REVIEW ARTICLE

Weird mammals provide insights into the evolution of mammalian sex chromosomes and dosage compensation

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Abstract

The deep divergence of mammalian groups 166 and 190 million years ago (MYA) provide genetic variation to explore the evolution of DNA sequence, gene arrangement and regulation of gene expression in mammals. With encouragement from the founder of the field, Mary Lyon, techniques in cytogenetics and molecular biology were progressively adapted to characterize the sex chromosomes of kangaroos and other marsupials, platypus and echidna—and weird rodent species. Comparative gene mapping reveals the process of sex chromosome evolution from their inception 190 MYA (they are autosomal in platypus) to their inevitable end (the Y has disappeared in two rodent lineages). Our X and Y are relatively young, getting their start with the evolution of the sex-determining *SRY* gene, which triggered progressive degradation of the Y chromosome. Even more recently, sex chromosomes of placental mammals fused with an autosomal region which now makes up most of the Y. Exploration of gene activity patterns over four decades showed that dosage compensation via X-chromosome inactivation is unique to therian mammals, and that this whole chromosome control process is different in marsupials and absent in monotremes and reptiles, and birds. These differences can be exploited to deduce how mammalian sex chromosomes and epigenetic silencing evolved.

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Introduction

'The Lyon hypothesis explains how males and females cope with having different numbers of X chromosomes.' I explained it all in great detail to a nice lizard physiologist as we set up next door posters at a meeting in Sydney in 1976. The only other onlooker in the deserted hall was a diffident little lady with a white cardigan and a shopping bag, diligently reading both our posters with equal interest. I had registered for 'Reproduction and Evolution' specifically to meet the great Mary Lyon, and when I was introduced to her that night, of course, I recognized her—even without the shopping bag. We both laughed about that encounter for years, and of course I soon recognized that she was not the sort of person to stick out her hand and say, 'Hi, I'm Mary Lyon and that's my hypothesis you're talking about'.

From my earliest interaction with her, Mary Lyon recognized that marsupials—and even the weirder monotremes—could even provide unique insights on sex chromosome organization and activity. Monotremes, marsupials and eutherian (placental) mammals constitute independent experiments in mammal evolution, and, with information from reptile and bird outgroups, help us deduce what happened at the outset. Weird rodents represent changes at the end of the lifetime of the Y.

There are three groups of mammals (figure 1). Subclass Theria (marsupial and placental mammals) and Prototheria (monotremes such as the egg-laying platypus) diverged 190 million years ago (MYA) and marsupials and eutherians last shared a common ancestor about 160 MYA (Luo *et al.* 2011). Thus, there has been plenty of time—more than twice the time that humans and mice have been evolving separately—for the three groups of mammals to evolve different genome arrangements, novel genes and different ways of regulating them. Within marsupials, there are also closely and distantly related species that can be analysed. In particular, Australian marsupials such as kangaroos diverged at about 70 MYA from American marsupials such as the opossum. Within eutherians too, there are deep divergences as well as recent radiations of rodents that are very instructive.

Of particular interest to me was the contributions nonmodel animals can make to our understanding of sex

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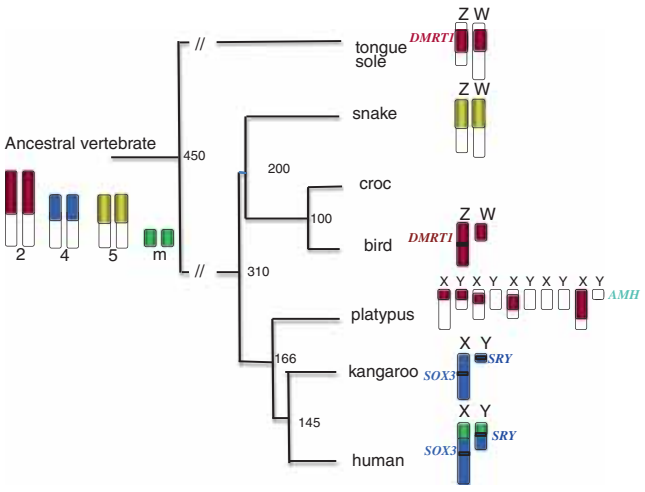

Figure 1. Relationships among vertebrate and their sex chromosomes. Comparative mapping demonstrates orthologous segments that are sex chromosomes in one lineage and autosomal in others. Four such segments are demonstrated here. The yellow segment has become sex chromosomes in snakes (sex determining gene remains unknown). The red segment defined by the DMRT1 gene, became sex determining in birds and independently in the tongue sole; the segment is represented on the sex chromosomes of platypus but the sex determining gene appears to be *AMH*, added from another block. The blue region defined by *SOX3* became sex chromosomes in mammals, and the green region fused with this ancient X before the radiation of eutherian mammals.

chromosome organization, function and evolution. Here, I summarize some of the highlights and emphasize the interactions with Mary Lyon that have enriched my work in this area.

Mammalian sex chromosomes

Eutherian (placental) mammals have an XX female XY male system in which the Y chromosome is male determining because of the action of the male-dominant testis determining gene *SRY*. The X and Y chromosomes of humans (and other eutherian mammals) are highly differentiated (Graves 2006). They pair at one end through a homologous pseudoautosomal region that contains about 24 genes. The X-specific region contains more than 1000 genes. The Y-specific region has only 27, but many genes are present in multiple copies, most of which are inactive. The X is extremely conserved among all eutherians, being about 5% of the haploid complement, and contains more or less the same set of genes; even when the order is highly conserved except for mouse, which has a generally much more rearranged genome. This conservation is enshrined as 'Ohno's law', after a suggestion that conservation was a consequence of selection against disruption of a chromosome-wide dosage compensation scheme (Ohno 1967).

In fact, marsupials provided an important test for the identity of the mammalian testis-determining gene. Mapping the first candidate gene *ZFY* in marsupials showed that it was not located on the Y, as would be expected of a sex-determining gene, but was autosomal. This was the first indication that *ZFY* was the wrong gene (Sinclair *et al.* 1988), and provided the trigger for the renewed search that led to finding the right gene, *SRY* (Sinclair *et al.* 1990). The presence of *SRY* on the marsupial Y was an important piece of evidence that it was the right gene (Foster *et al.* 1992), and the discovery of *SOX3* on the marsupial X was the first indication that the sex-determining gene, like other genes on the Y with male-specific functions, evolved from genes on the X (Foster and Graves 1994).

The basic X chromosome of kangaroos and other marsupials is smaller than the 5% that is highly conserved in placental mammals (Hayman and Martin 1969), and the Y is minute in some species. The X and Y do not undergo homologous pairing at male meiosis, but segregate from a proteinaceous plate (Fernandez-Donosa *et al.* 2010).

Gene mapping in kangaroos, at first by establishing sex linkage in families (VandeBerg *et al.* 1983), then by somatic cell genetics, radioactive *in situ* hybridization (Graves and Watson 1991; Wilcox *et al.* 1996), fluorescence *in situ* hybridization (FISH) (Deakin *et al.* 2012) and now by whole-genome sequencing (Mikkelsen *et al.* 2007; Renfree *et al.* 2011) has been established that the marsupial X is homologous to part of the eutherian X represented by the long arm of the human X and the proximal region of the short arm. However, most of the short arm of the human X is represented by an autosome in marsupials (Spencer *et al.* 1991; Graves 1995; Glas *et al.* 1999) and monotremes (Watson *et al.* 1991). This suggests either a fusion of an autosome early in the eutherian radiation, or a fission in marsupials; the latter is favoured by finding that these two blocks are present as separate autosomal blocks in the chicken. The finding that the fusion point corresponds to the centromere of the elephant X (Delgado *et al.* 2009) suggests that a centric fusion occurred sometime between the divergence of humans from marsupials 166 MYA and divergence of elephants 105 MYA.

This implies that the marsupial X chromosome represents the original X chromosome of a common therian ancestor.

Most of the genes on the human Y chromosome have partners on the X, from which they clearly diverged; even the sex-determining *SRY* gene has an X-borne partner, *SOX3* (Foster and Graves 1994). This homology supports the hypothesis that the Y is a degraded version of the X, and Y genes evolved from X genes.

The Y chromosome of eutherians is poorly conserved, representing, mostly, the paralogues of subsets of genes that lie on the X in all species. There are 27 unique protein-coding genes on the human Y; however, only four human Y genes (including *SRY*) have paralogues within the ancestral conserved region of X and thus were part of the original Y. The rest have partners on the recently added region, so were contributed by the fused autosomal region (Graves 2006). This explains the major difference between the oldest 'geological stratum' of the human X revealed by sequence changes between X and Y paralogues, and the more recently diverged strata (Lahn and Page 1999).

Strangely, several more genes have been discovered on the tiny marsupial Y (Pask *et al.* 2000; Murtagh *et al.* 2012) using strategies such as microdissection and BAC cloning with Y DNA, then BAC sequencing to discover genes on the marsupial Y. All these have partners on the X, so were present on the original therian XY pair.

We could go back further in time to the beginning of the mammal XY pair by mapping orthologues of marsupial X genes in birds and reptiles, frogs and fish. These genes lie in two autosomal blocks in all of these species (Nanda *et al.* 1999), implying that mammal sex chromosomes originated as an autosome sometime between the divergence of mammals and reptiles 310 MYA and the divergence of marsupials and eutherians 160 MYA.

However, to our astonishment, we discovered that genes that are on the therian X chromosome are located on autosomes in the basal mammal group, the monotremes such as platypus and echidna (Waters *et al.* 2005). Instead, the complex monotreme sex chromosomes have homology to the chicken ZW pair. This brings forward the time at which the therian sex chromosomes evolved only 190–166 MYA (Veyrunes *et al.* 2008), much more recent than had been supposed.

The sex chromosomes of monotremes were long an enigma because there were several unpaired chromosomes in males (Murtagh 1977; Wrigley and Graves 1988). Fluorescence *in situ* hybridization (FISH) mapping and cloning of genes on the multiple sex chromosomes of platypus established that the five platypus Xs and five Ys were products of translocation of a bird-like sex chromosome with orthologues of four other chicken chromosomes. Echidna X chromosomes share four of these, but the fifth is completely different (Rens *et al.* 2007), suggesting that four translocations occurred in the lineage of the common ancestor, but the fifth occurred independently in the two families and involved different autosomes. More recently whole-genome sequencing and comparison of the sequences of platypus X and Y chromosomes have delineated the nine pseudoautosomal regions. Comparison of the sequences of gametologues (paralogues on the differentiated segments of the X and Y) reveals six 'geological strata', some of which represented these translocation events (Cortez *et al.* 2014).

Sex determination in monotremes clearly does not involve *SRY*, since this gene cannot be demonstrated, and SOX3 maps on chromosome 6 with other human X orthologues (Wallis *et al.* 2007). Rather, the *AMH* gene, which lies on the tiny (and oldest) platypus Y5 is a strong candidate (Cortez *et al.* 2014).

X-chromosome inactivation in weird rodents

A great deal of information about X-chromosome inactivation comes from studies on mouse, starting with Mary Lyon's original observations of mosaic coat colour in heterozygous females. Mice may be our model mammal, but they are very atypical, having a high rearranged genome with many chromosome rearrangements and a Y chromosome that is more degraded than in other eutherians. Other rodent species have very atypical sex chromosomes that represent the end of the road for the degrading Y chromosome.

Here, I shall confine myself to two aspects of X inactivation in which I was directly involved, and in which Mary Lyon participated. The first was to test a theory that DNA methylation was involved in epigenetic inheritance, including X-chromosome inactivation (Holliday 1993). This idea was attractive because there was an obvious way that methylation patterns could be conserved through mitotic division. Fortuitously, I had been working with Gail Martin on lines of cells originally by Verne Chapman from a hybrid between *Mus musculus* and *M. caroli* in which three X-linked markers were differentiated between the two X chromosomes. I had made hypoxanthine-guanine phosphoribosyltransferase (HPRT) mutants from these lines that had negligible back-mutation rates. I had used these lines to make hybrids with Gail's mouse teratocarcinoma stem cell lines that retained two active Xs in an attempt to discover *trans*-regulation of X activation or inactivation (Graves and Young 1982).

I learned from Morgan Harris (who had been one of my major professors at UC Berkeley) that a DNA methylation inhibitor 5-azacytidine reversed the phenotype of thymidine kinase-deficient cell lines that we had used (Harris 1982). I tried out various concentrations on the $HPRT^-$ lines to see if I could reactivate the wild-type $HPRT^+$ locus on the inactive X. This was the easiest and most conclusive experiment I ever did, for the locus showed a spectacular dose-dependent turn-on (Graves 1982). Independent studies on the inactive human X in rodent–human somatic cell hybrids showed the same (Mohandas *et al.* 1981), although I am always a little leary of studying regulation in cell hybrids due to the many changes in genetic and epigenetic states. I presented this work at a meeting in Bengaluru in 1981, which Mary Lyon encouraged me to pursue similar experiments with marsupial cells. This was easier said than done, since we lacked marked and mutant cell lines, but these discussions set the stage for many investigations of marsupial X inactivation that I would do in the next decades.

My second foray into mouse work was to test the assumption that X-chromosome inactivation represented transcriptional silencing. This was a crucial tenet of the Lyon hypothesis and the basis for every speculation about the molecular mechanism, yet by the mid 1980s there was no direct evidence for transcriptional control.

I travelled to Seattle on sabbatical to work with Stan Gartler, who had derived lymphocyte cell lines from a boy suffering from Lesch–Nyhan's syndrome of HPRT deficiency, and from his normal mother, who must be heterozygous for the deficiency. I cloned two populations of cells from the mother's line, one $HPRT^+$ and able to grow in selective (HAT) medium, and one $HPRT^-$ and able to grow in thioguanine. Then, I prepared RNA from these lines as well as from the $HPRT^-$ son and a normal boy, and tested

these lines for the expression of HPRT messenger RNA using Northern blots. These were not easy experiments to do in 1985, but, with help of many people in the department, I managed to obtain Northern blots that showed definitively that HPRT RNA was transcribed in the $HPRT^+$ clones but not in $HPRT^-$ clones derived from the mother's lymphocytes.

I wrote a short paper with Stan to the effect that yes, X inactivation is, indeed, due to transcriptional repression, and, thinking this a rather crucial discovery, naively sent it to *Nature*. The baffling response I got with a second rejection letter, even after intervention from Mary Lyon, was, 'This is not very important because, well, what *else* could it be?' The paper appeared later in a now defunct journal (Graves and Gartler 1986) and is hardly referred to, the credit going to work in the 1990s when transcripts were more amenable.

The other weird rodents that I have championed, although I have worked with them only indirectly, are the species that have completely lost their Y chromosome. I knew from the classic work of Karl Fredga that the mole vole *Ellobius lutescens* has XO males and females (Fredga 1988), and I was aware that Walther Just had failed to find *SRY* in their cells. However, his submission to *Nature* had been rejected on the grounds that he just had not looked hard enough for a diverged vole *SRY*. We met at the International Genetics Congress in 1993, which I attended (and organized a symposium) at Mary Lyon's invitation, and with her kind assistance, since I was slowly recovering from neurosurgery at the time. Since my lab spends a lot of time looking for genes in marsupials that are not there (e.g. *ZFY* and *XIST*; Sinclair *et al*. 1988; Hore *et al*. 2007a, b), I could propose strategies to convince *Nature* referees that *SRY*—along with other Y genes that were not there—was really missing, essentially by cloning *SRY* from the closely-related species that retained a Y and using this as probe (Just *et al.* 1995). I also had pleasure and amusement in introducing two old friends and colleagues to Mary. Gail Martin, who is very animated and speaks very fast, found talking to Mary, was hard work; however, Mary found her match in Nobuo Takagi whose hesitant English exactly kept pace with Mary's thoughtful responses.

Since then many more details are available from these interesting rodents (Just *et al.* 2007), as well as from an independent loss of the Y in the Japanese spiny rat, which appeals to me particularly because gene mapping reveals that other fertility genes on the Y have been relocated, and also because there is now a plausible candidate gene that took over sex determination in these species (Kuroiwa *et al.* 2010). These rodents are a splendid demonstration that when things get really tough for the Y, experiments in novel sex determination are strongly selected for, a lesson there for humans.

X inactivation in marsupials

Animals with differentiated sex chromosomes share the problem that most genes on the X (or Z) are present in two doses in one sex but only one in the other (Graves *et al.* 1998). In placental mammals, the dosage imbalance of X chromosomes in XX female and XY male is mitigated by a system in which one X chromosome becomes inactive in somatic cells of females (Lyon 1961). This process is random, complete, stable and somatically heritable. The mammalian X-chromosome inactivation relies on a complex epigenetic system to silence one X in females and constitutes a splendid model system for studying epigenetic silencing on a grand scale. It was easy to imagine that all vertebrates with differentiated sex chromosomes would share problems of gene dosage, and would prove to share a similar epigenetic silencing system, but comparisons of dosage compensation systems in distantly-related mammals and other vertebrates shows that this is far from the case.

Work on marsupial X-chromosome inactivation began only a few years after the publication of Mary Lyon's seminal paper in 1961. It started in a very small way with my honours project, using the challenging, then new, technique of tritium autoradiography to look for a late replicating X in kangaroo lymphocytes (Graves 1967). I found that one X was significantly late replicating, and concluded that marsupials, too, used X inactivation to compensate for sex differences in the number of X chromosomes. 'Just like humans and mice,' I concluded.

Wrong, subsequent work on the gene content and activity of the marsupial X showed that X inactivation, like the gene content of the marsupial X and Y, differs in significant ways from that of eutherians. For a start, it is always the paternal X that is inactivated, the first demonstration of imprinting in a mammal. Not only was the process in marsupials imprinted, but also it was incomplete and tissue specific (Cooper *et al.* 1993; Deakin *et al.* 2012). These differences are not so extreme as first thought, since we now know that paternal inactivation occurs in the first rounds of inactivation in the embryos of at least some eutherian species, and we now accept that many genes on the human X (largely within the X-added region) escape inactivation fully or partially (Carrel and Willard 2005).

Subsequent studies of marsupial inactivation at the genome level by microarray analysis and RNAseq showed expression of the maternal allele and silencing of the paternal allele of most genes in opossum and kangaroo (Rodriguez-Delgado *et al.* 2014; Waters *et al.* 2005; Wang *et al.* 2014). However, many paternal genes partially escape inactivation (14% in opossum and 30% in kangaroo), although this might be partly due to a tissue-specific differences.

Studies of transcription at the level of single cells were performed using RNA-FISH to count the number of active alleles by observing signals of primary transcripts at one or two sites in interphase cells. Surprisingly, this revealed a mix of 2X-active and 1X-active cells, the proportion of which was specific for each gene (Al Nadaf *et al.* 2010). This suggested that the partial inactivation observed by biochemical assays on populations of cells is explained not by partial transcription of each allele but by a lower probability of transcription.

A similar pattern of stochastic transcription was observed for escaper genes on the human and elephant X (Al Nadaf *et al.* 2012), suggesting that this type of stochastic inactivation might be ancestral to both mammal groups.

DNA methylation differences at gene promotors could not be demonstrated by early work in marsupials (Kaslow and Migeon 1987; Chong and Piper 1996), and this has recently been verified by analysis of the promotor sites of many genes on the opossum X (Wang *et al.* 2014). However, histone modifications on the marsupial X reflect a partial set of those on the mouse and human X, including depletion of active marks H3 K4 me2, H3K9ac and H4Kac and enrichment of repressive marks H3K9me2 and H3K27me3, which is depleted in escaper loci (Wakefield *et al.* 1997; Koina *et al.* 2009; Chaumeil *et al.* 2011; Wang *et al.* 2014). It is interesting that these marks reflect modifications seen in centric heterochromatin, suggesting that they were reused in silencing facultative heterochromatin.

A major difference between X inactivation in eutherians and marsupials emerged when it became clear that there is no *XIST* gene in marsupials; instead the homologous region is disrupted and contains protein-coding genes that are present in birds and frogs but lost in placental mammals (Duret *et al.* 2006; Davidow *et al.* 2007; Hore *et al.* 2007a, b; Deakin *et al.* 2008). However, a total unrelated gene *RSX* has been discovered that seems to fulfill the same function, producing a noncoding RNA that coats the chromosome to be inactivated and primes further silencing changes (Grant *et al.* 2012).

Is there dosage compensation in monotremes and birds?

Monotremes, with their five pairs of X chromosomes, would seem to have an urgent need for dosage compensation, since at least 12% of the genome figures on the Xs but not the Ys (figure 1). Yet there were confusing data about whether any or all X's show the cytological symptoms of inactivation (Murtagh 1977; Wrigley and Graves 1988).

Biochemical studies using allele-specific probes and primers showed that for all genes both alleles were transcribed, but dosage differences ranged from 1:1 (complete dosage compensation) to 2:1 (no dosage compensation). RNA-FISH showed that genes on the unpaired regions of the five X chromosomes were subject to stochastic silencing. The fibroblast population was a mixture of 2X-active and 1X-active cells, the proportion of which differs among different genes (Deakin *et al.* 2008). This is similar to marsupial X-borne genes and escaper genes on the human X.

More recently, whole-genome sequencing and transcriptome analyses by RNAseq shows that genes on platypus X chromosomes are partially and variably compensated, adding up to a mean ratio of about 1.3, where 1.0 represents full compensation and 2.0 represents no compensation (Cortez *et al*. 2014).

What about birds and reptiles? Birds have highly differentiated Z and W chromosomes, so most of the nearly 1000 genes on the Z are present in two copies in males and a single copy in females. Yet there is no evidence for Z inactivation either from cytological studies or gene activity (Graves 2014). Rather, microarray analysis first showed that genes on the bird Z are compensated to a variable extent, averaging about 30% but varying from complete compensation to no compensation (Itoh *et al.* 2007). RNA *in situ* hybridization shows that chicken fibroblasts, like those of monotremes, are stochastically silenced in some cells but not others (Livernois *et al.* 2012). Whole transcriptome analysis and RNAseq now verifies that bird sex chromosomes are only partially and variably compensated (Julien *et al.* 2012).

Evolution of X-chromosome inactivation

It is tempting to consider monotremes and birds as a ground state of dosage compensation, and marsupials as an intermediate stage in building up the sophisticated multilayered inactivation complex of eutherian mammals. Perhaps, the ancestor of marsupials and eutherians already had evolved a global control of X inactivation. Elaboration of epigenetic control by other histone marks and DNA methylation occurred in the eutherian lineage to make it more complete, whereas gene-specific and tissue-specific inactivation was specifically selected for marsupials, perhaps as a form of female-biased gene expression. The absence of DNA methylation and some of the active and inactive histone marks from marsupials suggests that a basal level of inactivation was imposed on the X by recruiting inactive histone modifications from centromeric heterochromatin, then superimposing additional silencing mechanisms (active histone marks and DNA methylation) and coordinated by control by *XIST*.

This idea seemed to be supported by finding that paternal inactivation occurs in the extra-embryonic tissues of at least two eutherian lineages as well as in marsupials. Was paternal inactivation ancestral then, as proposed long ago (Cooper 1971)? An attractive hypothesis was that meiotic inactivation of both sex chromosomes (MCSI) might be the ultimate origin of XCI. The idea was that inactivation occurred at male meiosis and the paternal X simply stayed inactive (Huynh and Lee 2001). However, it is now clear that there is a two-X active stage in human and mouse, as well as marsupial embryos before paternal inactivation is imposed (Heard and Disteche 2006). Evidently, paternal inactivation evolved independently in eutherians and marsupials, perhaps as a means of mitigating the immunological mismatch between embryo and mother.

The control of whole X-chromosome inactivation by coating with a noncoding RNA is also a striking property of inactivation in eutherian and marsupial lineages; however, it cannot represent identity by descent, as they use nonhomologous ncRNAs. This implies that control by a ncRNA evolved independently by pressing into service of one of the numerous X-borne ncRNAs available in a therian ancestor.

Another similarity between eutherian and marsupial X inactivation, as well as dosage compensation in monotremes and birds, is the stochastic inactivation of partially expressed genes on the X. This supports the hypothesis (Ohlsson *et al.* 2001) that X inactivation initially arose from monoallelic silencing of many autosomal genes (Chess 2012).

Conclusion

Thus Mary Lyon's interest in what weird mammals can show us—both about the evolution of sex chromosomes and X inactivation–has therefore been more than justified. Marsupials are sufficiently diverged from eutherians that they can deliver informative genetic variation on conserved gene arrangements and regulation. Sequence comparisons have proved to be efficient in spotting conserved genes and potential regulatory regions, and comparisons of gene arrangement revealed major events in mammalian sex chromosome evolution.

The variety of sex chromosomes in birds and reptiles– and monotremes–reveals that the sex chromosomes of therian mammals are comparatively young, but that the Y chromosome has been degrading very rapidly. Rodents that have lost the Y chromosome present a foretaste of the sexual experimentation that will happen when the therian Y chromosome degrades completely. Comparisons of control by ncRNA, and by epigenetic marks, facilitated by a whole-genome approach, now provide us with a picture of the evolution of different ways to solve the sex chromosome dosage problem that uses molecular silencing mechanisms from a common molecular toolbox.

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