Role and control of X chromosome dosage in mammalian development
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Many species have evolved sex chromosomes with highly divergent gene content, such as the X and Y chromosomes in mammals. As most non sex-specific genes probably need to be expressed at similar levels in males and females, dosage compensation mechanisms are in place to equalize the gene dosage between the sexes, and possibly also between sex chromosomes and autosomes. In mammals, one out of two X chromosomes is inactivated early during development in a process called X-chromosome inactivation that has been investigated intensively in the 50 years since it was discovered. Less is known about the potential functional roles of X-linked gene dosage, for example in controlling development in a sex-specific manner. In this review, we discuss the evolution of dosage compensation and how it is controlled during embryogenesis of mammals. In addition we will summarize evidence on the potential role of X chromosome number during early development.

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Although in mammals, dosage compensation for most genes on the X is probably vital, dosage differences might also play a functional role at several levels. In particular, genes that escape X inactivation are thought to contribute to the phenotypic differences between the sexes [4]. In addition, before initiation of XCI during early development all genes on the X should be present as a double dose in females, but it is not known whether this results in sex-specific developmental differences and so far, this question has received little attention. In this review, we will discuss recent advances in our understanding of the evolution of dosage compensation in mammals, between the X chromosome and autosomes, as well as between the sexes. We will also discuss how dosage compensation is achieved and how X-chromosome dosage might contribute to sex differences, during mammalian development.

Evolution of sex chromosome dosage compensation in mammals
In recent years the question of whether the X chromosome was indeed upregulated during the evolution of the sex chromosomes (Ohno’s hypothesis) has received much attention. Initial studies comparing median expression levels of present day chromosomes, that is, X versus autosomes, lead to conflicting conclusions: when analyzing all expressed genes, the levels of X-linked genes were found to be indistinguishable from those of autosomal genes, while an analysis that excluded lowly expressed...
genes showed some degree of upregulation of the X chromosome [5–8]. As expression can vary significantly even between different autosomes, it has been difficult to draw definitive conclusions from these X-to-autosome comparisons [9**]. To try and circumvent this problem, two groups recently compared X-linked genes with orthologous genes in species, where these genes have remained autosomal, because their sex chromosomes evolved from a different pair of autosomes (e.g. birds) [9*,10]. These studies did not find any obvious upregulation of the X chromosome. However, there are indications that instead of chromosome-wide upregulation, initial dosage compensation was restricted to a subset of particularly dose-sensitive genes. For example, components of large protein complexes, whose stoichiometry probably needs to be maintained for proper function, show a ∼2-fold higher median expression when they are located on the X chromosome compared to autosomal protein complex genes [11*]. Moreover, the comparison of X-linked genes with autosomal orthologs in other species has suggested that autosomal genes, the protein products of which physically interact with those of X-linked genes, might have actually been downregulated to achieve dosage compensation [9***]. Therefore, the available evidence suggests that the X chromosome has not necessarily been upregulated in a chromosome-wide fashion. Instead, dosage compensation in relation to autosomal genes might have been restricted mainly to certain dosage sensitive genes. However, even the compensation of a subset of genes makes it necessary to equalize X-linked gene dosage between XY males and XX females, which, in mammals, is ensured by X inactivation.

X inactivation is found in all mammals with the exception of the most ancestral monotreme lineage, which, similarly to birds, do not appear to show global dosage compensation between the sexes [9**]. In marsupials, X inactivation is imprinted so that the paternal X is inactive in all cells, while placental mammals inactivate one randomly chosen X chromosome in each cell. Mice, which show random XCI in somatic tissues, have, in addition, evolved an imprinted form of X inactivation, which does not appear to be present in humans or rabbits, on the basis of existing data [12]. In mice, the paternal X is inactivated in the pre-implantation embryo and remains silent in extra-embryonic tissues, while it is reactivated in the embryo proper, before the onset of random XCI in the epiblast (Figure 1). In placental mammals XCI is controlled by the long non-coding RNA Xist, which is expressed from the inactive X and mediates gene silencing in cis [13]. In marsupials, Xist is not present, although another, apparently unrelated, non-coding RNA, called Rsx, seems to be involved in XCI. The exact role and mechanism of action of Rsx in marsupial dosage compensation remain to be defined [14*].

**X-chromosomal dosage defects**

The importance of X-linked gene dosage in mammals is illustrated by the symptoms associated with certain X aneuploidies. X monosomy (XO) for example, which leads to Turner syndrome in humans, is associated with congenital malformations and infertility [15]. Why should XO and XX females differ, when both have only one active X chromosome in theory? In fact, the observed defects are attributed to a subset of genes that escape X inactivation [4]. Some of these genes have a homolog on the Y (e.g. in the pseudoautosomal region), and are therefore present at reduced levels in Turner patients compared to normal males and females. Interestingly, XO mice show no phenotype except for being infertile [16]. This is probably due to the fact that far fewer genes escape XCI in mice (∼3%) compared to humans (∼15%) and also because the human X carries more pseudoautosomal genes than the mouse X chromosome [17,18]. Some behavioral symptoms of Turner patients are specific to cases where the paternal or the maternal X is present, suggesting a role of imprinted genes on the X. While Turner syndrome is associated with reduced levels of escape genes, the XXY Klinefelter syndrome presumably results from increased levels and manifests itself by tall stature, infertility and behavioral abnormalities [16]. Surprisingly, symptoms due to increased X-chromosome copy number are more severe in the male XXY context. Triple-X females (XXX) are often not even diagnosed because of their very mild phenotype [19]. This suggests a sex-specific function of escape genes.

In cases where X-chromosome dosage is disturbed due to the absence of XCI, more severe defects are seen. For example, ring X chromosomes that cannot undergo XCI because they lack the Xist locus cause multiple malformations and mental retardation due to a double dose of some of the (usually rather few) genes present on the ring [15]. The consequences of an abnormal dose of the entire X chromosome have been studied extensively in mice in different situations where XCI is not properly triggered. Defective imprinted XCI in the early embryo, leads to severe abnormalities in the extra-embryonic tissues and the embryos die around day 7 of gestation [20–22], while defective XCI in the embryo proper results in death around day 10 d.p.c. [23]. It is therefore clear that several developmental processes are sensitive to abnormal X dosage, but which genes actually require this stringent control? In general, genes participating in protein complexes and regulatory networks are thought to be especially dosage sensitive [24]. This is supported by the observation that most haplo-insufficiencies in humans involve transcription factors and protein complex genes, but rarely metabolic enzymes [25]. In the case of the X chromosome, however, the genes that need to be tightly controlled for normal development to occur remain to be identified.
Regulation of X-chromosome inactivation during early mouse development. Imprinted XCI is initiated by Xist upregulation (yellow) from the paternal X at the 2–4-cell stage, when the Xist activator Rnf12 is present as a large maternal pool (rose). Imprinted XCI is reversed in the inner cell mass of the blastocyst (E4.5), where Xist becomes downregulated. Subsequently, Xist (red) is upregulated from one randomly chosen X (Xm or Xp) in each cell, at least in part triggered by the downregulation of mostly autosomal (A), repressive stem cell factors (green). Female specific upregulation of Xist is partly ensured by a double dose of Rnf12 (blue) and maybe by reduced methylation levels of the Xist promoter (brown). On the inactive X (Xa) X-linked genes like Rnf12 are silenced and the Xist promoter loses methylation, while the Xist promoter on the active X (Xa) becomes fully methylated. The methylation state of the Xist promoter (brown) in male and female embryos is unknown, and was estimated from ES cell data. The depicted dynamics of stem cell factors are on the basis of [44,59]. Boxes indicate the time window when imprinted and random XCI are initiated.

Developmental regulation of X inactivation

In mice, imprinted XCI is initiated on the paternal X at the 2–4-cell stage [26]. In the inner cell mass (ICM) of the blastocyst the paternal X is then reactivated and random XCI occurs during the transition from the late blastocyst (E4.5) to the epiblast (E5.5) (Figure 1) [26,27]. The Xist imprint is established in the female germ line and prevents Xist upregulation [28,29]. Its exact nature is still unknown, but it is located within a 210 kb Xist-containing transgene that was shown to be sufficient to recapitulate imprinted XCI [30]. All X chromosomes (and Xist transgenes so far) that are not protected by the imprint are inactivated during imprinted XCI, independently of the number of X chromosomes in the cell [29,31]. Random XCI, by contrast, is regulated by more complex mechanisms than the imprinted version and cannot even be recapitulated by a large 460 kb Xist-containing transgene [32]. As the imprint on Xist is lost or overridden before the onset of random XCI, alternative mechanisms must ensure Xist expression from exactly one out of two identical chromosomes in a female-specific (i.e. X dose sensitive) fashion. Female specificity is in part ensured by the X-linked Xist activator Rnf12 (Rlim) present as a double dose in female XX cells (Figure 1) [33]. Rnf12 is a E3 ubiquitin ligase that might function by targeting the Xist repressor Rex1 (Zfp42) (see below) for degradation [34**]. During imprinted XCI, Rnf12 is provided as a large maternal pool independently of the sex of the embryo and allows the paternal Xist gene to be upregulated right after zygotic genome activation [35†]. The maternal Xist gene resists this due to its repressive imprint. Although additional regulators as well as trans-interactions have been proposed to contribute to female-specific Xist expression during random XCI (reviewed in [36]), Rnf12 remains the only factor whose overexpression leads to ectopic Xist upregulation in male cells. However, additional X-linked activators must exist, since even with a single dose of Rnf12 (in Rnf12+/− heterozygous ESCs or mice), females can still initiate XCI [33,35†].

With regard to the mechanisms that control the onset of random XCI, many insights have come from the use of embryonic stem cells (ESCs), which are derived from the ICM of the blastocyst and undergo XCI upon differentiation in vitro. These studies have led to a model whereby Xist is repressed by stem cell factors, which
are present at high levels in the ICM and ESCs. Xist upregulation is then triggered by the downregulation of these repressors, in addition to the expression of X-dosage sensitive factors such as Rnf12. A series of stem cell factors have been proposed to be implicated in Xist regulation (summarized in Table 1), as their depletion in ESCs results in upregulation of Xist [34**,37–39]. It is however difficult to identify the factor(s) that directly regulate Xist and are required for its repression, because the stem cell network is highly inter-connected and depletion of any one factor affects the expression of many others [40]. Often, binding of a candidate regulator to a locus is taken as an indication for direct regulation. In the case of Xist, however, nearly all stem cell factors tested so far, bind to one or more sites within or around the Xist gene (Figure 2, Table 1) [34**,37–39,41,42]. One of these binding sites (in Xist intron 1) has recently been tested for functionality by deletion and was found to have only a minor effect, if any, on Xist expression during differentiation [43]. This suggests either that only a subset of binding sites are functional, or that the sites and therefore also the factors that occupy them, act in a redundant fashion. To narrow down the list of potential relevant repressors of Xist, their expression kinetics are clearly important. Apart from binding to the locus and Xist upregulation upon deletion or depletion of the factor, a relevant Xist repressor should be downregulated before or concomitantly with Xist upregulation in vivo. While the core pluripotency factors Oct4, Nanog and Sox2 are still expressed in the E5.75 epiblast, some other stem cell factors such as Rex1 and Prdm14 are strongly down-regulated [44] (Table 1, Figure 1). Rex1 is indeed a likely Xist repressor both as a target of the Rnf12 ubiquitin ligase and as an activator of Tsix, the antisense repressor of Xist [34**,38]. Also a potential role of Prdm14 in Xist regulation merits further investigation, as its ectopic expression in combination with Klf2, increases the efficiency of Xist reactivation during reprogramming of epiblast stem cells [45**]. Taken together, the emerging picture is that the core pluripotency factors may not directly control the initiation of XCI but rather stem cell factors, such as Rex1 or Prdm14 may play important roles in Xist regulation, the precise nature of which needs to be explored further.

**Sex-specific functions of X-chromosome dosage in development**

The two time windows during mouse development when X-chromosome number would be predicted to have the strongest effect, are in the ICM and in the female germ line, when both X’s are active. At least in ES cells, X-linked genes show on average a 2-fold higher expression in female cells compared to males [6]. Interestingly, X dosage also affects global levels of DNA methylation, such that XX cells are hypomethylated at repeats and imprinted regions compared to XY and XO cells [46,47]. The fact that female primordial germ cells, which also have two active X chromosomes, are similarly hypomethylated, supports a role of X-chromosomal dosage in controlling global DNA methylation levels [48]. The X-linked gene(s) responsible for this effect remain to be found however. These sex-specific differences in methylation levels might also contribute to ensuring the female-specificity of random XCI. Mirroring global methylation differences, the Xist promoter is fully methylated in undifferentiated male ES cells, but only partially methylated in females [49,50]. During differentiation of female ESCs, the promoter gains methylation on the active X (where Xist is silenced) and loses methylation on the inactive X from which Xist is expressed [51]. Xist promoter methylation on the active X is important to maintain its silent state during differentiation, since a loss of methylation in embryonic fibroblasts results in Xist derepression, and male ES cells deficient for DNA methyltransferases erroneously upregulate Xist at later stages of differentiation [52–54]. Given these sex-specific differences in ESC DNA methylation levels, it is interesting to speculate that DNA hypomethylation in XX cells might be required to allow X inactivation in females. This hypothesis however remains to be investigated.

A hint that X-chromosome dosage, and the transient double dose of X-linked genes in the ICM of the female blastocyst (see above) might indeed affect development

<table>
<thead>
<tr>
<th>Potential Xist repressors in stem cells</th>
<th>Xist upregulation upon depletion</th>
<th>Binding to the Xist/Tsix locus</th>
<th>Fold downregulation in the epiblast [44]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct4/Pou5f1</td>
<td>+ [39,37]</td>
<td>+ [60,37,39]</td>
<td>1×</td>
</tr>
<tr>
<td>Sox2</td>
<td>– [37]</td>
<td>+ [60,37,39]</td>
<td>4×</td>
</tr>
<tr>
<td>Nanog</td>
<td>+ [39]</td>
<td>+ [60,39]</td>
<td>16×</td>
</tr>
<tr>
<td>Rex1/Zfp42</td>
<td>+ [34**,38]</td>
<td>+ [34**,38]</td>
<td>&gt;64×</td>
</tr>
<tr>
<td>Klf4</td>
<td>n.d.</td>
<td>+ [41,38]</td>
<td>64×</td>
</tr>
<tr>
<td>Esrrb</td>
<td>– [61]</td>
<td>+ [41]</td>
<td>32×</td>
</tr>
<tr>
<td>Prdm14</td>
<td>+ [42]</td>
<td>+ [42]</td>
<td>&gt;64×</td>
</tr>
<tr>
<td>Klf2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>64×</td>
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</table>

For each stem cell factor it is indicated, whether knockdown or knockout of the respective factor result in Xist upregulation (first column, n.d.: not determined). The second column summarizes the available ChIP-Seq data shown in Figure 2, and the third column indicates the fold downregulation of the respective factors in the E5.75 epiblast compared to ES cells.
of the epiblast, came from a series of studies performed by Paul Burgoyne in the 1990s. It was previously known that in several mammalian species male embryos grow faster during early development [55]. By comparing the growth rates of various XX, XO and XY karyotypes, Burgoyne and colleagues could show that the Y chromosome (of most mouse strains) accelerates embryonic development before implantation (<E3.5), while the presence of two X chromosomes slows down embryonic development just after implantation into the uterus [56–58]. As a consequence it could be hypothesized that either sex-specific DNA methylation differences and/or the double dose of X-linked genes might slow down development of XX embryos compared to XY or XO embryos, shortly after the time window when both X’s are active in the ICM of the blastocyst and when random XCI is first initiated.

Conclusions

Questions surrounding the extent and mechanisms of X-chromosome dosage compensation relative to autosomal genes, as well as to differences between the sexes, have been debated for decades. Since the advent of genome-wide approaches, several efforts have been recently undertaken to test Ohno’s hypothesis. As so often however, the devil seems to be in the detail and different groups come to opposite conclusions depending on the specificities of their analysis. The degree to which X chromosome-wide dosage compensation is necessary seems to depend on how many genes actually require such compensation. Indeed, whether the well-known phenotypes associated with dosage defects are caused by a few highly dose sensitive genes or whether they are rather a cumulative result from the dosage imbalance of many genes remains an open question. The identification of the genes involved represents an important challenge for the future.

The molecular mechanisms underlying dosage compensation between the X and autosomes, as well as between the sexes remain areas of active investigation. In the case of XCI, although the role of X-linked gene dosage in regulating X inactivation is better understood, even here the genes involved (apart from Rnf12) remain to be identified and the mechanisms of such fine tuned regulation elucidated. It also remains a challenge to disentangle the complex links between the stem cell network and XCI, and to define the different actors that ensure female-specific, monoallelic Xist expression. Apart from XCI, the only developmental sex difference that has been attributed to X dosage in vivo so far is differential growth.
during post-implantation development. Sex differences at the molecular level (DNA methylation, transcription) have only been analyzed in the ESC in vitro system, but it is now becoming technically possible to test their in vivo relevance and hopefully to elucidate their potential impact on development and female versus male growth rates. Hopefully, our knowledge about the impact of X-chromosomal gene dosage on different processes will increase rapidly in coming years.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


In this comprehensive study, the authors use RNA-sequencing data from several tissues of different species to gain better insight into dosage compensation mechanisms. Among other results they show that, in higher mammals, X-linked genes have not been upregulated compared to their autosomal orthologs in other species.


This study shows that X-linked genes, whose protein products participate in larger protein complexes and which are therefore expected to be dosage sensitive, are dosage compensated relative to autosomal genes.


This study identifies an X-linked gene called Rsx in the marsupial Monodelphis domestica, which exhibits a similar expression pattern to the Xist RNA in placental mammals and can initiate gene silencing in cis.


Role and control of X chromosome dosage


In this study a variety of stem cell factors are tested for their ability to promote X reactivation during reprogramming of epiblast stem cells. The authors show that the combined ectopic expression of Prdm14 and Klf2 strongly increases the reprogramming efficiency.


