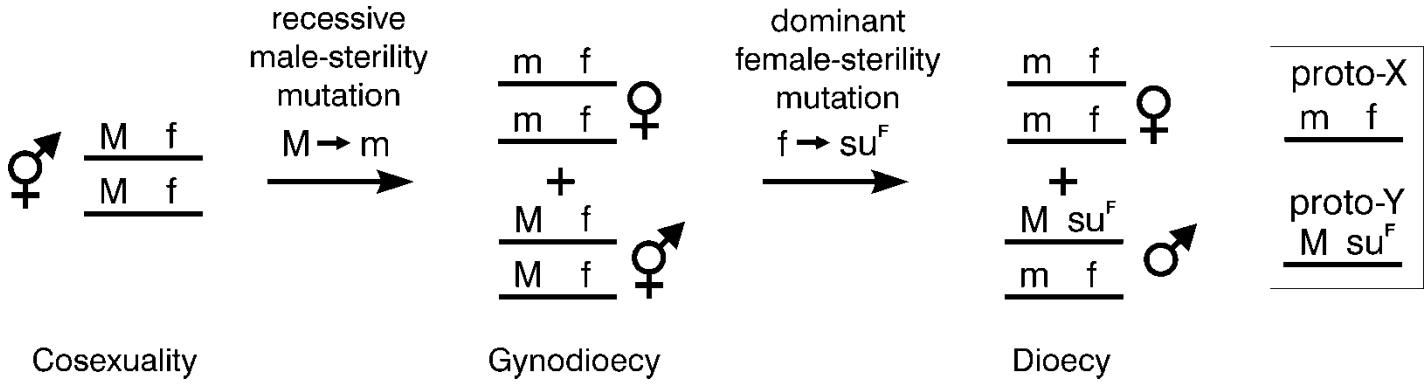

FIGURE 1 **One possible mechanism of evolution of chromosomal sex determination from the initial cosexual state. The important feature of the process outlined is the need for two suppressor mutations. The creation of double suppressor mutant chromosomes is strongly disfavored by selection and is the primary reason for the initial inhibition of recombination between primary sex determining loci. M and f are male and female fertility loci, respectively. Upper case and lower case characters denote dominant and recessive alleles, respectively. Figure is adapted from Charlesworth (Charlesworth, 1991).**

EVOLUTION OF THE CURRENT STRUCTURE

The modern heteromorphic sex chromosomes of mammals differ greatly from each other in both sequence and structure. For example, the human X chromosome is about three times longer than the Y chromosome and contains a nearly 10-fold excess of active genes (Ross *et al.*, 2005; Skaletsky *et al.*, 2003). However, there is overwhelming theoretical and experimental support for the idea that the mammalian sex chromosomes are derived from a single ancestral autosomal pair.

Although the fundamental forces driving the evolution of the sex chromosomes are well established, the details of the process are less understood. Currently it is believed that the evolutionary process that led to the current sex chromosomes was a stepwise process that began between 300 MYR and 210 MYR ago, at the times of the split of birds and monotremes from the mammalian lineage, respectively (see Figure 2). Monotremes have sex chromosomes that are related to mammalian sex chromosomes, but the Z and W chromosomes found in birds are completely unrelated to the X and Y (Graves, 1996; Rens *et al.*, 2004). The independent origin of XY and ZW chromosome pairs has been decisively established by a comparison of mammalian genomic sequences with the chicken genomic sequence (Hillier *et al.*, 2004; Kohn *et al.*, 2004). The X chromosome of mammals is related to autosomes in chickens and vice versa, that is, the chicken Z chromosome consists of segments homologous to human autosomes.

The key driving force for diversification is the suppression of recombination between the X and Y chromosomes. The suppression of recombination around the sex-determination locus is a mandatory element of modern theories of sex chromosome differentiation. It was recognized long ago that there is an evolutionary advantage in suppressing recombination between sex-determining genes (Nei, 1969). Next, there is an advantage in establishing and maintaining a strong linkage of sex-determining genes with either male- or female-beneficial sexually antagonistic mutations. The logic here is the same as that for the initial development of the suppression of recombination between two ancestral sex-determining genes (see Figure 1). For example, the linkage of female-deleterious gene with a primarily sex-determining female-beneficial region would lead to a decreased fitness of the offspring and would be selected against. Over time, the sequential addition of interacting genes would gradually lead to the suppression of recombination on the whole chromosome. The non-recombining portion of the Y is restricted only to males. Selection only in males results in the accumulation of male-specific functions, such as those for spermatogenesis or sex determination, on the Y chromosome (Rogers *et al.*, 2003; Vallender & Lahn, 2004). Many of those male-beneficial mutations can be harmful to females and as such these mutations are sexually antagonistic. The degradation of the Y also promotes further divergence of the X from its autosomal predecessor. After some time, when the non-recombining Y has sufficiently diverged and some of the genes on the Y become inactive, the X chromosome also starts accumulating sexually antagonistic mutations (for a more detailed discussion, see below and "Gene content" section) (Fisher, 1931; Rice, 1984, 1992). The process is essentially unidirectional, and it is nearly impossible to restore recombination between suppressed regions after a relatively short time. Let us consider the likely sequence of events for mammals. In the early stages the sex-determination region was quite small, perhaps just a single gene, *e.g.*, the sex-determining region Y gene (*SRY*), (Graves, 2001) and its vicinity. Suppression of recombination between the proto-Y with the X chromosome over this sex-determination region led to the accumulation of a number of linked male-beneficial mutations on the proto-Y and thus to further divergence (Figure 3).

A non-recombining chromosome (or, more generally speaking, any non-recombining region) should degenerate purely on statistical grounds ("Muller's ratchet") (Muller, 1918). For sex chromosomes, it is impossible to rescue a degenerate sequence by recombination with the other copy of the chromosome, as is the case with autosomes. A similar logic explains the accumulation of mobile DNA elements on the Y chromosome. Once these elements insert themselves into the Y chromosome, it is impossible to remove them by simple and relatively benign recombination with a homologous chromosome derived from the other parent. Therefore, a stepwise regional loss of recombination should have resulted in the progressive degeneration of the proto-Y chromosome. While the process is more complicated than originally thought (Charlesworth, 2002, 2003; Charlesworth & Charlesworth, 2000), the end result is the same—the degeneration and accumulation of satellite sequences on the non-recombining chromosome. The degeneration

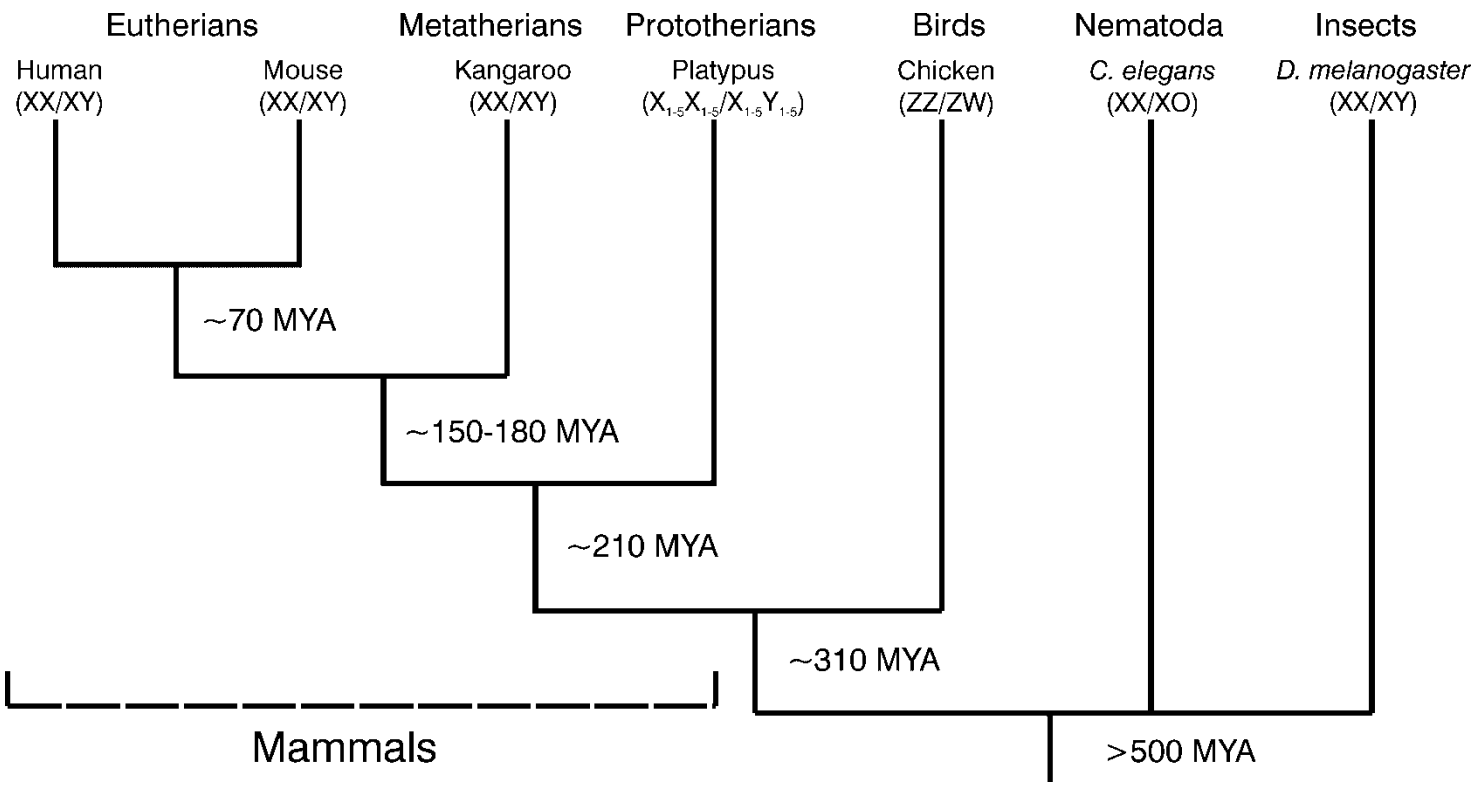

FIGURE 2 **Outline of mammalian evolution. XY chromosome pair emerged after the split of bird and mammals.**

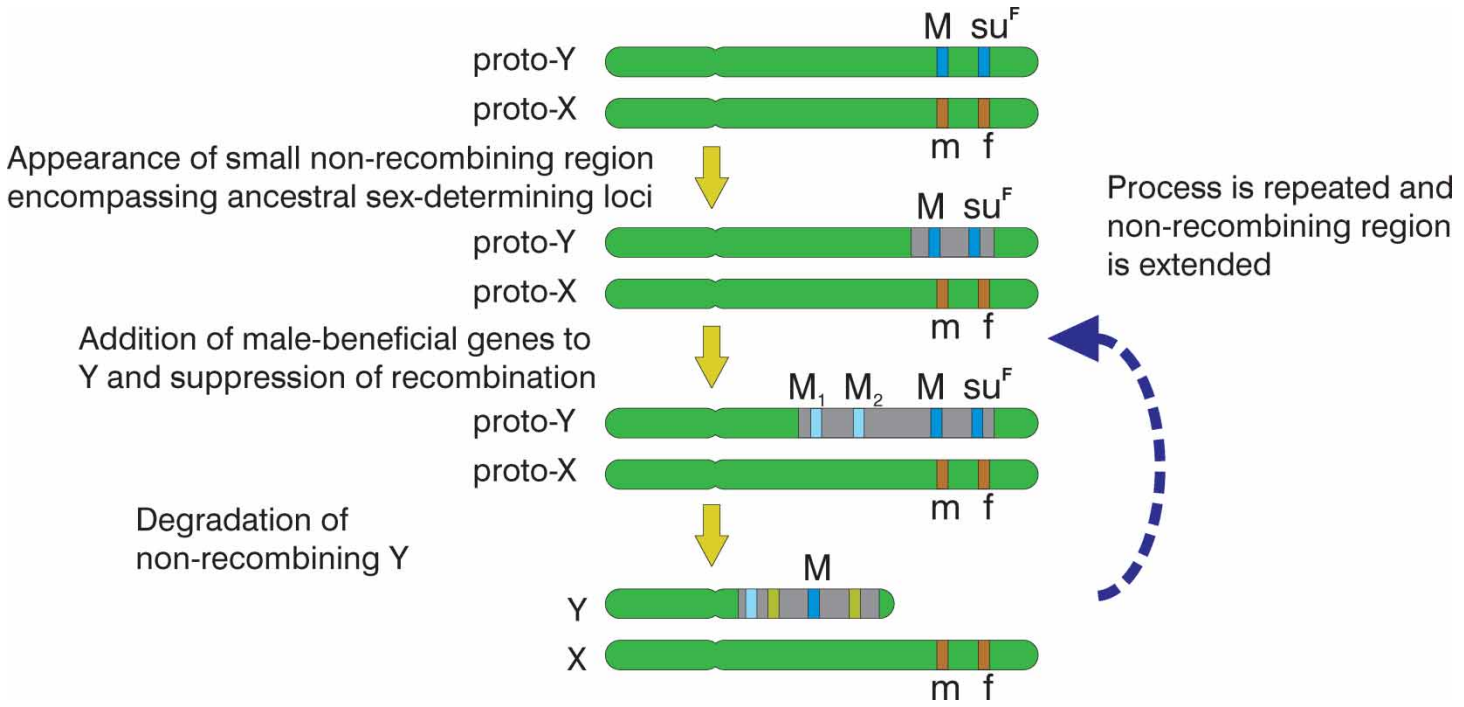

FIGURE 3 **Major stages in the evolution of heteromorphic sex chromosomes. After the initial suppression of recombination between primary sex-determining loci, additional male-beneficial genes are added and the region of suppression is extended. Selection of the male-beneficial loci is strongly favored on the proto-Y chromosome. The suppression of recombination may be achieved by either an inversion-mediated or other mechanism. The suppression of recombination leads to the degradation of the non-recombining Y chromosome. The process is reiterated and more and more regions are converted into the non-recombining state and they degrade.**

has its natural end-point in some species in which the Y chromosome has completely disappeared. On the other hand, the X chromosome has become a lone bearer of essential genes previously shared by the X and Y chromosomes. The consequence of this strong selection is an unusually high structural conservation of the X chromosome ("Ohno's Law") (Ohno, 1967). It is the most conserved human chromosome and is preserved in an intact state in nearly all mammalian species (Kohn *et al.*, 2004; Wienberg 2004).

Simple degeneration of the Y chromosome is probably not sufficient to explain the structure of modern heteromorphic sex chromosomes. Most likely segments of autosomes were added to one or both sex chromosomes (attrition-and-addition hypothesis) (Graves, 1995). There is also strong evidence of additions to the X chromosome (for detailed analysis see (Kohn *et al.*, 2004)). Examples of recently added segments are the pseudoautosomal regions. Only a very small portion of the sex chromosomes (the so-called pseudoautosomal region, PAR) is identical. The PAR region serves as a substrate for the mandatory crossover between the X and Y chromosomes. There is one pseudoautosomal region in the mouse and two in humans. The PAR1 is shared by all eutherian mammals and is located in the terminal region of the short arms of the X and Y chromosomes (Graves *et al.*, 1998). The PAR2 in humans is a more recent addition to the opposite end of both of the sex chromosomes (Charchar *et al.*, 2003). The recent resolution of the platypus karyotype also supports the ubiquity of large-scale additions in the evolution of the sex chromosomes (Charlesworth & Charlesworth, 2005; Rens *et al.*, 2004).

One line of evidence supporting the gradual evolution of the sex chromosomes comes from an analysis of intermediate stages of the evolution of the sex chromosomes. Several species have sex chromosomes that are still evolving and have preserved a much higher degree of similarity than that found in the human X and Y. For example, in the papaya, sex chromosomes are identical over nearly 90% of their length (Liu *et al.*, 2004). The remaining small 4.4 Mb region contains the sex-determining genes. A similar organization for the proto-X and proto-Y chromosomes is found in threespine stickleback fish (Filatov, 2005; Peichel *et al.*, 2004). Another example of neo-sex chromosomes is found in *Drosophila pseudoobscura*, where sex chromosomes appear to be a very recent development (Charlesworth & Charlesworth, 2005).

Independent molecular evidence in support of this hypothesis of a stepwise diversification of the sex chromosomes comes from the sequence comparison of the modern X and Y in humans. It was noted by Lahn and Page (1999) that a relationship exists between the position of the common genes on the X and Y chromosomes and their sequence divergence. 19 common genes that can be traced to both chromosomes can be subdivided into four groups with a similar degree of sequence divergence (evolutionary strata). The members of each group are located in separate regions on the X chromosome. However, a recent in-depth analysis of the completed sequence of chromosome X suggests the presence of a fifth evolutionary stratum (Ross *et al.*, 2005). The analysis was facilitated by the virtual absence of large scale rearrangements of the X chromosome in mammals and the nearly perfect co-linearity of gene order in the modern X and its autosomal precursor. Recently, the presence of evolutionary strata was confirmed by sequence analysis in the mouse as well (Sandstedt & Tucker, 2004).

One possibility proposed by Lahn and Page (1999) is that the stepwise process was mediated by series of large inversions and other rearrangements on the proto-Y chromosome. These large-scale rearrangements have led to the suppression of recombination over the region involved and to the stepwise degradation of the Y chromosome. An alternative to the "suppression driven by inversions" model is the gradual spread of regions with inhibited recombination induced by a mechanism alternative to large-scale rearrangements. There is some evidence in support of both models. One well studied example is the *AMELOGENIN* (*AMEL*) locus (Iwase *et al.*, 2003; Marais & Galtier, 2003). This gene had been predicted to be located on the boundary of strata 3 and 4 (Lahn and Page, 1999). Comparative sequence analysis uncovered a striking pattern. Nucleotide sequence divergence between the X and Y copies is three times higher in the 5′ region of this gene, compared to that at the 3′ end. Moreover, when analyzed separately, the 5′ and 3′ parts of the gene have different phylogenies! This pattern of divergence suggests that the pseudoautosomal boundary was located inside this gene, leading to a situation where one part of the gene was recombining while the other was not (Marais & Galtier, 2003) without any evidence for an inversion. On the other hand, the gene order of common genes differs significantly between the X and Y chromosomes suggesting multiple inversions (Skaletsky *et al.*, 2003). Moreover, the Y copy of the XG gene located at the

current PAR1 boundary is truncated by a pericentric inversion (Iwase *et al.*, 2003). It is likely that both mechanisms were used in the evolution of the X and Y chromosomes.

The rapid progress in sequencing and molecular analysis of novel genomes is an invaluable source of additional evidence for the evolutionary history of the sex chromosomes. Numerous eukaryotic genomic sequencing projects are currently underway. They include sequencing of genomes of apes, numerous other mammals and several non-mammalian vertebrates. Of particular interest are genomic sequences of the platypus, kangaroo, and threespine stickleback fish, all being currently sequenced (see www.genome.gov). A detailed analysis of the genomic sequences of species representing intermediate steps in the differentiation of the sex chromosomes and a comparative analysis of multiple related mammalian sequences will help clarify the evolution of sex determination and sex chromosomes.

WHEN AND WHY MSCI

One of the unique features of the X chromosome is its behavior in male meiosis. While the autosomes have a sister chromosome with which to pair and recombine in the course of meiotic progression, the X chromosome is alone. It does not share extended segments of sequence homology with the other chromosomes, except for that located on the very ends at the PAR regions. Recombination with non-homologous sequences would lead to catastrophic consequences and is therefore prevented. There are several ways to solve this problem. In flies, for example, recombination does not occur at all in males (McKee, 2004). In mammals sex chromosomes are condensed in male meiosis and a cytological structure called the "XY body" or "sex body" is formed (Handel, 2004; Hoyer-Fender, 2003; McKee & Handel, 1993; Solari, 1974). This process is called meiotic sex chromosome inactivation (MSCI). In addition to its role in suppressing recombination, MSCI results in the repression of transcription from the sex chromosomes. Both MSCI and somatic X-chromosome inactivation (XCI) are chromosome-wide epigenetic phenomena in which the whole X chromosome is condensed. Therefore, the natural expectation was that they would arise through similar mechanisms. The major regulator of somatic XCI is the XIST gene (Heard, 2004; Plath *et al.*, 2002). The non-coding XIST transcript marks an inactive copy of X chromosomes and is thought to spread and maintain the inactive chromatin state (Heard, 2004). Even though the XIST gene is expressed in spermatogenesis and its RNA is associated with the sex body (Ayoub *et al.*, 1997; Richler *et al.*, 1992; Salido *et al.*, 1992), XIST it is not required for X chromosome silencing in meiosis. It has been shown that the disruption of the XIST gene does not influence the onset of MSCI (McCarrey *et al.*, 2002; Turner *et al.*, 2002). Therefore, the mechanisms regulating MSCI and somatic X inactivation are different, at least in mammals.

Although the benefits of having MSCI in a species with advanced heteromorphic sex chromosomes are clear, the evolutionary history of MSCI is far from being completely understood. Several hypotheses with varying degrees of detail have been put forth to explain the origins of MSCI (Lee, 2005; Lifschytz & Lindsley, 1972; McKee & Handel, 1993; Wu & Xu, 2003). One popular view suggested initially by McKee and Handel (1993) is that the MSCI is mostly required to suppress recombination of non-homologous chromosomes to prevent illegitimate recombination between unpaired chromosomes and accumulation of double-strand breaks. Without a doubt, an active recombination between the modern highly divergent X and Y would be incompatible with genomic stability and efficient sperm production. The method of choice for this suppression to occur in mammals is formation of the sex body. The condensation of chromatin in the sex body inevitably leads to transcriptional silencing of X-linked genes. But was the suppression of recombination a driving force in the evolution of germline inactivation? Let's analyze this situation from an evolutionary perspective. In the initial state, before sex chromosomes diverged, there was just one autosomal pair recombining normally. The need for suppression of recombination to prevent genome instability emerges as the proto-X and proto-Y become increasingly divergent. The probability of highly deleterious consequences to recombination between sex chromosomes increases with their degree of divergence and, conversely, this probability was negligibly small at the early stages of the evolution of sex chromosomes, when proto-X and proto-Y had only a very few differences. Therefore, it is unlikely that the need to maintain genome stability led to the gradual spread of germline inactivation on the proto-sex chromosomes. Moreover, it was the suppression of recombination and the maintenance of the non-recombining state for a prolonged period that led to the divergence of the X and Y, and this suppression precedes the divergence.

Therefore, it appears that the initial development of suppression of recombination between the proto-X and proto-Y was promoted, not by the need to avoid deleterious consequences of recombination between X and Y, but by independent selective forces, such as the need to preserve sexually antagonistic genes in the same linkage group. In the discussion above, we assume that the suppression of recombination is achieved through MSCI-like germline inactivation. We cannot exclude the utilization of alternative mechanisms for the development of this recombinational suppression, at least at the early stages of the evolution of the sex chromosomes. Regardless of the mechanisms, the overall conclusion remains the same: the driver in the evolution of the suppression was not the need to maintain genomic stability.

A recent model called SAXI (an acronym for "sexual antagonism and X inactivation") (Wu & Xu, 2003) utilizes an opposite logic. It suggests that the accumulation of sexually antagonistic female-beneficial genes on the X and the removal of male-beneficial genes from the X drives the evolution of meiotic inactivation. The prediction of the SAXI hypothesis is that there should be a redistribution of sexually antagonistic genes prior to the appearance of inactivation. However, current data neither contradict nor unambiguously support this theory. Perhaps gene expression studies and genomic sequencing of species that have not evolved germline X inactivation will help to validate the SAXI model.

Another recent hypothesis proposes that MSCI is an adaptation of a pre-existing mechanism for preservation of genome integrity called meiotic silencing of unpaired DNA (MSUD) (Turner *et al.*, 2005). MSUD is an ancient biological process pre-empting the origin of sex chromosomes (Shiu & Metzenberg, 2002; Shiu *et al.*, 2001). Although MSUD was initially described for the fungus *Neurospora crassa*, it is widespread among animals including *C. elegans* (Bean *et al.*, 2004) and the mouse (Baarends *et al.*, 2005; Turner *et al.*, 2004; Turner *et al.*, 2005). MSUD modifies the chromatin structure of unpaired genomic sequences into heterochromatin, prevents them from recombining, and deems them transcriptionally inactive. It has been suggested that MSUD may have been used initially to prevent excessive transpositions and control the invasion of foreign DNA.

This hypothesis can be integrated well with the current view of the evolution of the sex chromosomes. Recombination between X and Y was initially suppressed over a small region and then gradually spread to the nearly whole chromosome. One can imagine that the trigger event promoting the suppression of recombination between two suppressor mutations at the very beginning was the integration of a transposon near the sex determination genes and a resultant MSUD-mediated inactivation. Because of the evolutionary advantage of suppressing recombination, this small non-recombining region quickly spread in the population. Alternatively, recombination between early genes was inhibited via an independent pathway, for example by mutations inside a recombinational hotspot, and then spread to neighboring regions via mobilization of the pre-existing MSUD system. This progressive expansion can be promoted by genomic duplications or inversions providing additional substrates for MSUD-mediated inactivation.

THE RELATIONSHIP BETWEEN XCI AND MSCI

Despite the obvious differences in mechanisms of MSCI and XCI in the mouse (McCarrey *et al.*, 2002; Turner *et al.*, 2002) they might have a deep evolutionary relationship. Integrating several recent advances in our understanding of the dynamics of X chromosome inactivation in the preimplantation embryo with the evolutionary evidence has led to the development of a unified view linking XCI with MSCI and imprinting (Huynh & Lee, 2005; Reik & Lewis, 2005). The link appears to be provided by the inactivation of the paternal X in male meiosis.

Until recently, it was believed that both copies of the X chromosome are transcriptionally active at early stages of embryo development and that one of them is then randomly silenced to achieve dosage compensation. This view was supported by the higher expression level of a handful of X-linked genes in the XX versus XY zygote and the normal replication timing of both X chromosomes in the pre-implantation embryo. At the same time, the paternal X chromosome is inactivated in extra-embryonic tissues of placenta in many mammals, including the mouse. Transcriptional activity of both copies of the X chromosome in the zygote implies a rather cumbersome sequence of multiple inactivation/reactivation rounds for the X chromosome in eutherians. Initially the paternal X chromosome is inactivated by MSCI, then it is reactivated in the zygote and inactivated again in the paternally imprinted form, as found in extraembryonic tissues, such as trophoblasts.

In the epiblast cells, destined to become the embryo, there is yet another round of reactivation in preparation for the random inactivation of X characteristic in adult females. Several recent papers suggest a simpler alternative: the X chromosome is transmitted from the father in a pre-inactivated form and is never completely reactivated in the XX zygote (Huynh & Lee, 2003; Mak *et al.*, 2004; Okamoto *et al.*, 2004). This paternal imprint is likely established during MSCI. The inheritance of a pre-inactivated X chromosome significantly simplifies the necessary sequence of events that lead to the establishment of somatic X chromosome-inactivation. In extra-embryonic tissues, weak inactivation of the paternal X is strengthened, while in the embryonic tissues, a single round of complete re-activation occurs, followed by random X-inactivation.

According to Hyunh and Lee (2005), the sequence of events in the evolution of this major epigenetic phenomena appears to be the following: When sex chromosomes started to diverge, the need for dosage compensation emerged. At the same time, progressively divergent sex chromosomes became inactivated in the male germline by a MSUD-mediated mechanism, and the ancestral MSCI system was established. MSCI marked the X chromosome with inactive chromatin and provided XX zygotes with a pre-inactivated paternal X chromosome. The maintenance of a partly pre-inactivated X provides a very simple and automatic dosage compensation system. Later this imprinted inactivation was stabilized and is found currently in extraembryonic tissues, such as the placenta. Such a model suggests that the ancestral state of mammalian X inactivation was paternally imprinted. In agreement with this view, it is known that somatic X inactivation in marsupials is paternally imprinted. Later, an alternative regulatory mechanism was invoked, and modern eutherians use XIST/TSIX transcripts to control random X inactivation (Heard, 2004).

A random X-inactivation system was established at the same time as the appearance of placenta and autosomal imprinting. All these processes were evolving simultaneously. An alternative hypothesis suggests that imprinted XCI was established in response to the evolution of the placenta and should be considered a form of imprinting (Reik & Lewis, 2005). Evolution of imprinting is believed to be driven by a conflict for energy investment between maternal and paternal genes (Wilkins & Haig, 2003). The critical difference that may help to differentiate these two models may be the dosage compensation system of egg-laying monotremes. If they have an imprinted X chromosome inactivation system similar to that of marsupials, this finding will support the model of Huynh & Lee (2005), on the other hand, if an upregulation mechanism for dosage compensation is found in monotremes, this finding will support the hypothesis suggested by Reik and Lewis (2005). Perhaps the recent resolution of the platypus karyotype will help to resolve this issue (Rens *et al.*, 2004).

The evolution of the dosage compensation system used in mammals is quite puzzling if considered alone (Charlesworth, 1996). Eutherians use a complicated mechanism of dosage compensation involving X chromosome counting, random selection of a single copy of the X to inactivate and chromosome-wide inactivation. The evolution of such a complicated system cannot be completed in one step. It is much easier to evolve a dosage-compensation mechanism where transcription is upregulated from a single X in males, such as found in *Drosophila* (Kelley, 2004; Straub *et al.*, 2005). Why do mammals use a more complicated dosage-compensation system? Perhaps there is a simple explanation for this contradiction. If X chromosomes were provided in the pre-inactivated form and dosage compensation was automatically achieved in XX females, such a scenario would perhaps be a simpler alternative to upregulation. The switch from an imprinted form of X inactivation to random X inactivation can be explained by evolutionary selection. There is a significant evolutionary advantage to having random XCI. It provides both a greater phenotypic variation and partially masks the effect of deleterious maternal X-linked mutations in female offspring. Thus, random XCI is a major mechanism of dosage compensation in soma (Heard, 2004; Heard *et al.*, 1997; Lee, 2003). But why don't all species having MSCI use X chromosome-inactivation for dosage compensation? Perhaps the difference in dosage-compensation mechanisms reflects differences in the mechanisms of sex determination. Mammals use a dominant sex determination system where the development of males is controlled by the Y linked gene *SRY*. Flies and worms utilize the X to autosome ratio to determine sex. The X/A ratio is estimated based on the relative expression levels of specific X-linked and autosomal genes. Thus, both copies of the X need to be transcribed early in the development of the embryo, and the MSCI/MSUD imprint in the paternal germline cannot be extended to provide

somatic X-chromosome inactivation. Consistent with this prediction, the dosage compensation in *C. elegans*, a species where MSCI is clearly present (Kelly *et al.*, 2002) but in which sex is determined by the X/A ratio, is achieved by downregulation of both copies of the X in females.

GENE CONTENT

So far, we have primarily discussed the evolution of the structural and epigenetic features of the X chromosome. In addition to its unique structure, the X chromosome possesses quite a special set of genes. The X chromosome is enriched for genes involved in brain function, sex and reproduction, genes expressed in extra-embryonic tissues, skeletal muscle, and retroposed genes (Graves *et al.*, 2002; Khil *et al.*, 2005; Reinke, 2004; Vallender & Lahn, 2004). Several models have been suggested to explain the accumulation of a non-random subset of genes on the sex chromosomes (Rogers *et al.*, 2003; Schlotterer, 2003; Vallender & Lahn, 2004). Again, as often happens with sex chromosomes, the common theme is sexual selection and sexual antagonism. These are believed to be among the major driving forces for specialization of the X chromosome. The most obvious difference between sex chromosomes and autosomes is that the former are not equally represented in males and females. The Y chromosome is limited exclusively to males and is present in a single copy. Exposure to selection only in males results in the accumulation of male-specific functions on the Y chromosome, either sexually antagonistic or not. This selection is facilitated by the fact that the Y chromosome is present in a single copy in males, and the mutations on it are not masked by the intact copy of a gene on the second chromosome (Rogers *et al.*, 2003; Vallender & Lahn, 2004). In agreement with this selective pressure, genes on the Y chromosome are, for the most part, involved in sex determination and spermatogenesis.

It was suggested long ago by Fisher (1931) that sexually antagonistic genes should accumulate on the X chromosome. A more recent analysis confirmed this prediction (Rice, 1984, 1992). The logical argument is again based on unequal dosage. The X chromosome is present in both sexes, but spends two thirds of its evolutionary time in females (Figure 4). As a consequence, a sexually antagonistic female-advantageous mutation has a higher chance for fixation if located on the X (Figure 3a). Therefore, the X chromosome should be enriched for female-beneficial functions. Somewhat paradoxically, masculinization of the X chromosome should be expected as well (Fisher, 1931; Rice, 1984; Rogers *et al.*, 2003; Vallender & Lahn, 2004). This masculinization comes from the hemizygous exposure of the X chromosome, which is present in males in only one copy. A rare recessive mutation that is beneficial to males would be readily selected on the X chromosome but not on the autosomes (Figure 4b). The predictions of these models are the enrichment on the X of sexually antagonistic genes beneficial to either females or males. For mammals, there is agreement with Rice's hypothesis and most male-biased genes are indeed enriched on the X chromosome. It has been reported that genes expressed in spermatogonia, a male premeiotic germ cell type, are over-represented on the X chromosome of mice (Divina *et al.*, 2005; Khil *et al.*, 2004; Wang *et al.*, 2001) and that sex- and reproduction-related genes (Saifi & Chandra, 1999) and prostate-specific genes (Lercher *et al.*, 2003) are over-represented on the human X chromosome. The enrichment of female-biased genes has been reported for flies (Ranz *et al.*, 2003) and mice (Khil *et al.*, 2004) but not for humans (Lercher *et al.*, 2003).

Surprisingly, recent data indicated that in worms (Reinke *et al.*, 2004, 2000) and flies (Parisi *et al.*, 2003; Ranz *et al.*, 2003) germline male-biased genes are underrepresented on the X-chromosome. Similarly, mammalian germline genes expressed late in spermatogenesis are sparse on the X (Divina *et al.*, 2005; Khil *et al.*, 2004). The explanation here, at least for worms and mammals, is selection by MSCI. Transcriptional inactivation of sex chromosomes drives genes expressed late in meiosis off the X. The consequence is their removal to autosomes, or, perhaps the removal precedes inactivation, as suggested by the SAXI model. It is not clear if MSCI is present in flies, where some data support its presence but some observations appear to be incompatible with transcriptional silencing in male germ cells (McKee & Handel, 1993; Rastelli & Kuroda, 1998). There is no need to provide a specific mechanism to maintain the integrity of the X in male flies, since there is no recombination between any of the chromosomes in males. Aside from the presence of MSCI in flies, there is the question of whether a dosage-compensation mechanism is active in the male germline. In flies, dosage compensation is achieved via an upregultaion of the single X. The dosage-compensation complex on the X includes several specific proteins and non-coding

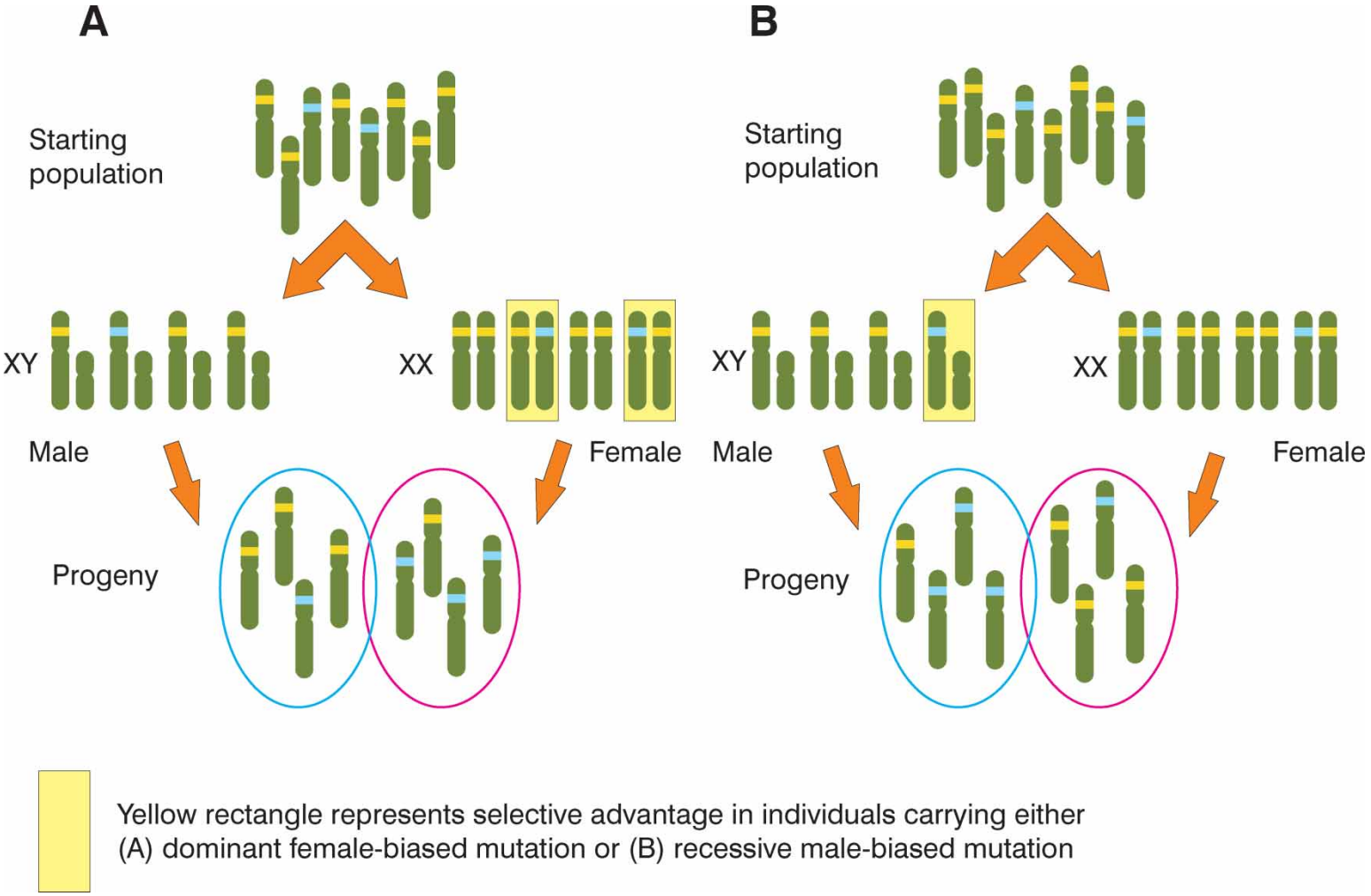

FIGURE 4 **Mechanism of selection for sexually-antagonistic genes on the X chromosome. Sexually—antagonistic mutations (either male- or female-beneficial) are shown as blue rectangles. A) Dominant female-biased genes are more efficiently selected when they are located on the X chromosome. B) Male-biased recessive genes are selected on the X chromosome.**

transcripts (Kelley, 2004; Straub *et al.*, 2005). In male germ cells in *Drosophila*, these specific factors are not seen on the X chromosome (Rastelli & Kuroda, 1998). Therefore, it appears that the X chromosome is not upregulated by the mechaninsm operating in the soma (Kotlikova *et al.*, 2005; Rastelli & Kuroda, 1998; Straub *et al.*, 2005). We must note, however, that the upregulation of transcription by a yet unknown mechanism cannot be excluded. The situation when there is no dosage compensation in the male germ cells of flies resembles incomplete inactivation by MSCI and may lead to similar evolutionary consequences. Thus, the integration of genetic and epigenetic features of the X can explain all the major trends in the distribution of sex-biased genes.

Another intriguing observation made initially for *Drosophila*, and then confirmed for mammals, is the excess of retrotransposition off and onto the X chromosome (Betran *et al.*, 2002; Emerson *et al.*, 2004; Khil *et al.*, 2005; Wang, 2004). The retroposition of genes off the X is explained by the transcriptional silencing of sex chromosomes in male meiosis. The presence of several autosomal intronless genes having a closely related, intron-containing gene on the X chromosome and expressed exclusively in the testis was noted more than ten years ago (McCarrey & Thomas, 1987). The original explanation for this phenomenon is referred to as the compensatory hypothesis (McCarrey & Thomas, 1987). The compensatory hypothesis states that such autosomal retrogenes were generated to allow for the expression of genes essential for male germ cell function that would be otherwise silenced by MSCI. Indeed, the majority of genes that retroposed from the X have evolved testis-specific expression (Wang, 2004). A modification of the compensatory hypothesis does not require the autosomal copy to be an essential gene. This hypothesis proposes that these newly retroposed genes now located on autosomes express novel meiosis-specific functions, while the sex chromosomes are silenced by MSCI (Wang, 2004), an idea supported by several recent observations (Betran & Long, 2003; Bradley *et al.* 2004). For example, Utp14b, a protein-coding retrogene located on chromosome 1 in the mouse, and that was apparently generated by retroposition of an X-linked gene, is required for spermatogenesis (Bradley *et al.*, 2004). Both hypotheses support the preferential selection for genes retroposed from the X. It is also possible that the excess of X-linked male germline genes results in a relative over-representation of X-originated RNA substrates for retrotransposition (Khil *et al.*, 2005). The movement in the opposite direction, onto the X, is most likely explained by a combination of preferential integration of retrotransposed sequences onto the X chromosome and the sexually antagonistic selection for male-beneficial mutations (Khil *et al.*, 2005; Vallender & Lahn, 2004).

One of the most interesting features of the X chromosome is the strong association of X-linked genes with the development and function of the brain (Arnold, 2004; Skuse, 2005). Nearly 30% of all described mental disorders are X-linked, and there is a significant excess of males among mentally retarded individuals (Ropers & Hamel, 2005). The prevalence of X-linked disorders in males, as is frequently the case for mental retardation, is easily explained by the hemizygous exposure of recessive mutations. In addition, there are more brain-specific genes on the X. For example, it has been suggested the selection for higher cognitive ability was driven by female mate preference (Zechner *et al.*, 2001). The preferential accumulation on the X of genes conferring higher cognitive ability is then explained by selection for sexually antagonistic genes.

Multiple mechanisms are responsible for this link between the X chromosome and the brain. One of the major effects of the sex chromosomes on the brain is indirect. Brain morphology is different in males and females, and sex hormones play a significant role in brain development. In addition to this indirect influence, sex-linked genes appear to be directly involved in the functioning of the brain. Numerous sex-linked genes are expressed in brain including the major regulator of male development, the *SRY* gene (Graves, 2001). The functional importance of *SRY* expression, however, is not clear. These expression patterns suggest a direct involvement of sex-linked genes in cognitive functioning and brain development. Another important mechanism may be related to the differences in imprinting of X-linked brain genes (Davies *et al.*, 2005). One example of the influence of imprinting on the brain comes from studies of the brain in Turner syndrome patients. It was found that maternally expressed genes are responsible for hippocampal development and that paternally expressed genes influence the development of the caudate nucleus and thalamus in females (Skuse, 2005). Another recent observation shows that the disruption of the imprinting pattern of a group of genes on the X chromosome has a major

effect on cognitive functions (Raefski & O'Neill, 2005; Skuse *et al.*, 1997). Another unique X chromosome-specific effect is somatic mosaicism due to random X chromosome inactivation. It is believed to be responsible for differences in color perception between males and females in primates (Dulai *et al.*, 1999; Webb *et al.*, 2004) and for the strong prevalence among females of a form of severe mental retardation called Rett syndrome (Arnold, 2004; Gibson *et al.*, 2005; Segawa & Nomura, 2005).

CHROMATIN MODIFICATION OF THE X CHROMOSOME

Suppression of recombination and silencing of sex chromosomes in the male germline via MSCI has a number of important consequences. A unique feature of the Y chromosome, an extreme enrichment with long palindromes is tolerated only because of suppression of recombination of the Y chromosome. On autosomes, such palindromes are generally unstable. These palindromes are very important to maintain the functional activity of the Y chromosome and are used to prevent the degeneration of functional genes (Rozen *et al.*, 2003). Another important consequence of MSCI is an apparent lack of late meiotic genes on the X. MSCI simply prevents their expression, thus effectively excluding them from the X. Similarly, it is likely responsible for the excess of retrotransposed genes originating from the X.

One long-known feature of the X chromosome is its significant enrichment with LINE-1 (long interspersed nuclear element 1) (Deininger *et al.*, 2003) family of retrotransposons and other retroposed sequences. There is an approximately a 1.5- to two fold excess of both processed pseudogenes and LINE-1 elements (Lander *et al.*, 2001; Waterston *et al.*, 2002) on the X. One hypothesis suggests that the LINE sequences may help to spread and stabilize somatic X chromosome inactivation (Lyon, 1998, 2003), but the mechanism of such stabilization is unclear and remains subject to debate (Bailey et al., 2000; Hansen, 2003; Ke and Collins, 2003). An alternative hypothesis is that the excess of LINE-1 elements on the X is explained by the structure of the chromatin in the sex body. Retropseudgenes and LINE-1 retrotransposons integrate mostly in the male germline and utilize the same L1 machinery for their integration into the genome (Deininger *et al.*, 2003; Esnault *et al.*, 2000). Thus, the excess of sequences on the X that are generated by retrotransposition could be the result of preferential targeting of the retrotransposing sequences toward the X (Khil *et al.*, 2005). The fact that there is still an excess of recently retrotransposed L1 elements on the X chromosome provides support for at least some preferential targeting (Myers *et al.*, 2002; Ostertag & Kazazian, 2001).

CONCLUSIONS

We can clearly see that, in the evolution of the X chromosome, epigenetic and evolutionary forces were and remain deeply intertwined. Some of the evolutionary forces were acting on early stages of evolution, some are still functional, and some are emerging. MSCI is one of the current forces and perhaps one that permitted the evolution of modern sex chromosomes. The integration of functional information in evolutionary models leads to a deeper understanding of the underlying mechanisms. The relatively well studied mammalian sex chromosomes are a prime proving ground for testing evolutionary theories.

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