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The compleX balancing act of controlling X-chromosome dosage and how it impacts mammalian germline development

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In female mammals, the two X chromosomes are subject to epigenetic gene regulation in order to balance X-linked gene dosage with autosomes and in relation to males, which have one X and one Y chromosome. This is achieved by an intricate interplay of several processes; X-chromosome inactivation and reactivation elicit global epigenetic regulation of expression from one X chromosome in a stage-specific manner, whilst the process of X-chromosome upregulation responds to this by fine-tuning transcription levels of the second X. The germline is unique in its function of transmitting both the genetic and epigenetic information from one generation to the next, and remodelling of the X chromosome is one of the key steps in setting the stage for successful development. Here, we provide an overview of the complex dynamics of X-chromosome dosage control during embryonic and germ cell development, and aim to decipher its potential role for normal germline competency.

Introduction

The emergence of distinct sex chromosomes from autosome pairs during evolution, and the subsequent degradation of one sex chromosome by meiotic recombination in the heterogametic sex, would lead to a potential gene dosage imparity between autosomes and sex chromosomes and between males and females [1,2]. Different species have developed diverse dosage compensation mechanisms to overcome this problem [3,4]. In placental mammals, at least three mechanisms are at play; X-chromosome upregulation (XCU), X-chromosome inactivation (XCI) and X-chromosome reactivation (XCR). To balance X-chromosome gene dosage with autosomal levels, genes on the single active X in XY males and XX females are transcriptionally upregulated by XCU [5]. X-dosage parity between males and females is achieved through epigenetic silencing of one of the two X chromosomes in females, in a process called XCI [6]. Although the mechanisms of XCI demonstrate variation between mammalian subclasses [7,8], in eutherians the long noncoding RNA X-inactive specific transcript (XIST) is the indispensable master regulator of the process [9–12]. XIST becomes monoallelically upregulated and acts *in cis* to coat the inactivating X chromosome, resulting in the recruitment of a cascade of effectors that elicit heritable large-scale epigenetic gene silencing and heterochromatization [13]. Finally, this leads to the emergence of the Barr body, with the inactive X chromosome becoming a morphologically characteristic hallmark of the female cell nucleus [14]. XCI is maintained in somatic cell lineages, however, in the germline it is reversed, resulting in the emergence of female germ cells with two active X chromosomes, by a process coined XCR [15–17].

XCR co-occurs with genome-wide epigenetic reprogramming in the female germline, where a clean slate is generated for the propagation of genetic material to the next generation [18–20]. This, in turn, prepares the cells for the initiation of meiosis during oogenesis, and ultimately the acquisition of totipotency upon fertilisation [21]. Currently, the mechanisms and biological roles of XCI, XCU and XCR in female germ cell development and oogenesis remain elusive.

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In this review, we aim to discuss the significance of the intricate interplay of the different X-chromosome dosage compensation mechanisms and their unique dynamics and role in female embryonic and germ cell development. Particularly, we want to address to which degree the status of the X chromosome is a cause, or a consequence, of normal germ cell development. We discuss the current understanding within the field with a focus on the mouse model system, and suggest gaps in our knowledge that would benefit from further investigation, including in humans.

X-chromosome inactivation

In female mice, XCI is first initiated at the 2–4 cell stage of embryonic development and occurs specifically on the paternal X chromosome, while the maternal X chromosome remains active [22–25]. This imprinted form of XCI (iXCI in Figure 1) is continuously maintained in the mouse extraembryonic lineages of the primitive endoderm and trophectoderm and later on in the placenta, but is reversed in the developing epiblast of the inner cell mass of the E(mbryonic day) 4.5 blastocyst, which gives rise to all embryonic cell lineages including germ cells [23,26–28]. Imprinted XCI is controlled by the paternal expression of *Xist* and essential for female

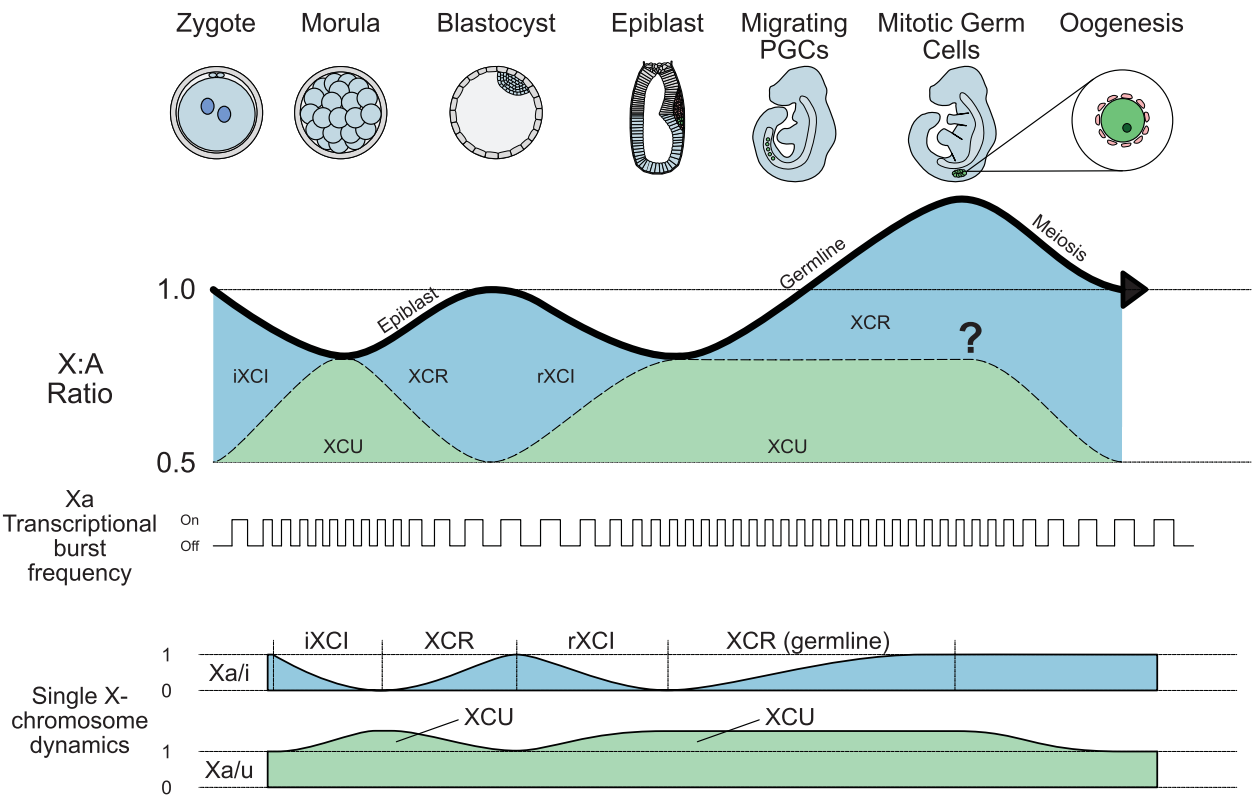

Figure 1. X-chromosome dosage control during female mouse early embryonic and germ cell development.

The zygote is established with two active X chromosomes. As the embryo develops beyond the 2–4 cell stage, the paternal X chromosome undergoes imprinted XCI, meanwhile the maternal X partially balances the X-dosage with autosomes through upregulation (XCU). XCU is controlled by an increased transcriptional burst frequency on the active X (Xa), symbolised by the square wave pattern by which promoters switch between ON and OFF states. As the embryo develops from the morula to the preimplantation epiblast, the inactive X chromosome rapidly reactivates, while the XCU of the active X diminishes. After implantation, the epiblast undergoes random XCI and X-chromosome dosage is again partially compensated by XCU. XCR then occurs in the developing germline, when primordial germ cells (PGCs) migrate towards and enter the embryonic gonads. A possible delayed reduction (question mark) of XCU may lead to hyperexpression of X-chromosomal genes prior to meiotic entry, which subsequently subside to normal XaXa levels. The top graph depicts the combined gene dosage from both X chromosomes in relation to autosomes (X:A ratio). The graphs at the bottom show the proposed gene dosage in blue from the X chromosome switching between active (Xa) and inactive (Xi) states by XCI and XCR and in green from the constitutively active X, which tunes its expression between a regular active (Xa) and a hypertranscribed upregulated (Xu) states by XCU.

embryonic development, as paternal *Xist* deletion leads to postimplantation lethality [12]. A non-canonical H3K27me3 imprinting mark is established during oocyte growth at the *Xist* locus, which ensures that *Xist* remains off on the maternal X chromosome during preimplantation development [29–31]. Subsequently, *Xist* remains repressed in the extraembryonic lineages by maternal expression of *Tsix*, the noncoding antisense repressor of *Xist* [32,33]. In addition to suppression of *Xist* on the maternal X chromosome, imprinting of the paternal X chromosome in the male germline might be at play, which mediates *Xist*-independent silencing of repeats [24] and promotes *Xist* expression during mouse preimplantation development [34]. The process of imprinted XCI is not conserved among all placental mammals and does not occur in humans [35] (Figure 2), whereas it is the only form of XCI in marsupials [39], where it is controlled by a different noncoding RNA called *Rsx*, the marsupial equivalent to *Xist* [8,40].

Upon differentiation of the mouse postimplantation epiblast, ultimately giving rise to the embryo proper, a second wave of so-called random XCI (rXCI) is initiated, where either the maternal or paternal X is inactivated and clonally maintained throughout life in somatic cell lineages [6,41–43] (Figures 1 and 2). The timing of rXCI initiation upon differentiation of the epiblast indicates a link between naive pluripotency and the status of the X chromosomes. The presence of two active X chromosomes in mouse embryonic stem cells (ESCs) favours pluripotency through inhibition of GSK3 and MAPK signalling, thereby stabilising the pluripotency network [44]. *Dusp9*, an X-linked negative regulator of the MAPK pathway, has been shown to play an important role in establishing lower global DNA-methylation levels in XX mouse ESCs [45], compared with XY or XO cells, through its increased dosage from the two active X chromosomes [46–48]. A second X-linked gene, *Klhl13*, has been recently identified to be involved in promoting pluripotency factor expression, thereby causing delayed differentiation of XX cells due to higher *Klhl13* dosage when compared with XO or XY cells [48]. Thus, XCI of dosage-sensitive pluripotency regulators contributes to the differentiation kinetics during female pluripotency exit. In the absence of rXCI due to *Xist* mutation, XX ESCs show delayed pluripotency exit, cell death and frequent X-chromosome loss [44,49]. *In vivo*, surprisingly, rXCI might not be an absolute requirement for mouse embryonic development according to a study using conditional *Xist* deletion in the postimplantation epiblast, in which some embryos survived to term [50]. Nevertheless, partial dosage compensation has been observed, indicating either delayed deletion of *Xist* after initiation of XCI, or potential alternative dosage compensation mechanisms at play, such as a lack of XCU (see chapter below). It will be important to address in future studies the importance and biological function of rXCI for mouse development in detail and why imprinted XCI in the mouse placenta is essential for viability and cannot be compensated for by other mechanisms [12].

After down-regulation of the naive pluripotency programme during the transition from the pre- to postimplantation epiblast, the programme becomes re-established in the nascent primordial germ cell (PGC) lineage, which is specified in the proximal epiblast around embryonic days 6 and 7 in mice [51–53]. The proximal epiblast cells with competence for PGC fate thereby show high expression of pluripotency genes and a more heterogeneous degree of XCI compared with non-germ cell-competent epiblast cells, with proximal epiblast cells displaying either an absence or incomplete level of X-chromosomal gene silencing [43,54]. In addition to the expression of pluripotency factors such as PRDM14, OCT4, SOX2 and NANOG [55,56], PGCs reacquire an underlying pluripotent-like capacity, which can be uncovered by their potential to give rise to embryonic germ cells (EGCs) in culture. Similar to ESCs, EGCs exhibit self-renewal in addition to the ability to contribute to the three germ layers; ecto-, meso- and endoderm when differentiated *in vitro* or when injected into mouse blastocysts to form chimaeras [57,58]. Furthermore, female XX EGCs display DUSP9-mediated DNA-hypomethylation when compared with male XY EGCs, indicating the same X dosage-dependent mechanisms being at play as in ESCs [46]. Recently we found that similar to the germ cell-precursors in the epiblast *in vivo* [43,54], female *in vitro*-derived primordial germ cell-like cells (PGCLCs) undergo attenuated and heterogenous XCI, with part of the PGCLCs retaining two active X chromosomes (XaXa cells) and another population showing incomplete XCI (XaXi cells) when compared with somatic cells [54]. XaXa PGCLCs exhibited increased expression of naive pluripotency genes such as *Zfp42/Rex1* or *Esrrb*, and a shortened ESC-like cell cycle, when compared with X-inactivated XaXi PGCLCs [54]. Furthermore, XaXa PGCLCs displayed a higher EGC derivation capacity under naive 2i + LIF culture conditions compared with their XaXi counterparts. These data may suggest an enhanced readiness for pluripotency reacquisition as a result of having two active X chromosomes, which would align with the stabilisation of the pluripotency network in ESCs by double X-dosage [44,47,48]. When we further differentiated XaXa and XaXi PGCLCs *in vitro* towards early meiotic stages and the onset of oogenesis, unexpectedly the cells, which had two active X chromosomes at the PGCLC

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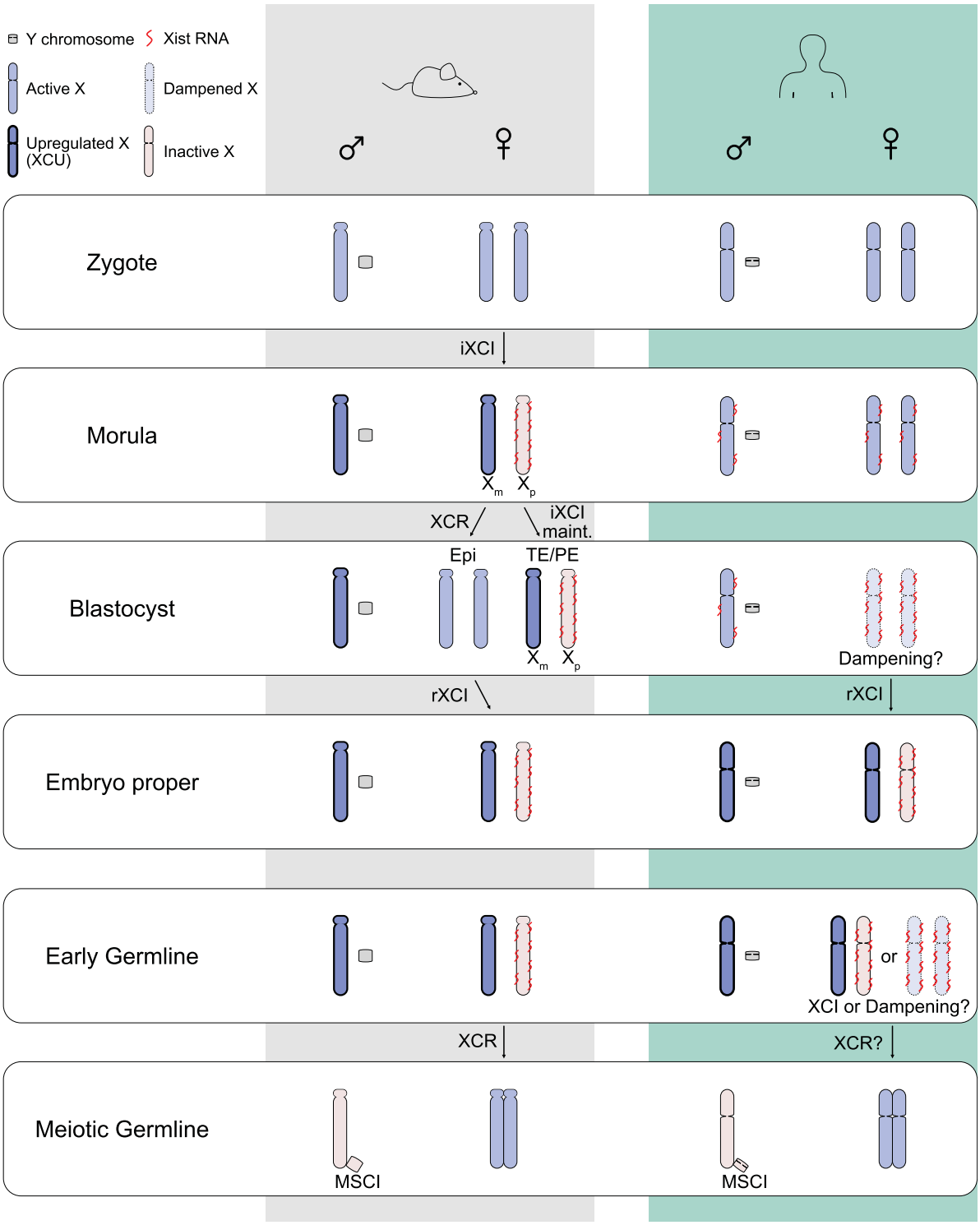

Figure 2. Comparison of X-chromosome dynamics between male and female mice and humans/primates. Part 1 of 2

In the male mouse, the single active X chromosome becomes transcriptionally upregulated via XCU shortly after zygotic genome activation, and this upregulated state is maintained throughout embryonic development. In meiotic germ cells, the X and Y chromosomes partially synapse, while unsynapsed regions undergo meiotic sex chromosome inactivation (MSCI), which does not require *Xist* [36]. In the female mouse, the paternal X chromosome (X_p) undergoes imprinted XCI (iXCI) progressively from the 2- to 4-cell stage onwards, while the maternal X chromosome (X_m) compensates dosage with autosomes through XCU, resulting in a XaXi morula. Cells of the developing epiblast (Epi), which will give rise to all embryonic lineages including

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Figure 2. Comparison of X-chromosome dynamics between male and female mice and humans/primates. Part 2 of 2

germ cells will subsequently reactivate the X_p. On the contrary, the extraembryonic lineages of trophectoderm (TE) and primitive endoderm (PE) and subsequently the placenta maintain imprinted XCI. Upon differentiation of the postimplantation epiblast, one of the two X chromosomes undergoes random XCI (rXCI) while the Xa demonstrates XCU, which is maintained in the embryo proper and primordial germ cells. During the migration of PGCs towards the gonad, the Xi reactivates to produce two active X chromosomes before the cells enter meiosis. In humans, the situation is less clear than in mice and the figure shows a speculative model partially based on inference from non-human primate data. In both males and females, XIST is expressed at low levels on the X chromosomes during the morula and blastocyst stages, which is not sufficient to cause XCI. After implantation, XCU occurs in males, followed by MSCI in meiotic germ cells. In female preimplantation embryos, initially dampening of X-linked gene expression by biallelic XIST expression [37] occurs, although this view has been challenged [142]. The postimplantation epiblast and placenta then undergo random XCI (rXCI) of one X chromosome and XCU of the active X [38]. In the developing human germline, both dampening [150] and XCI [147] have been reported. Prior to meiosis, the germ cells undergo XCR.

stage, demonstrated a lower meiotic competence compared with their XaXi counterparts [54]. Specifically, they were less likely to enter and progress through meiotic prophase I under culture conditions that promote meiosis of PGCLCs, either by aggregation with gonadal somatic cells or by culture on feeders with the addition of meiosis-inducing cytokines [59,60]. These data hint towards a link between the X-chromosome status in PGCLCs and their later developmental competence. Further functional testing will be necessary to establish if a lack of XCI in PGCLCs is the cause or a consequence of abnormal germ cell development and how mechanistically temporal control of X-dosage might be important. Furthermore, while PGC-competent precursor cells frequently show incomplete or a lack of XCI also *in vivo* [43,54], it has not yet been determined by allele-specific RNA-Seq, at which frequency XaXa PGCs might exist. Stainings for X-inactivation marks such as H3K27me3 or Xist RNA suggest that they could comprise ∼20% of PGCs at day E7.5 of development, after PGCs are specified [16,17]. Such XaXa PGCs might disappear eventually, perhaps due to selection mechanisms such as apoptosis. However, in the case that such cells can persist *in vivo*, we would speculate that XaXa PGCs could be a source of germ cell tumours, due to the rapid self-renewal and pluripotency properties that are conferred by possessing two active X chromosomes [54]. It remains to be tested whether all PGCs *in vivo* eventually undergo XCI, and if not, what would be the fate of such aberrant cells.

X-chromosome reactivation

XCR, the reverse process to XCI, by which the inactive X chromosome gets reactivated into an active state, occurs naturally at two points in mouse development (Figures 1 and 2). Firstly, imprinted XCI is reversed by XCR in the epiblast of the E4.5 blastocyst [23,26–28], and the subsequent rXCI is erased by XCR specifically in the germline [15–17]. Reversal of XCI related to pluripotency can also be studied *in vitro*, for example by cell fusion of somatic cells with pluripotent stem cells (reviewed in [61]), or through the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) [62], which has become an effective model for investigation of XCR dynamics and mechanisms [28,63–67]. Furthermore, *in vitro* model systems for X-inactivation and its subsequent reactivation in the germ cell lineage have been developed [59,68] and recently applied to gain insight into the biological role of X-dosage compensation in the germline [54].

A critical step during XCR, common to all systems in which it occurs, is the down-regulation of *Xist* expression, which is a prerequisite for the structural and epigenetic remodelling of the inactive X into an active state. Multiple control elements feed into *Xist* regulation at the *X-inactivation centre* (*Xic*), the region which harbours the *Xist* locus itself and its *cis*-regulators. The *Xic* contains both *Xist* activators, such as the noncoding *Jpx* [69,70], *Ftx* [71,72], *Xert* [73] and the protein-coding *Rnf12/Rlim* [74,75], but also *Xist* repressors such as its antisense transcript *Tsix* [33,76] and the noncoding element *Linx* [77]. It is, therefore, not surprising that *Xist* down-regulation during XCR may be achieved by a combination of different mechanisms. During iPSC-reprogramming, the down-regulation kinetics of *Xist* and its activator *Jpx* correlate tightly, and Jpx knockdown in somatic cells is sufficient to decrease *Xist* expression, suggesting *Jpx* down-regulation to be one of the mechanisms to shut down *Xist* during XCR [65]. Recently, GATA transcription factors have been reported as activators of *Xist* expression by binding to distal enhancers, whereby *Xist* upregulation is impaired in GATA1/4/6 triple mutant embryos during preimplantation development [78]. As GATA factors are specifically down-

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regulated in the mouse blastocyst epiblast [79], it will be interesting to examine if this could contribute to the *Xist* down-regulation during XCR. Furthermore, Xist extinction is mirrored by the upregulation of the naive pluripotency network during reprogramming, which mediates both direct and indirect repression of *Xist* [63–65]. Pluripotency factors such as PRDM14, NANOG, ZFP42/REX1, SOX2 and OCT4 bind in different combinations at regulatory hubs along the *Xic* [80], and thereby cause repression of the *Xist* activator *Rnf12* [28,81], upregulation of the *Xist* repressor *Tsix* [82,83] and repression of *Xist* itself [28,75,84]. Indeed, deletion of *Nanog* or *Prdm14* resulted in XCR defects in mouse blastocysts and during iPSC-reprogramming [28,85], indicating the importance of the pluripotency network in shutting down *Xist* during XCR.

In the mouse germline *in vivo*, XCR is initiated in PGCs in the hindgut as they migrate towards the developing gonads. Xist expression and the associated H3K27me3 accumulation on the inactive X thereby both get erased gradually in a cell-heterogeneous manner between E8.5 and E11.5 [16,17,86,87]. Like in blastocysts and during iPSC-reprogramming [28], the germ cell and pluripotency factor PRDM14 plays an important role in this process in addition to its function for genome-wide epigenetic reprogramming, which occurs concomitantly during PGC migration [88]. This includes global upregulation of the H3K27me3 and down-regulation of the H3K9me2 histone marks and repression of the DNA-methyltransferase machinery in PGCs, thereby contributing to their low global DNA-methylation state [56,88–91]. Reactivation of X-linked genes occurs subsequently between ∼E10.5 and E14.5 as mouse PGCs colonise the gonads and enter meiosis, and based on the few X-linked genes studied thus far, the reactivation kinetics differs from gene to gene and is heterogenous between cells [16,92]. An X chromosome-wide study using allele-specific single-cell RNA-Seq, as it has been done for XCR in mouse blastocysts [27] or for XCI and XCU kinetics in pre- and postimplantation embryos [43,93,94], will be needed to obtain a full picture of the XCR kinetics in mouse PGCs *in vivo*. Studies on XCR in mouse blastocysts during iPSC-reprogramming and germ cell *in vitro* differentiation have shown that XCR is a progressive process where genes are reactivated at different times [27,54,64–66]. During iPSC-reprogramming, early reactivated genes are located within a 3D-cluster in close proximity to constitutively active escapee genes, suggesting that reactivation initiates nearby already active open chromatin regions [64,65]. Furthermore, it has been reported that genes reactivating early during XCR in mouse epiblast show enrichment in MYC binding sites [27], and proposed that the pluripotency factor ZFP42/REX1 may also facilitate reactivation of X-linked genes during iPSC-reprogramming [66]. Functional studies will be needed to confirm if pluripotency factors, besides their established role in repressing *Xist*, also play a direct role in reactivating X-linked genes during XCR. Furthermore, it will be interesting to find out to which degree the order of genes, in which they are reactivated, is conserved between the different systems where XCR occurs. For example, the cell type of origin, from which iPSCs are derived, could influence the epigenetic state of the inactive X and additionally express different sets of transcription factors during reprogramming, thereby affecting the kinetics and order (early vs. late) in which X-linked genes are reactivated. It might be different again for XCR in PGCLCs, which are derived from early epiblast cells, which show incomplete and heterogenous XCI [43,54] and might be, therefore, more prone to reactivation than somatic cells used for iPSC-reprogramming experiments. Comparing the kinetics of XCR in these different systems will reveal if gene reactivation propensity is hard-wired into the X-chromosome sequence and mediated by a common set of *trans*-acting factors, or if it depends on the cell type-specific context.

Despite its importance, *Xist* down-regulation by itself is not sufficient for XCR to take place. In addition, multiple layers of epigenetic silencing including DNA methylation and repressive histone marks such as H3K27me3 need to be erased and properties of active chromatin such as histone acetylation and active RNA-Polymerase II need to be regained [27,63,64,95]. Another important step during XCR is the remodelling of the 3D X-chromosome structure, from a compact and transcriptionally inactive state into an open active chromatin configuration [65,96]. Cohesin is an essential factor in this process, as knockdown of the cohesin complex member Smc1a during iPSC-reprogramming results in abnormalities during the restructuring of the X chromosome and in defects in X-linked gene reactivation [67]. XCR occurs with markedly different kinetics in the blastocyst epiblast (within 1 day) [27] in contrast with the germ cell lineage (several days) [16], when considering the time it takes from *Xist* down-regulation to X-linked gene reactivation. This suggests important mechanistic differences between the reactivation of imprinted XCI in the epiblast versus the reversal of rXCI in germ cells. Potential explanations could be a different epigenetic status of the inactive X after imprinted and rXCI, or a difference in the factors mediating the epigenetic remodelling. For example, DNA methylation of X-linked gene promoters is critical for the maintenance of random, but not imprinted, XCI [63,95,97], and the DNA-methylation states between the active and inactive X chromosomes are equally low in mouse

preimplantation embryos already prior to *Xist* down-regulation and XCR [98]. In the germ cell lineage, however, DNA-demethylation occurs slowly and gradually on the reactivating inactive X chromosome mostly after *Xist* has been down-regulated, and is only completed once germ cells have entered the gonads. This is in line with the slower X-linked gene reactivation dynamics when compared with the epiblast [99]. Further studies will be needed to dissect the specific mechanism of DNA-demethylation on the inactive X chromosome and its contribution to gene reactivation during XCR in the germ cell lineage.

In addition to how XCR is regulated mechanistically, it remains an open question of what may be its biological function in female germ cell development. After germ cells complete XCR, they enter oogenesis and initiate the meiotic programme until the diplotene stage of prophase I, at which point the early oocytes are held in a state of the meiotic arrest until oocyte maturation and meiosis resume during adolescence. It has been demonstrated that the presence of two X chromosomes contributes to the proper progression through meiosis in oogenesis. In the case of cells with a single X-dosage, either as XO or XY, meiotic progression is perturbed [100–102]. This, at least in part, is due to the mispairing of sex chromosomes during prophase I, and may also be a result of differential dosage of X-transcripts [87,102,103], although more evidence is required before this can be confirmed. In relation to the XCR process, a question that remains to be answered is whether an active, euchromatic X chromosome could pair with an inactive, heterochromatic X in the case that XCR fails, or if this would lead to meiotic pairing defects and trigger a meiotic check-point, leading to a potential loss of oocytes. Our recent study on X-chromosome dynamics during mouse germ cell development *in vitro*, further corroborated an association between the XaXa state and the meiotic programme [54]. We observed that germ cells, which had gradually reactivated the X chromosome subsequently initiated meiosis, whereas cells that had undergone XCR too rapidly rather showed a mitotic phenotype. This suggests that XCR in itself is not sufficient to drive cells through a meiotic programme, but that the correct kinetics of XCR may play a role in order for meiosis to succeed. Thus, precocious reactivation of certain X-linked dosage-sensitive genes might be detrimental to normal germ cell development and meiotic entry, and might need to be controlled in sync with meiosis-inducing signals from gonadal somatic cells. As in pluripotent stem cells, double X-dosage-dependent repression of DNA methylation [46–48,104] may also aid the activation of meiotic genes, and thereby contribute to the right timing of meiotic entry [105,106]. Our understanding of these processes would benefit greatly from further allele-specific single-cell RNA-Seq of the reactivating germline *in vivo*, to better resolve the temporal control of X-linked genes during XCR. With this, we may be able to pinpoint key X-linked genes required for the correct establishment of meiotically competent germ cells.

Finally, XCR might also be needed for creating an epigenetic clean slate in preparation for the transfer of genetic material to the next generation, thereby ensuring offspring survival. Without XCR, maternal inheritance of an inactive X chromosome to male embryos, which have only a single X chromosome, and as well to female embryos, which undergo imprinted XCI of the paternal X, might potentially lead to lethality due to the absence of X-linked gene expression. After XCR has occurred, H3K27me3-based non-canonical imprinting of the *Xist* gene during oocyte maturation further safeguards the activity of the maternally inherited X chromosome during preimplantation development [29,30] and later in extraembryonic tissues by *Xist's* antisense regulator *Tsix* [32]. Direct functional perturbation of XCR will be needed to reveal its importance for meiosis specifically and for germ cell maturation, oogenesis and fertility in general.

X-chromosome upregulation

Apart from the dosage compensation mechanism of XCI, which ensures X-linked gene dosage parity between XX females and XY males, another level of dosage control exists for the regulation of gene expression from the active X. First proposed by Susumo Ohno in his seminal book 'Sex Chromosomes and Sex-linked Genes' [1], XCU is the result of transcriptional hyperactivation of the active X chromosome in order to attain X-to-autosome dosage balance, and occurs in both males and females. According to Ohno's hypothesis, XCU developed during the evolution of distinct sex chromosomes from an autosome pair, which are the X and Y chromosomes in placental mammals. Degradation and the associated gene-loss from the Y chromosome, therefore, would lead to a potential underdosage of genes that are only expressed on one X chromosome in males, in comparison with autosomal genes expressed from two chromosome copies. While XCU of the single X in males would lead to the dosage balance with autosomal genes, it could potentially lead to an overdose in XX females, which led to the evolution of XCI to counteract XCU [2,3]. Although XCU has been extensively studied as the dosage compensation mechanism of the single male X chromosome in the fruit fly *Drosophila melanogaster* [107], its existence has been under debate in mammals, due to a previous lack of allele-specific

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analysis methods and differences in approaches when comparing X-chromosomal with autosomal gene expression levels [5,108–112]. The discrepancy between studies providing evidence in favour and against Ohno's hypothesis might have also stemmed from the fact that species-specific differences exist in the fraction of dosage-sensitive genes on the sex chromosomes, which are dosage compensated depending on their evolutionary history [113,114]. However, many recent studies investigating the epigenetic and gene expression states of individual X chromosomes with allelic resolution, by which chromatin accessibility and transcripts can be assigned to the active or inactive X, have brought major advances to our understanding of X-chromosome regulation, confirming that XCU in mammals indeed exists [66,94,115–119]. Interestingly, the new data suggest that XCU has an elastic relationship with XCI and XCR, whereby the level of upregulation of the active X chromosome during XCU is tuned to the overall X-dosage, acting as a counterbalance to XCI, and therefore in direct relationship with the status of the inactivating X chromosome (Figures 1 and 2). During early mouse development, ESC-differentiation and iPSC-reprogramming, when dynamic regulation of X-chromosome activity by XCI and XCR occurs, XCU in response to XCI ensures maintenance of the overall balance of X-linked and autosomal gene expression [66,94,117–119].

Mechanistically, XCU regulation has been demonstrated to operate through increased transcriptional burst frequency, i.e. the frequency at which a gene switches between transcriptionally active and inactive states, thus resulting in higher X-linked gene expression [66,94,117,119]. This is associated with increased chromatin accessibility on the active X chromosome [66], although this has not been detected on the single-cell level [94]. Furthermore, enhanced transcription on the active X is correlated with an enrichment of active chromatin marks and phosphorylated active RNA-Pol II [115,116], although it is not clear if this is cause or consequence of the hyperactive state during XCU. Moreover, it has been proposed that the half-life of X-chromosomal transcripts may be enhanced, as they would be less prone to nonsense-mediated decay [116,120], suggesting that also potential posttranscriptional mechanisms may be involved in XCU. In *Drosophila*, the male X chromosome becomes epigenetically upregulated [107] by the histone acetyltransferase MOF, which is part of the male-specific lethal (MSL) complex. MSL gets recruited to the single male X chromosome through two long noncoding RNAs; roX1 and roX2 [121] and acts to deposit H4K16ac marks, resulting in the upregulation of X-linked genes [122–124]. Also in mouse cells, enrichment of MSL and MOF has been observed at specific X-chromosomal regions and has been put forward to play a role in regulating X-linked genes [116,125]. Interestingly, MSL2 has been shown to bind and regulate *Tsix* expression in order to repress *Xist* in ESCs, demonstrating an involvement in X chromosome regulation [125]. With regards to XCU, MOF and its associated H4K16ac mark have been shown to be enriched at a subset of X-linked gene promoters, and MOF depletion led to their down-regulation [116]. A continued dissection of the mammalian homologues of the *Drosophila* dosage compensation system would be invaluable, and may be the key to achieving a greater understanding of the mechanism of mammalian XCU. Additional insight into the XCU mechanism comes from a recent study, which identified the chromatin factor BRD4 as a critical regulator of XCU during mouse preimplantation development [126]. Embryos treated with BRD4 inhibitor or injected with siRNAs to knock down BRD4 showed defective XCU and reduced developmental potential after being transferred as blastocysts into recipient mothers [126]. Due to its dual function both as a reader and writer of histone lysine acetylation marks [127], BRD4 could thereby potentially provide a downstream mediator of histone acetylation by MOF homologues in mammals. Overall, however, as it is the case for XCI and XCR, the mechanisms of XCU remain far from being understood.

XCU has been shown to result in the transcriptional hyperactivation of the single active X chromosome of around 1.6 fold of average expression compared with one copy of autosomal genes (resulting in an approximate 0.8 X:autosome ratio) in mouse embryos and somatic cells [5,66,94,108,116,117,119,128]. This incomplete dosage compensation might be due to the fact that not all X-linked genes are dosage-sensitive and therefore compensated by XCU, and might explain why some previous studies did not detect XCU in mammalian cells when looking at X-chromosomal gene expression globally [109,112,119,126,129]. It also explains why, after XCR in mouse pluripotent stem cells, in which XCU is absent from females but present in XO females and XY males, X-chromosome dosage-dependent differences regarding DNA methylation and pluripotency exit kinetics become apparent between XX and XY or XO pluripotent stem cells. XX cells display an X to autosome ratio of around 1 while XY or XO cells reach around 0.7–0.8 [44,46–48,66,108]. Finally, the incomplete X-dosage compensation level achieved by XCU compared with full double X-dosage might also provide an explanation as to why there are deleterious effects on female development in *Xist*-knockout embryos in the absence of XCI and XCU [12,94]. However, it still remains to be tested if XCU, while it is absent from female *Xist*-knockout

preimplantation embryos [94], is also missing during postimplantation stages when the *Xist*-KO lethality phenotype emerges. In the case that XCU might occur, an absence of XCI in the presence of XCU could in turn generate an even higher overdose of X-linked genes. In particular, it would be interesting to examine if XCU is regulated differently between the placenta and in embryonic tissues. This might explain why a failure in imprinted XCI is tolerated in preimplantation embryos [94] but is lethal in the placenta [12], and why a lack of rXCI in postimplantation embryonic tissues can be partially compensated for [50].

In the mouse germ cell lineage *in vivo*, the current lack of allele-specific datasets only allows inference of the contributions of XCI, XCR and XCU to the overall X-chromosomal gene dosage from the dynamic changes of X:autosomal ratio in XX, XO and XY data. The X:A ratios in early XX, XO and XY mouse germ cells start out at similar levels around or below 1 due to the combined action of XCI and XCU in XX females and XCU in XO females and XY males [87,103]. Interestingly, XCU in the germline appears to be down-regulated only after the occurrence of XCR once germ cells have entered the gonad, resulting in an X-to-autosome ratio that exceeds 1 in XX females due to the combined expression from both X chromosomes in addition to XCU (Figure 1) [87]. Gonadal germ cells are believed to remain in this state for approximately 3 days, coinciding with meiotic entry and not returning to an X-to-autosome ratio of 1 until the pachynema stage of prophase I. Allelic RNA-Seq analysis would be required to disentangle the exact kinetics of XCR in relation to XCU, and to measure their contribution to the overall X-dosage. Whether this elevated dosage of the X chromosome in the XX germline is functionally important for female meiosis to occur remains to be tested. Further investigations will deepen our understanding of the relationship between the X-chromosome status and the switch from mitosis to meiosis in developing germ cells.

X-chromosome dynamics in the primate germline

While a lot has been learned about X-chromosome dosage mechanisms from the mouse model system, it is becoming increasingly apparent that not all findings from the mouse are applicable to primates and humans in particular [130,131]. For example, the dynamics of XCI during human embryonic development differs from mice (Figure 2) due to the absence of imprinted XCI and a later onset of gene silencing in humans [35,37,132].

As outlined above, in the mouse, the noncoding RNA *Tsix* acts as an antisense repressor of *Xist* on the active X chromosome, thereby preventing *Xist* expression and XCI of the active X both during rXCI in the embryo and imprinted XCI in the placenta [32,33,76]. In primates, however, *TSIX* does not seem to play such a role, as its transcript terminates prior to overlapping with the *XIST* promoter [133,134], explaining why in human placentas random, but not imprinted XCI has been found [135,136]. In preimplantation mouse embryos, imprinted XCI is not controlled by *Tsix* but rather by a H3K27me3 imprinting mark of the *Xist* locus [30,137]. Also, this imprint of the *XIST* locus seems to be absent from human preimplantation embryos, as XIST RNA becomes expressed after embryonic genome activation from both female X chromosomes and the single male X, although this does not lead to immediate gene silencing [35,37,132]. XACT, a hominoid-specific noncoding RNA, which coats the active X chromosome in human pluripotent stem cells [138], is initially co-expressed with XIST in early embryos from both X chromosomes and has been proposed to potentially interfere with XIST spreading or in recruiting its silencing effector proteins [37,132,139]. However, the function of XACT RNA during early development still needs to be tested, as a recent study did not detect effects on X-linked gene expression after the deletion of *XACT* in human pluripotent stem cells [140]. Single-cell RNA-Seq analysis of female blastocysts suggested a biallelic reduction in X-linked gene expression, termed dampening [37]. Dampening conceptually resembles the dosage compensation mechanism in the nematode *Caenorhabditis elegans*, where gene expression is down-regulated from both X chromosomes by half in XX hermaphrodites compared with XO males [141]. However, when different analysis parameters were used, a switch from biallelic to monoallelic expression was observed in human embryos, rather suggesting the onset of rXCI instead of dampening [132,142,143]. In an implantation model of *in vitro* cultured human embryos, XCU in males and rXCI and XCU in females occurred progressively and asynchronously in different cell lineages of epiblast, trophectoderm and primitive endoderm [38]. A recent study used Cynomolgus monkeys (macaques) as a non-human primate model system for X-chromosome dynamics that more closely resemble humans than mice [144]. Similar to humans, in monkey preimplantation embryos XIST becomes expressed from both X chromosomes in females and the single X in males, and does not lead to the silencing of X-linked genes. This is despite the recruitment of polycomb repressive marks H3K27me3 and H2AK119ub, which has not been observed in human embryos at that stage [35]. After implantation, XIST expression becomes monoallelic in female monkey embryos and eventually leads to gene silencing [144]. Another recent paper comparing single-cell RNA-Seq

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data from mouse, monkey and human foetal oocyte development concluded that overall X-dosage regulation is similar across these species [145]. It remains an open question, why XIST expression in early human and monkey embryos does not lead to immediate gene silencing and how the switch from bi- to monoallelic X-linked expression is mediated.

The germ cell lineage features commonalities and differences between the X-chromosome dynamics in mice, monkeys and humans. In mice, early PGCs show XCI [15–17] but to a lesser degree when compared with somatic cells or early embryonic cells without PGC competence [43,54], and subsequently go through XCR during migration and colonisation of the gonads. Also in monkeys, early PGCs show partial XCI after specification, with some genes being more efficiently silenced than others, and most female PGCs initially showing monoallelic XIST expression [144]. Afterwards, during PGC migration, XIST gets down-regulated, H3K27me3 erased and X-linked genes progressively reactivated. Once monkey germ cells have entered the gonads, XCR is complete and X-linked genes biallelically expressed, however, XIST becomes reexpressed from both X chromosomes in females and the single X in males, without fully coating the X chromosomes and without leading to gene silencing, similar to the state during preimplantation development.

In the human germline, the X-chromosome dynamics have been studied so far during the migratory (week 4–5 of development) and gonadal germ cell stages (week 5 onwards). Allele-specific single-cell RNA-Seq analysis of X-linked genes initially suggested that biallelic expression and thereby XCR have already occurred at the first time point observed (4 weeks) [146]. However, a later study with further in-depth single-cell analysis concluded that XCR in human germ cells was rather a gradual process, which appears to occur in a heterogeneous manner [147] and is accompanied by erasure of H3K27me3 and DNA-demethylation of X-linked gene promoters [147,148]. As currently data on the X-status of human PGCs at their specification time (week 2) are missing, it is not clear to which extent XCI has already occurred in the early human germline before XCR takes place. Interestingly, XIST RNA has been detected in both male and female germ cells and its overall expression levels do not predict the extent of X-linked gene reactivation during XCR [147,149]. A recent study reported that X-linked gene expression is reduced in female germ cells prior to their meiotic entry when XIST becomes down-regulated [150]. In RNA-FISH experiments, XIST frequently showed a biallelic dispersed expression pattern and was accompanied by biallelic coexpression with XACT RNA in premeiotic germ cells, resembling the situation in human preimplantation embryos [132,150]. While XIST and XACT are expressed, X-linked gene expression is attenuated compared with autosomal genes, which has been interpreted as an XIST-dependent dampening mechanism [150]. Nevertheless, it still remains unclear, if biallelic dampening really occurs and what the contributions of XIST and XACT RNA may be. An alternative hypothesis for reduced X-linked gene dosage in female germ cells could be incomplete XCR, as it has been previously proposed [147]. From the currently available data, many different possible interpretations for X-linked gene dosage regulation in human germ cells exist; ranging from the possibility of continuous dampening in the absence of XCI and XCR, over XCI and XCU followed by XCR, to a sequence of all XCI, XCU, XCR and dampening [103,151]. Needless to say, further investigations including allele-specific single-cell RNA-Seq and multi-omics analyses will be necessary to bring clarity to the complex control of X-linked gene dosage during human germ cell development *in vivo*.

From a functional perspective, *in vitro* germ cell derivation from human pluripotent stem cells could shed light on the requirement of X-dosage control for the different steps from PGC specification to meiotic entry and oogenesis. At the moment human *in vitro* germ cell development is less far advanced than the mouse model system [152] and only early meiotic stages have been achieved so far [153]. Nevertheless, in two recent studies, it has been tested whether the original X-chromosome status of the pluripotent stem cells used as the starting material would affect human PGCLC-specification efficiency [154,155]. While the first study did not observe any significant impact, but rather that cell type of origin might affect PGCLC-differentiation efficiency [154], the second reported that stem cells with an eroded XCI state showed reduced germ cell competence when compared with cells with more complete XCI [155]. In the study with the most advanced *in vitro* development into oogonia-like cells, progressive demethylation of X-linked gene promoters and partial XCR has been observed [153]. At this point, it is difficult to assess if the X-status is deterministic or rather diagnostic for the germ cell differentiation potential of pluripotent cell lines. In particular, the general cell line-to-cell line variability can affect germ cell development, which is influenced by many confounding parameters beyond the often ill-defined X-status, such as genetic background, cell type of origin and culture conditions. Functional experiments modulating the X-status in a well-controlled, isogenic cell background, will be needed to directly address the potential roles of X-chromosome dosage for human germ cell development.

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Concluding remarks

Despite all the recent progress in deciphering the dynamics of the X chromosome in the germline, studies that allelically resolve the contribution of the different dosage compensation mechanisms and functionally interrogate their importance are still missing. Here we have highlighted some of the key findings that help us gain better understanding of this relationship, however, significant questions remain to be addressed (Box 1). With these questions addressed, we would hope to better understand the role of the X chromosome in the establishment of the germline, and whether its dynamics are a cause, or simply a consequence, of correct female germ cell development. Importantly further understanding of what is normal during germ cell development *in vivo* will help recapitulate these processes during germ cell development from pluripotent stem cells *in vitro*. Achieving faithful epigenetic reprogramming will be a critical step towards the generation of high-quality human oocytes in a dish.

Box 1 — Major questions to be answered

- What is the kinetic relationship between XCI, XCR and XCU in regulating the X-dosage in the mouse and human germline *in vivo*?
- Is dosage compensation in the human germline achieved by dampening, and what is its mechanism?
- Do mouse or human PGCs without XCI exist *in vivo* and what is their fate?
- Do genes reactivate at the same time and in the same order in imprinted XCR as they do in germline XCR?
- What factors drive the XCR process?
- Is an XaXa state through XCR required for meiosis to take place?
- Is X-chromosome hyperexpression necessary for meiosis?
- What mechanisms underlie XCU?

Perspectives

- The interplay and roles of different X-chromosome dosage compensation mechanisms in the mammalian female germline remain elusive.
- Strict temporal control of X-linked gene expression dynamics suggests a link between the X-chromosome status and germ cell developmental competence, but the extent of this is unknown.
- Further work aiming to resolve the allele-specific regulation of X-chromosome dosage and the functional perturbation of the X-chromosome status during germline development would help to better resolve the details of this relationship.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Bernhard Payer: Conceptualization, Funding acquisition, Writing — original draft, Writing — review and editing.
Tom Mattimoe: Conceptualization, Visualization, Writing — original draft, Writing — review and editing.

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Abbreviations

ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; PGCLCs, primordial germ cell-like cells; PGCs, primordial germ cells; Xa, active X chromosome; XACT, X-active coating transcript; XCI, X-chromosome inactivation; XCR, X-chromosome reactivation; XCU, X-chromosome upregulation; Xi, inactive X chromosome; XIST, X-inactive specific transcript

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