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RESEARCH ARTICLE

Mammalian X-chromosome inactivation: proposed role in suppression of the male programme in genetic females

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Why does development of a normal mammalian female involve inactivation of one of her two X chromosomes? An early view, which had its origin in *Drosophila* genetics, was that it is a means of dosage compensation, to balance the activity of X-linked genes between males (AAXY) and females (AAXX) (Muller and Kaplan 1966; Stern 1973; reviewed in Gartler 2014). Ohno (1967) linked the origin of X inactivation to the viability of the single-X (monosomic) condition and proposed a two-step evolutionary hypothesis, also involving dosage compensation: (i) enhancement of X-chromosome activity so that the product levels of genes on the single X in the male and those of genes on the two sets of autosomes would be similar; and (ii) inactivation of one of the two X chromosomes in the female to bring down the enhanced levels of X-coded products to disomic levels. Support for the hypothesis came from genomewide expression studies using microarrays and RNA sequencing (Nguyen and Disteche 2006; Gupta *et al.* 2006), but other studies were not in support (Xiong *et al.* 2010; Julien *et al.* 2012; Lin *et al.* 2012; Chen and Zhang 2015; reviewed in Birchler and Veitia 2012), raising the question whether X inactivation serves other functions which might explain its evolution (Lin *et al.* 2012). Recent work employing allele-specific methods for the study of transcription in single cells suggest that upregulation of the active X occurs, and that the extent of upregulation is similar in the two sexes, in accordance with Ohno's postulate (Larsson *et al.* 2019).

Here I provide evidence in support of the hypothesis that, in addition to its role in dosage compensation, X inactivation performs a key function in female sex determination (Chandra 1985, 1986). We discuss three lines of evidence: (i) human and mouse X chromosomes contain a large number of genes related to male sexual development and reproduction (Saifi and Chandra 1999; Lercher *et al.* 2003; Zhang *et al.* 2010 and others); (ii) development of the eutherian female involves active suppression of genes for maleness from embryonic stages to adulthood (Chassot *et al.* 2008; Uhlenhaut *et al.* 2009; Matson *et al.* 2011; reviewed in Sinclair and Smith 2009; Veitia 2010); and (iii) many, perhaps most, mammalian genes controlling sex determination and sexual differentiation are dosage sensitive (Bardoni *et al.* 1994; Foster *et al.* 1994; Huang *et al.* 1999; Achermann *et al.* 2002; Maatouk *et al.* 2008 and others). I argue that a function of X inactivation is to enable the ovary and the female phenotype to develop in genetic females by lowering the product levels of X-linked genes for maleness (figure 1). The term 'genetic female' is used here to indicate the absence of *SRY*, the Y-linked gene which, by activating the pivotal autosomal gene *SOX9,* triggers testis development and the male pathway (Goodfellow and Lovell-Badge 1993; Sekido and Lovell-Badge 2008).

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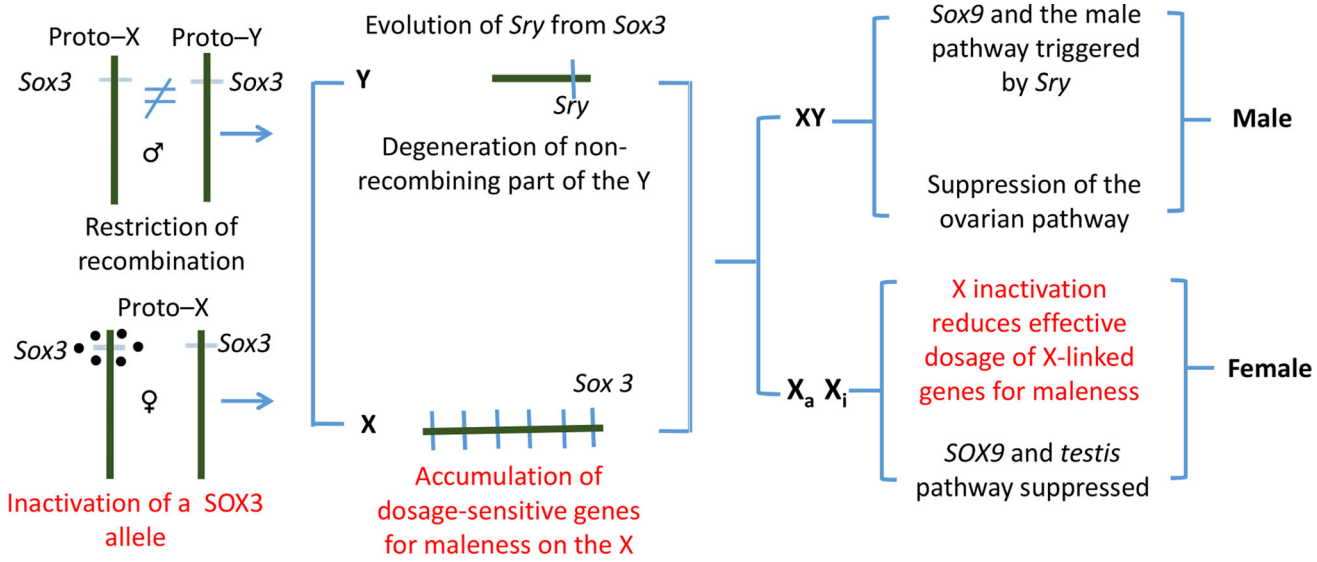

Figure 1. Hypothetical steps in eutherian sex-chromosome evolution showing (in red) the proposed connection between X inactivation and the female phenotype. It is suggested that at an early stage in the evolution of heteromorphic sex chromosomes, X inactivation arose as a means of promoting development of the ovary and the female phenotype by reducing the effective dosage of one or more X-linked genes for maleness such as *SOX3*. The presence of a significant number of genes for maleness on the X, their dosage-sensitive nature, and the finding that testis development and the male pathway are actively suppressed during female development are cited in support of the proposal. X_a and X_i denote, respectively, active and inactive X chromosomes.

The data upon which the above three summary statements (i – iii) are based are as follows:

(i) In a study of the distribution of sex-related and reproduction-related (SRR) genes among individual human chromosomes, Saifi and Chandra (1999) noted that a significantly high proportion of such genes are located on the X chromosome. Lercher *et al.* (2003) made similar observations on a different dataset and showed further that a majority of SRR genes on the X chromosome are related to maleness. Subsequent studies, cited below have confirmed and extended these findings. In mice, the number of X-linked genes with male-specific expression is significantly higher than those with female-specific expression (Wang *et al.* 2001). Mouse and human X chromosomes appear to have acquired about 50 million years ago a burst of male-biased protein-coding and miRNA genes, and in numbers greater than those acquired by the autosomes (Zhang *et al.* 2010). *Rhox*, an X-linked cluster of over 30 mammalian homeobox genes, is expressed in the testis, ovary and other reproductive tissues. Several lines of evidence suggest that *Rhox* may be part of a programme that promotes maleness (MacLean and Wilkinson 2010). *SOX3*, the progenitor of *SRY*, is X-linked (Foster and Graves 1994; Stevanović *et al.* 1993), as is the gene for androgen receptor (*AR*), whose activity is essential for male sexual differentiation. Two other X-linked genes, *ATRX* and *ARX*, are involved in differentiation of Leydig cells which synthesize testosterone; mutations in the former cause urogenital abnormalities ranging from micropenis to complete XY sex reversal (Pask *et al.* 2000; Huyhn *et al.* 2011). Among the 1098 genes identified in the human X-chromosome sequence, as many as 99 are expressed in the testis (Ross *et al.* 2005). The X may also be a preferred location for genes influencing sex-specific behaviours. In an unbiased screen, Xu *et al.* (2012) identified 16 genes showing sexually dimorphic expression in the mouse brain, of which seven are X-linked ($P<1 \times 10^{-4}$). Upon targeted disruption, six of the seven resulted in changes in sex-specific reproduction-related behaviours.

(ii) The mammalian gonad is a bipotential organ whose fate is determined by competitive interactions among factors regulating the testis and ovarian pathways (Capel 2000). In the embryonic gonad, the contest is primarily over control of expression of *SOX9*, the pivotal autosomal gene whose activity is essential for male development. Following its activation by SRY, the SOX9 protein, together with Steroidogenic factor 1 (SF1) binds to TES —the testis enhancer of SOX9— and sets up an autoregulatory system of enhanced and continuous SOX9 expression (Sekido and Lovell-Badge 2008), tilting the balance in favour of testis development and the male pathway. Reprogramming of the testis in to an ovary is prevented by *Dmrt1*, an ancient sex determination gene closely related to *Doublesex* in *D. melanogaster* (Matson *et al.* 2011). In XX embryos, *Sox9* expression is prevented by the action of R-spondin 1 and Wnt 4, acting via β-catenin, an anti-testis, pro-ovarian signalling molecule (Chassot *et al.* 2008). There is active suppression of testicular

development from embryonic stages to adulthood (Uhlenhaut *et al.* 2009). This involves continuous repression of *Sox9* by *Foxl2*: targeted loss of *Foxl2* alone is sufficient to initiate conversion of an adult ovary into a testis, illustrating the plasticity of the gonad. It appears that competing networks, with *Sry*, *Sox9*, *Dmrt1* and others on one side and *WNT/β-catenin* signalling and *Foxl2* on the other determine sexual fate (Chassot *et al.* 2008; Uhlenhaut *et al.* 2009; Matson *et al.* 2011).

(iii) Correct gene dosage is critical at most steps in the mammalian sex determination pathway. We consider first examples of dosage sensitive genes located on autosomes: in humans, duplication of a region containing *SOX9* causes female-to-male sex reversal in XX individuals (Huang *et al.* 1999), whereas a defective *SOX9* copy leads to dominant XY sex reversal in heterozygotes (Foster *et al.* 1994). Autosomal genes involved in the activation of *SOX9* are individually dosage-sensitive in both humans and mice. Development of the gonads and adrenals is regulated by *Sf-1* in a dosage-dependent manner (Achermann *et al.* 2002). Transgenic expression in the gonad of *Sox10*, a close relative of *Sox9*, results in complete female-to-male sex reversal in XX mice (Polanco *et al.* 2010). The extent of sex reversal was correlated with levels of *SOX10* expression. Duplication of the distal segment of human chromosome 1p, which includes both *WNT4* and *RSPO1*, disrupts the male pathway and causes male-to-female sex reversal (Maatouk *et al.* 2008), whereas targeted deletion of Wnt4 causes masculinization of XX mice (Jordan *et al.* 2001). Loss of the fibroblast growth factor Fgf9 results in XY sex reversal (Siggers *et al.* 2014). *DMRT1* deletions cause XY feminization in humans (Muroya *et al.* 2000; Ounap *et al.* 2004). The transcriptional factors Fog2 and Gata4 exert a dosage-dependent influence on gonad development in mice (Bouma *et al.* 2007).

The phenotypic consequences of variation in the actual or effective dosage of sex-determination genes on the X chromosome are of direct relevance to the present proposal. We examine below data from humans and experimental results from mice on six X-linked genes: *SOX3*, *DAX1*, *AR*, *RHOX*, *ATRX* and *Eif2s3X*.

SOX3, the likely progenitor of SRY, codes for a transcription factor very similar to *SRY*: the two proteins share 90% similarity in their DNA-binding HMG domains (Stevanović *et al.* 1993; Bowles *et al.* 2000). To study the functional equivalence of SOX3 and SRY, Sutton *et al.* (2011) developed a line of transgenic mice overexpressing Sox3 and showed that it can substitute for Sry and cause XX sex reversal by activating Sox9. Sex reversal was attributed to ectopic expression of the transgene because native Sox3 expression levels are very low in the developing gonad and single nucleotide changes in SOX3 are not known to affect sex determination in the mouse. In humans, sex reversal has been reported in 46,XX SRY-negative individuals carrying SOX3 duplications (Woods *et al.* 2005; Sutton *et al.* 2011; Moalem *et al.* 2012; Vetro *et al.* 2015; Grinspon *et al.* 2016; Tasic *et al.* 2019) and in a few showing genomic rearrangements in SOX3 regulatory sequences (Woods *et al.* 2005; Sutton *et al.* 2011; Moalem *et al.* 2012), but the mechanism by which these rearrangements induce sex reversal could not be delineated further because studies on gene expression cannot be done on the human gonad. However, since these observations raise the possibility of a dosage effect, we make use of cytogenetic data on human X-chromosome aneuploids to ask whether the sex reversal observed in these XX individuals could be a consequence of increased SOX3 dosage. Both the 45,XO condition and the 47,XXX condition are female. Very few phenotypic features distinguish 47,XXX women from 46,XX women. Gonadal function in 47,XXX women is normal, and many are fertile. SOX3 dosage in 47,XXX women would be three, the same as in XX men carrying a SOX3 duplication. In triplo-X women, one X is expected to be active and the other two inactive, whereas in the XX male, if X inactivation is random, the X carrying the duplication would be active in roughly 50% of the cells, and sex reversal could be a consequence of the expected increase in SOX3 levels. These observations allow us to suggest that increased SOX3 levels support testis development and, further that in the normal 46,XX embryo the functionally monosomic condition induced by X inactivation keeps SOX3 levels below a threshold and curbs SOX3's capacity to trigger the male pathway.

Dosage sensitive sex reversal, adrenal hypoplasia congenita critical region on X, gene 1 (*DAX1*) codes for an unusual orphan nuclear receptor (NROB1). It lacks a DNA-binding domain and acts as a dominant-negative regulator of *SF1* and other nuclear receptors (Iyer and McCabe 2004). It has an important role in Sertoli cell survival and differentiation. The gene is necessary for normal gonadal and adrenal development (Swain *et al.* 1996). It is coexpressed with *Sf1* during gonadal differentiation and represses Sf1-mediated upregulation of *Amh*, the gene for anti-Müllerian hormone. Mutations in *DAX1* are responsible for X-linked adrenal hypoplasia congenita (AHC) in humans. In XY patients with a duplication of the Xq21.2 region, there are two active copies of *DAX1* which results in male-to-female sex reversal despite the presence of a normal *SRY* (Bardoni *et al.* 1994; McClelland *et al.* 2012; Ludbrook *et al.* 2012). Since 47,XXY individuals are male, clearly this gene is dosage sensitive and subject to X inactivation. Mice and humans differ in sensitivity to variations in DAX1 dosage. In sensitized genetic backgrounds, *DAX1* knockout mice show XY sex reversal (Park *et al.* 2008). In cultured cells, *DAX1* overexpression antagonises SF1/SOX9-mediated activation of TES—the testis-specific enhancer of *SOX9* (Ludbrook and Harley 2004; Ludbrook *et al.* 2012). *DAX1* has been described as having both 'antitestis' and 'protestis' capacities (Ludbrook and Harley 2004; McClelland *et al.* 2012).

Experimental work on cells from a female patient with AHC suggests that DAX1 has the capacity to undergo a dosage-dependent shift from being a repressor to an activator (Wilson *et al.* 2001). On the basis of experimental observations on mice and patient data on humans, it has been suggested that there may be two DAX1 concentration thresholds such that DAX1 activity in excess of an upper threshold antagonizes testis differentiation whereas its activity below a lower threshold allows ovarian differentiation to occur (Ludbrook and Harley (2004). Since one of the two copies of *DAX1* is expected to be inactive in normal females, the role of X inactivation could be inhibition of DAX1's protestis capacity and promotion of its pro-ovarian capacity by keeping DAX1 levels below a lower threshold.

Androgen receptor: *AR* acts during sexual differentiation, after gonadal determination. It is required for male reproduction, but its role in female reproductive physiology is less understood. The actions of testosterone and dihydrotestosterone are mediated by *AR*. The gene is subject to X inactivation. In female, excessive androgen levels are associated with polycystic ovary syndrome, anovulation and infertility (Davey and Grossman 2016). Female mice heterozygous for a targeted deletion of *Ar* showed significant dosage-dependent reduction in fertility (Zhou 2010). The only known androgen-inducible and AR-inducible transcription factor in Sertoli cells is Rhox5, which is also X-linked and subjected to X inactivation (MacLean and Wilkinson 2010).

The RHOX cluster: Like several other genes in this cluster, *Rhox5* is responsive to androgen and selectively expressed in the Sertoli cells of the testis (MacLean and Wilkinson 2010). It is subject to X inactivation in the early embryo and expressed only in the paternal X chromosome (Kobayashi *et al.* 2006). Targeted disruption of Rhox5 results in reduced fertility (MacLean and Wilkinson 2010). In mice that have undergone sex reversal as a result of targeted deletion of *Foxl2*, there is a dramatic upregulation of *Rhox8* (Uhlenhaut *et al.* 2009), suggesting that *Rhox* may be part of a programme that promotes maleness and that X inactivation is part of the process that keeps Rhox8 levels low in females (MacLean and Wilkinson 2010).

ATRX: This is an ancient ultraconserved sex-determining gene essential for normal testis development (Ion *et al.* 1996). It is subject to X inactivation. *ATRX* mutations lead to XY sex reversal in humans. In marsupials, orthologs of this gene are present in both X and Y chromosomes (Pask *et al.* 2000). The Y-linked orthologue has been lost among eutherians. *Atry* is expressed in the developing and adult testis of the tamar kangaroo whereas *Atrx* is more broadly expressed. These observations have led to the proposal that *Atry* may be the primary trigger of testis determination in marsupials and that the evolution of *Sry* from *Sox3* in eutherians occurred later (Pask *et al.* 2000; Huyhn *et al.* 2011).

Eif2s3Y and Eif2s3X: *Eif2s3Y* codes for subunit 3 of the eukaryotic translation initiation factor 2. It is a spermatogonial proliferation gene which may be the only Y-linked gene necessary for spermatogenesis (Yamauchi *et al.* 2016). There is some evidence that it may also have a role in testis differentiation and gonadal development. *Eif2s3Y* is conserved on the Y during eutherian evolution but has been lost from the human Y and the Y chromosomes of other simian primates (Ehrmann *et al.* 1998). In mice, *Eif2s3Y* and *Eif2s3X* are functionally interchangeable (Yamauchi *et al.* 2016), which means that this X–Y homologous gene is present in two copies of both males and females. *Eif2s3X* is not subject to X inactivation, and there is no evidence for the compensatory enhancement of *Eif2s3X* that one would expect from Ohno's hypothesis (Yamauchi *et al.* 2016). Instead, what appears to be important are the global levels of *Eif2s3Y/Eif2s3X* expression. In the absence of X inactivation, there appears to have evolved a novel way of bringing about inequality between XX and XY embryos in *Eif2s3Y/Eif2s3X* product levels. The expression levels of the X-copy and the Y-copy are distinct: overexpression of Eif2s3Y, ostensibly to meet the needs of spermatogenesis, and low-level expression of Eif2s3X to facilitate successful oogenesis.

The six X-linked genes described above code for constituents of large multimeric complexes which are part of signalling pathways and transcriptional networks. Such genes tend to be dosage sensitive because of overexpression (as a result of duplication or trisomy) or underexpression (due to deletion) of a protein or subunit forming a multimeric complex, would lead to stoichiometric imbalance and the normal functioning of the complex and the regulatory network would be compromised (Veitia and Birchler 2010; Birchler and Veitia 2012; Pessia *et al.* 2012). Among the more than 40 sex-determination and gonadal development genes known among mammals, more than half are transcription factors, signalling molecules and receptors (data from Wilhelm *et al.* 2007; Eggers and Sinclair 2012).

The similarity between the proteins encoded by SOX3 and SRY, and their functional equivalence (Stevanović *et al.* 1993; Foster and Graves 1994; Sutton *et al.* 2011) provide strong support to the hypothesis that SRY evolved from SOX3 during differentiation of the X and Y as heteromorphic sex chromosomes. Typically, restriction of recombination between proto-X and proto-Y chromosomes in the heterogametic sex leads to degeneration of the nonrecombining part of the Y chromosome. During the initial stages of transition to the heteromorphic state, there would be a substantial number of X–Y homologous genes, but they would become progressively fewer as erosion of the Y continues (Charlesworth 1978, 1991; Charlesworth *et. al.* 2005). The resulting imbalance between XX and XY individuals in the dosage of X-linked genes would lead to the evolution of compensatory mechanisms to overcome the detrimental effects of haplo-insufficiency (Ohno 1967). As many as 12

to 20% of human and 3 to 7% of mouse X-linked genes escape inactivation (Balaton and Brown 2016), and about 30 genes located in the two pseudoautosomal regions of the human X have homologs on the Y chromosome (Bellott *et al.* 2014). Thus, there are four possible types of dosage relationship between XX and XY individuals: (i) X–Y homologous genes, with the X copy subjected to inactivation (XX = 1 effective copy; XY = 2 copies); (ii) X–Y homologous, and the X copy not subjected to inactivation (XX = 2; XY=2); (iii) X-specific genes subjected to inactivation (XX = 1 effective copy; XY=1); (iv) X-specific, not subjected to inactivation (XX = 2; XY = 1) (Chandra 1994). Among the four, type (i) is of evolutionary interest because it suggests a role for X inactivation in initiating functional inequality between X-borne and Y-borne alleles of a sex-determining gene.

Therian mammals (marsupials and eutherians) and monotremes (egg-laying mammals) shared a common ancestor ∼168 million years ago (Mya) (Bininda-Emonds *et al.* 2007). The sex chromosomes emerged in this common ancestor. *SRY* is a therian innovation; it is not present in other vertebrates. X inactivation too is a feature of therian biology; it is not found among monotremes, which diverged from the therian lineage ∼148 Mya, or in other vertebrates. This suggests that both *SRY* and X inactivation evolved in a common therian ancestor sometime between ∼168 and ∼148 Mya (Bininda-Emonds et al. 2007; Patel *et al.* 2010). It has been suggested that sex-related forces played a major role in shaping X-chromosome functions and that this 're-modelling' of the X occurred within about 90 million years of its origin (Potrzebowski *et al.* 2010).

A number of sex-related forces have been investigated for their possible roles in the evolution of X inactivation. These include imprinting (Brown and Chandra 1973; Chandra and Nanjundiah 1990, 1993; Hurst 1997), conflict between parental genomes (Moore and Haig 1991; Hurst 1997; Haig 2006), sexual antagonism (Wu and Xu 2003; Haig 2006) and sex-specific selection (Iwasa and Pomiankowski 2001). Imprinting is of particular interest because there is a large body of evidence linking this unusual phenomenon to sex determination in certain insect taxa. In the dipteran *Sciara coprophila*, the zygote contains two sets of autosomes (Am Ap) and three X chromosomes, of which two are maternal and one is paternal in origin (Xm Xm Xp). In early embryonic development, one Xp is lost from the germline of both sexes, two Xps from the male soma, and one Xp from the female soma. As a result, the chromosome constitution of the germline (Xm Xp) is the same in the two sexes, but the male soma (XO) and the female soma (XX) differ from each other into X-chromosome dosage. This difference determines whether the gonad develops into an ovary (XX) or a testis (XO) (Metz 1938; Crouse 1960). In the sexually reproducing diaspidid coccid *Pseudaulacaspis pentagona*, there are no sex chromosomes, but at an early stage of embryonic development the paternal chromosomes are eliminated from some embryos but not in others. The former develop into haploid males and the latter, diploid females (Brown and Bennett 1957). Developmental inactivation of a chromosome, when its homolog in the same nucleus is active, is known only from mammals and certain coccid insects, the mealybugs and their relatives. In sexually reproducing mealybugs, the paternal set of chromosomes is inactive in sons but not in daughters. As a result, the male is functionally haploid, and the female, diploid (Brown and Nelson-Rees 1961; Nur 1963, 1971; Chandra 1963). Comparative accounts of imprinting in *Sciara*, mealybugs and mammals can be found in Chandra and Brown (1975), Brown and Chandra (1977) and Chandra and Nanjundiah (1990, 1993).

Among marsupial mammals, X inactivation is incomplete, tissue-specific and paternal. It may also be stochastic, in the sense that it is the probability of expression rather than the degree of expression that is regulated (Nadaf *et al.* 2010). There is no evidence of an X-inactivation centre. A dosage effect of the X chromosome on sexual differentiation has been reported in certain marsupial species, but its relationship to X inactivation, if any, is not known (Pask and Graves 2001). The eutherian system of random X inactivation is thought to offer certain advantages over the marsupial system of paternal X inactivation (Brown and Chandra 1973; Chandra and Brown 1975; Chandra and Nanjundiah 1993). For instance, the cellular mosaicism (mosaic heterozygosity) which is a consequence of random X inactivation confers protection to heterozygotes from X-linked recessive disorders.

In summary, it is proposed that X inactivation plays a key role in female sex determination by suppressing the activity of X-linked genes for maleness. This role in sex determination may have provided a selectionist basis for its evolution. As a consequence of X inactivation, the effective dosage of X-linked genes in XX embryos would be the same as their actual dosage in XY embryos. In terms of the present hypothesis, this dosage-compensatory process results in two dissimilar sexual phenotypes, female and male, because X-linked genes for maleness are suppressed in the former, while *SRY* activates *SOX9* and the male pathway in the latter (figure 1). If this line of reasoning is valid, it suggests that sex determination and dosage compensation have evolved as interconnected processes.

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