Title

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Journal

Philosophical Transactions of the Royal Society B Biological Sciences, 372(1733)

ISSN

0962-8436

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Publication Date

2017-11-05

DOI

10.1098/rstb.2016.0363

Peer reviewed

PHILOSOPHICAL TRANSACTIONS B

rstb.royalsocietypublishing.org

Review

Cite this article: Sahakyan A, Plath K, Rougeulle C. 2017 Regulation of X-chromosome dosage compensation in human: mechanisms and model systems. *Phil. Trans. R. Soc. B* **372**: 20160363. http://dx.doi.org/10.1098/rstb.2016.0363

Accepted: 15 July 2017

One contribution of 12 to a discussion meeting issue 'X-chromosome inactivation: a tribute to Mary Lyon'.

Subject Areas:
genetics, developmental biology, cellular biology

Keywords:
X-chromosome inactivation, pluripotent stem cells, X-chromosome dampening, Xist, Xact

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Regulation of X-chromosome dosage compensation in human: mechanisms and model systems

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The human blastocyst forms 5 days after one of the smallest human cells (the sperm) fertilizes one of the largest human cells (the egg). Depending on the sex-chromosome contribution from the sperm, the resulting embryo will either be female, with two X chromosomes (XX), or male, with an X and a Y chromosome (XY). In early development, one of the major differences between XX female and XY male embryos is the conserved process of X-chromosome inactivation (XCI), which compensates gene expression of the two female X chromosomes to match the dosage of the single X chromosome of males. Most of our understanding of the pre-XCI state and XCI establishment is based on mouse studies, but recent evidence from human pre-implantation embryo research suggests that many of the molecular steps defined in the mouse are not conserved in human. Here, we will discuss recent advances in understanding the control of X-chromosome dosage compensation in early human embryonic development and compare it to that of the mouse.

This article is part of the themed issue 'X-chromosome inactivation: a tribute to Mary Lyon'.

1. The X-chromosome state of the human pre-implantation embryo

Somatic cells of an adult female human have two X chromosomes, but most genes on one of them are silenced at the level of transcription, so that the X-chromosome gene dosage in female XX cells is equal to that of male XY cells. The silenced X chromosome can be either the paternally or the maternally inherited one, making the adult female a natural mosaic. This random pattern of X-chromosome inactivation (XCI) is established in early embryogenesis. The X chromosomes inherited from the egg (maternal) and the sperm (paternal) are both active in very early female development [1,2] before each cell commits to transcriptionally silencing one X chromosome for the rest of the cell's and its progeny's life. It is not known exactly when this choice is made in human development, but based on mouse studies it is hypothesized to happen shortly after the embryo implants [3]. Surplus pre-implantation embryos from *in vitro* fertilization clinics donated to research have made *ex vivo* studies of human pre-implantation development possible. Combined with advances in single-cell transcriptome profiling, these have recently enabled a closer look at the X-chromosome biology in early human development [1,2,4–6].

Petropoulos and colleagues studied the transcriptome of the largest number of human pre-implantation embryos reported to date, and performed sex-specific analysis of human development at days 3–7 post fertilization (E3–E7) at the single-cell level [2]. Their analysis revealed that immediately after zygotic gene activation (ZGA) at E4, female embryos had almost double expression of X-linked genes compared with males, consistent with females

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having two active X chromosomes (figure 1). However, with increasing developmental time from E4 to E7, this roughly 2 : 1 female : male ratio decreased, reaching nearly 1 : 1 in all cells of the embryo at E7 (figure 1), just in time for the commencement of implantation. Surprisingly, this drop in X-linked gene expression level was not due to the onset of X-chromosome-inactivation, because allelic expression analysis by single-cell RNA-sequencing revealed that both X chromosomes were active at all times [2]. Evidence for the presence of two active X chromosomes in female human pre-implantation embryos was extended further by RNA fluorescent *in situ* hybridization (RNA-FISH) [1,2,5,6]. Thus, Petropoulos *et al*. uncovered a novel mechanism of X-chromosome dosage compensation, at the mRNA level, in human pre-implantation development where female to male expression is equalized not by inactivating one of the two X chromosomes in the female, but rather by dampening the expression of both female X chromosomes (figure 1). This X-chromosome dampening (XCD), which has not been observed in mice, is reminiscent of the dosage compensation system occurring in a model organism further removed from the human on the evolutionary scale—the roundworm *Caenorhabditis elegans*. Both X chromosomes of XX hermaphrodite *C. elegans* undergo condensin-mediated three-dimensional structural remodelling, resulting in reduced transcriptional output to match X-linked gene dosage to that of the single X in XO males [7,8]. However, whether XCD in human and *C. elegans* are mechanistically similar remains an open question (see §2). In any case, together these findings indicate that X-chromosome dosage compensation in human is regulated by two different and sequential processes: first XCD and later XCI. Interestingly, moderate but significant expression asymmetry between the two X chromosomes was detected from E5, suggesting that X-linked gene silencing may initiate in a progressive manner at this developmental stage [5].

2. *XIST* expression correlates with X-chromosome dampening

A hallmark of the inactive X chromosome (Xi) is expression and accumulation of the *cis*-acting long non-coding RNA (lncRNA) *XIST* (X inactive specific transcript) [9–11], which, as its name suggests, was thought until recently to always correlate with the inactive status of the X chromosome. However, an unexpected finding was made in 2011, when Edith Heard's group used RNA-FISH to demonstrate that both male and female human pre-implantation embryos express the lncRNA *XIST* without any evidence of X-inactivation (figure 1) [1]. This was the first report of long-term expression (over several days) and accumulation of *XIST* RNA that does not lead to chromosome-wide silencing, and was indeed very intriguing. This finding inspired further studies of the X-chromosome state in the human pre-implantation embryo, which validated the presence of *XIST*-expressing active X chromosomes [2,4–6]. While *XIST* was expressed from both X chromosomes in the majority of cells in female blastocysts, a proportion of the cells, however, displayed mono-allelic *XIST* expression pattern [1,2,5]. In RNA-FISH studies, *XIST* was also found accumulating on the single X in male embryos, although contrasting results were obtained between studies in the proportion of *XIST*-expressing cells—from a majority of male cells in the blastocyst expressing *XIST* [1,5] to most cells being devoid of *XIST* expression [2]. This discrepancy is perhaps due to differences in the sensitivity of the RNA-FISH assays employed, and might be related to the fact that *XIST* was found at much lower amounts in male cells compared with female cells in RNA-sequencing experiments [2].

Human *XIST* expression initiates as early as at the 4–8-cell stage of the embryo and coincides with the onset of ZGA [2,4,5]. *XIST* levels increase over time up to E7, in a manner that correlates with X-linked dampening (figure 1). This correlation is also observed in naive human embryonic stem cells (hESCs), where cells with two active chromosomes and no *XIST* expression have overall higher X-linked gene expression compared with cells with two active Xs and *XIST* expression (see §6) [12]. Whether XCD in human is mediated by *XIST* remains an open question, but in the worm other mechanisms are involved as *XIST* is not conserved beyond placental mammals [13]. Should *XIST* mediate XCD in the female human pre-implantation embryo, one would have to assume that the lower level of *XIST* in male embryos is not sufficient for the induction of XCD on the male single X chromosome.

3. Differences between mouse and human XCI

In contrast to the human, mouse embryos are more easily attainable in larger numbers; hence our understanding of mouse pre- and post-implantation development, including the regulation of X-chromosome dosage, is more advanced. It is well established that female mice undergo X-chromosome dosage compensation via XCI in two waves. At the 4-cell stage mouse embryos initiate paternally imprinted XCI, which is completed by the morula stage; hence only the maternally inherited X chromosome is active in all cells (figure 1; reviewed by Takagi [14]). Imprinted XCI is maintained in the cells of the trophectoderm, which will eventually give rise to extra-embryonic tissues such as the placenta [15]. By contrast, as the embryo develops into the mid-stage blastocyst, the inactive X chromosome is reactivated in cells of the inner cell mass (ICM) that give rise to the epiblast [16–18], resulting in cells with two active X chromosomes (figure 1). These cells then undergo a second wave of XCI, which is not imprinted, but rather the maternally or the paternally inherited X chromosome is chosen at random. Both imprinted and random XCI depend on *Xist*, which acts *in cis* in both cases to silence the X chromosome from which it is expressed [19–21], and the reactivation of the imprinted Xi is accompanied by *Xist* silencing (figure 1) [16–18].

Early reports addressing the question of whether human early development follows what is observed in the mouse with respect to imprinted XCI have been mixed, but recent studies using more advanced techniques and larger sample sizes agree that human pre-implantation embryos lack imprinted XCI [1,2,22], and that, instead, human pre-implantation embryos reduce X-linked gene dosage by XCD on both X chromosomes [2]. Thus, in addition to XCD and *XIST* expression from an active X chromosome, the lack of imprinted XCI in human pre-implantation embryos is a key difference between mouse and human embryonic development. Interestingly, *XIST* expression and lack of imprinted XCI are also observed in rabbit pre-implantation development, despite the closer evolutionary distance

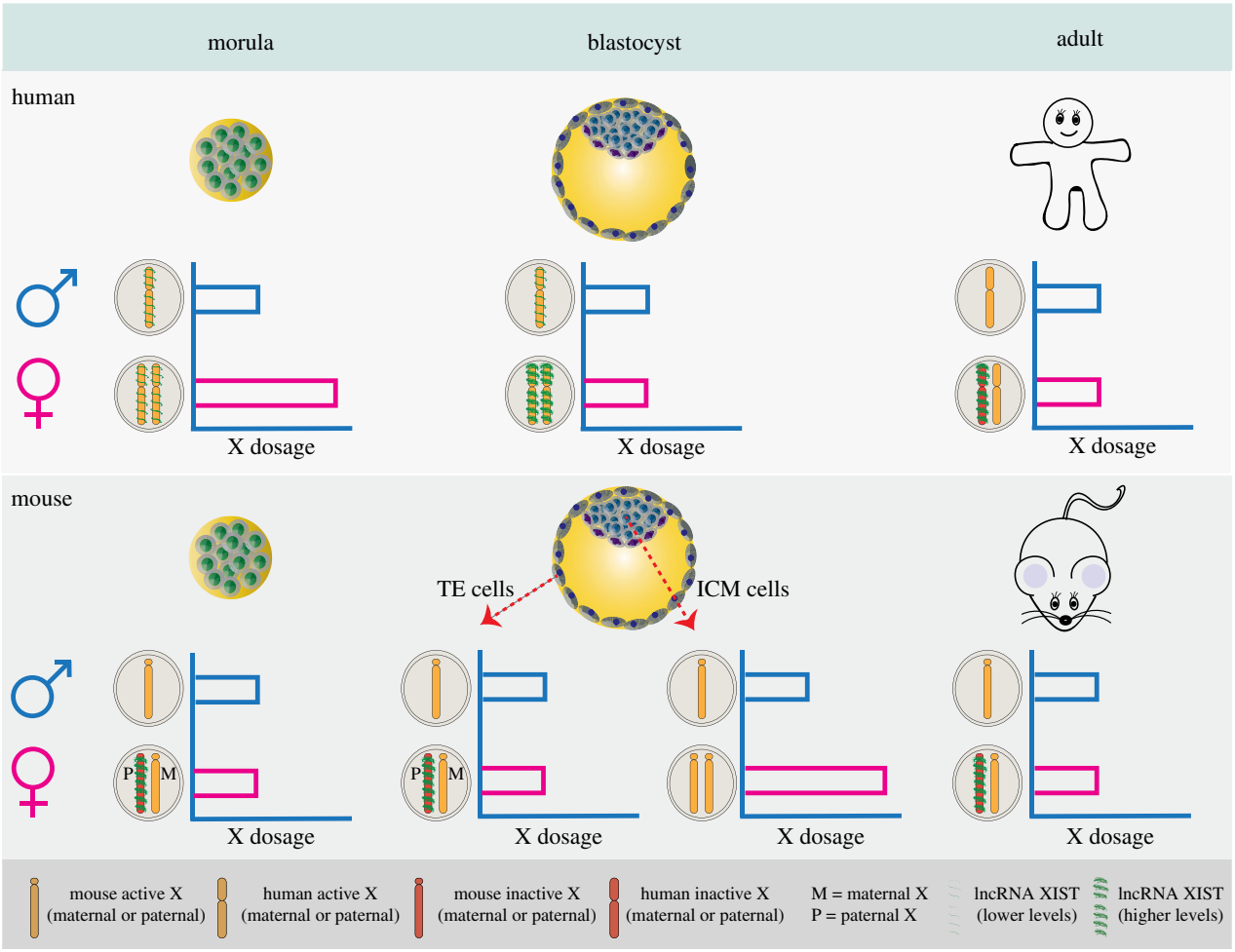

Figure 1. X-chromosome dosage compensation in mouse and human. In human pre-implantation development, *XIST* becomes expressed from all X chromosomes upon zygotic gene activation. As pre-implantation development progresses, *XIST* expression from both female X chromosomes increases, but remains low in males. The former correlates with dampened gene dosage from both X chromosomes of the blastocyst, equalizing X-linked gene dosage of females to that of males. Upon implantation, all cells undergo random XCI, again resulting in dosage-compensation. In mice, XCI happens in two waves. First, *Xist* is induced only on the paternally inherited X chromosome (P), causing imprinted XCI in all cells of early pre-implantation embryos (morula). As the blastocyst forms, *Xist* expression becomes suppressed in the ICM cells (but not in the TE), and the Xi reactivates, leading to increased X-linked gene dosage in females compared to males. As the embryo implants, the maternal or the paternal X chromosome becomes randomly chosen to undergo XCI, similar to humans.

between mouse and rabbit compared with rabbit and human [1].

Another distinguishing feature between mouse and human in the epigenetic regulation of the X chromosome is the presence of the long non-coding RNA *Tsix* in mice but not in humans. *Tsix* is transcribed antisense to *Xist* and, in imprinted XCI, is expressed from the active, maternal X chromosome in mouse pre-implantation embryos and extra-embryonic annexes, where it is required to maintain *Xist* repressed on this chromosome [23,24]. Similar to imprinted XCI, *Tsix* represses *Xist* expression from the active X chromosome during random XCI [25,26]. Despite the role of *Tsix* in both imprinted and random XCI, there is a *Tsix*-independent repression of *Xist* at play during embryo cleavage stages of mouse development, because the maternal *Xist* is repressed in the absence of *Tsix* expression [24]. Although a *TSIX* gene has been annotated in the human genome, a recent study shows that it is not transcribed in human pre-implantation embryos [2]. The lack of *TSIX* expression and function may be related to the expression of *XIST* from the active X chromosomes in the human pre-implantation embryo. Thus, human cells seem to have evolved a different mechanism to control the function of *XIST* during the initiation of random XCI and to cope with *XIST* expression in the pre-implantation embryo: it is the silencing ability of *XIST* rather than *XIST* expression that is prevented in these cells. This contrasts to the mouse, where *Xist* expression systematically leads to silencing, unless certain regions of the *Xist* gene are deleted [27]. A strong candidate for repressing *XIST*'s ability to silence the X chromosomes in the pre-implantation embryo is the recently identified human- and pluripotency-specific lncRNA *XACT* (X active coating transcript; see §7) [28].

4. Mouse ESCs perfectly recapitulate the X-chromosome state of the mouse blastocyst

Much of our understanding of XCI comes from mouse studies mainly because mouse embryonic stem cells (mESCs), derived from the pre-implantation blastocyst, perfectly capture the X-chromosome state of *in vivo* development [29]. Cells of the ICM and mESCs have two active X chromosomes and, upon implantation *in vivo* or differentiation *in vitro*, *Xist* expression is induced from one of the two X chromosomes, chosen at random, which leads to

chromosome-wide inactivation *in cis* (figure 1). The *in vitro* model system has been ideal for unravelling the molecular mechanism behind the initiation of random XCI and the transition from the XaXa (Xa for active X chromosome) to the XaXi^{Xist+} state (Xi for inactive X chromosome). For instance, mESCs were used to perform extensive *Xist* RNA domain deletion studies that suggested a modular structure of *Xist* RNA, with different RNA domains mediating different functions [27]. More recently, mESCs were used to reveal that, at the onset of XCI, *Xist* spreads to regions on the X chromosome spatially closest to the *Xist* transcription locus, highlighting the importance of three-dimensional modelling of the X chromosome [30]. Moreover, two groups independently identified protein partners of *Xist* at the onset of XCI [31,32], beginning to provide a detailed mechanistic understanding of how *Xist* function is mediated [31–34].

The mouse model has also contributed immensely to our understanding of pluripotency—the ability to differentiate into all three germ layers. Pluripotent cell identity is not fixed but rather represents a spectrum of states, perhaps because pluripotency *in vivo* spans multiple days of development instead of a fixed singular time point [35]. This became obvious when pluripotent stem cells (PSCs) with characteristics rather distinct from those of mESCs were isolated from the mouse post-implantation epiblast (EpiSCs for epiblast stem cells) [36,37]. Although both are pluripotent, mESCs capture the naive pluripotent state of the pre-implantation blastocyst and EpiSCs the developmentally more advanced primed pluripotent state of the post-implantation embryo [35].

5. Limitations of conventional human ESCs in modelling the pre-implantation X-chromosome state and initiation of XCI

Unlike mESCs, conventional hESCs, which are derived in the presence of basic fibroblast growth factor, do not recapitulate the X-chromosome state of the naive pluripotent cells in the human blastocyst. When comparing to what we know from mouse studies, conventional hESCs resemble mouse EpiSCs instead of naive mESCs, although, like mESCs and unlike mouse EpiSCs, they are derived from the pre-implantation and not the post-implantation blastocyst (see [38] for a detailed review). This resemblance extends to cell morphology, signalling pathway dependence with global transcriptional signature, and the post-XCI state [38]. Hence, similar to mouse EpiSCs, conventional hESCs are in primed pluripotency [35].

The X-inactivation status of hESCs has been very controversial, likely due to the epigenetic instability of the inactive X chromosome in primed hESCs. Two X-chromosome patterns can be observed at early passage, when hESCs are derived from the pre-implantation embryo in conventional conditions: one with two active X chromosomes (XaXa) and no *XIST* expression, and one with one active and one inactive X chromosome from which *XIST* is expressed (XaXi^{XIST+}), the latter being more frequent (figure 2) [6,39–43]. The *XIST*-negative XaXa state was initially reported to be the pristine state, due to its resemblance to the mouse situation [40,41]. However, in our study, induction of differentiation of XaXa hESCs is accompanied neither by *XIST* induction nor by XCI [6] (figure 2). Because of this, we classified this *XIST*-negative XaXa state as an abnormal state, probably due to the permanent silencing of the *XIST* gene during the derivation of primed hESCs [6]. Previous studies contradicting this conclusion and reporting de novo XCI from such cells [40,41] may be explained by the heterogeneity of most hESC lines, with both XaXa and XaXi^{XIST+} cells present in the same culture before induction of differentiation. Following cells through the derivation process from human blastocysts by the analysis of a few time points suggested that the transition from the pre-implantation embryo state with two active, *XIST*-expressing X chromosomes to a post-XCI state involves transient silencing of *XIST* on both X chromosomes and its subsequent reactivation from one X only, to induce XCI [6] (figure 2). In this model, it may be possible that effective upregulation of *XIST* is only possible in a brief developmental window and, in cases when this window is missed *in vitro*, both *XIST* alleles become permanently silenced, leading to the stabilization of the XaXa state without *XIST* expression.

The other, more common XaXi^{XIST+} state in early passage hESC lines appears to resemble the post-XCI state of somatic cells, as shown, for example, by the occurrence of methylation of CpG islands on the Xi [6,39,44]. However, it changes in culture over time: in nearly all cases, *XIST* expression on the Xi is gradually lost in these cells, and the inactive X is partially reactivated, resulting in double dosage of a subset of X-linked genes (figure 2) [6,39,44–47]. This erosion of XCI is accompanied by the loss of DNA methylation specifically in the CpG islands of affected genes, and its extent varies in different cell lines, ranging from only a handful of genes to almost the entire inactive X chromosome [6,44]. The determinants of XCI erosion are currently poorly understood, but certain regions on the Xi are more likely to erode than others [44]. Interestingly, chromatin signatures, such as H3K27me3 and H3K9me3 modifications, are good predictors of erosion, with genes enriched for H3K27me3 and relatively depleted for H3K9me3 on the Xi having an increased likelihood of reactivation upon XCI erosion [47]. A defining feature of XCI erosion is that it cannot be undone, even during differentiation [6,44,46] (figure 2). In other words, the aberrant X-chromosome state of these cells is locked in place so that, upon differentiation, the reactivated parts of the inactive X chromosome cannot be re-silenced, resulting in differentiated cells with a double dose of the X-linked genes that fall in eroded regions. This has not only been problematic for basic researchers who wish to study the onset of XCI in the human system, but also influences studies of X-linked diseases and use of female induced pluripotent stem cells (iPSCs) for disease modelling (see below) [46]. Furthermore, XCI erosion may affect cell replacement and regenerative therapies, because inappropriate dosage compensation of X-linked genes is a hallmark of female-specific cancers [48].

The X-chromosome state of human iPSCs, and whether reprogramming of somatic cells to pluripotency is accompanied by Xi-reactivation, has been heavily debated in the literature. Data from us and others argue that human iPSCs are XaXi with *XIST* at early passage, but over time in culture *XIST* expression is lost and the Xi is partially reactivated due to XCI erosion, similar to *XIST*-expressing XaXi hESCs [6,44,45,49,50]. Thus, despite various reports of complete Xi-reactivation in human iPSCs [51–54], our data suggest that the XaXa state is not achieved in human iPSC

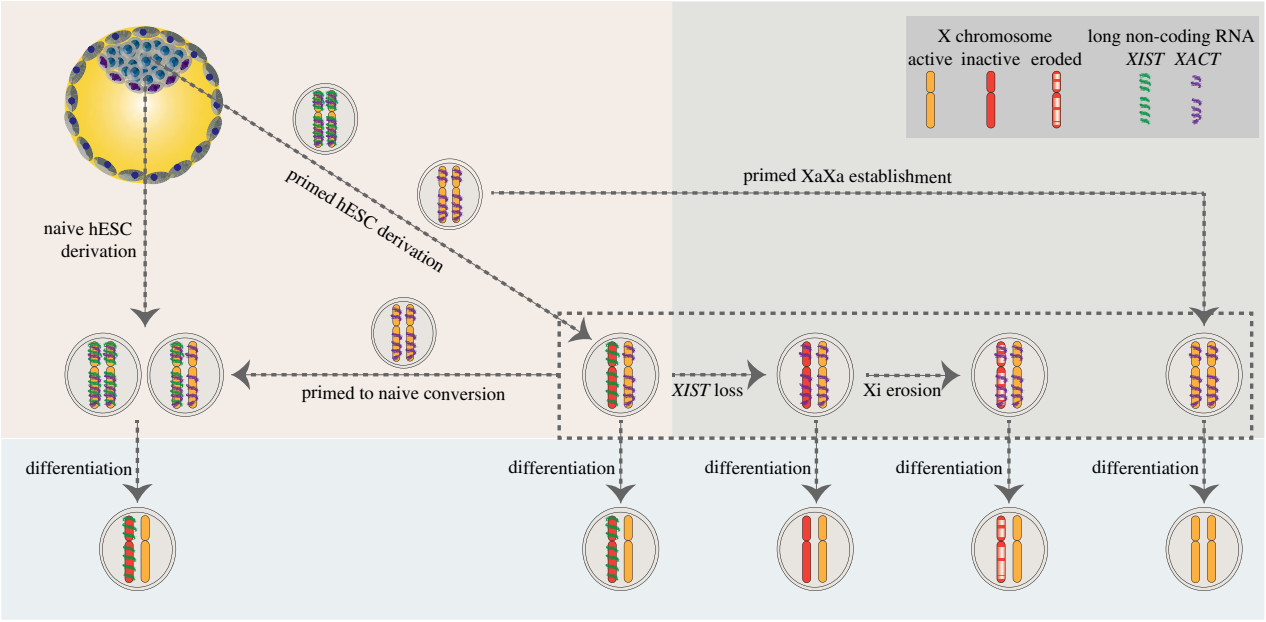

Figure 2. X-chromosome states of human pluripotent stem cells. The X chromosome state of conventional (primed) hESCs differs from the ICM of human pre-implantation blastocysts from which they are derived. Primed hESCs are in a post-XCI state with an *XIST*-coated Xi. Over time in culture, the Xi loses expression of *XIST* and partially reactivates, undergoing XCI erosion. Primed hESCs with two active X chromosomes can also be derived from ICM outgrowths (far right), potentially capturing an intermediate state of the X chromosome in the transition to XCI. Differentiation does not change the X-chromosome state of any of these primed hESCs. When hESCs are derived from the blastocyst under naïve culture conditions, or when primed hESCs, regardless of their X state, are converted to naïve pluripotency, the X-chromosome state resembles that of the blastocyst, with two active X chromosomes and *XIST* expression (on one or both X chromosomes). Like normal development, differentiation of naïve hESCs induces XCI. Similar to primed hESC derivation, an *XIST*-negative state with two active X chromosomes is an intermediate in the primed to naïve hESC conversion, suggesting stepwise reversal of events. The lncRNA *XACT* is co-expressed with *XIST* in naïve pluripotency and might be responsible for inhibiting *XIST*-mediated silencing. *XIST* and *XACT* occupy non-overlapping territories on the active X chromosome (green and purple) in naïve hESCs. *XACT* is also expressed in primed hESCs both from active and eroding/eroded X chromosomes, and it might be driving erosion by interfering with *XIST* expression or accumulation. *XACT* is not expressed in differentiated cells.

cultures but is unique to hESCs, consistent with the idea that it is due to the expansion of this transient state unique to the transition from the blastocyst to primed pluripotency [6].

6. Naive human PSCs capture features of the X chromosome of the blastocyst

Mouse PSCs can transition from one pluripotent state to the other *in vitro*. For instance, over-expression of specific transcription factors, such as Klf4 [55] or deriving stem cells from post-implantation epiblasts in leukaemia inhibitory factor (LIF) and fetal calf serum [56] achieves primed to naive conversion. The ability to convert mouse cells *in vitro* from one pluripotent state to the other inspired researchers to screen for naive culture conditions appropriate for hESCs, with the idea that establishment of the primed pluripotent state was due to culture conditions and not intrinsic to the pre-implantation human blastocysts from which these cell lines are derived. Different approaches were used in the search for media formulations supporting naive pluripotency, with most of them using small molecule inhibitors, building upon naive condition of the mouse. Hanna and co-workers [57] demonstrated that the serum-free naive culture formulation for mESCs on its own—inhibition of both glycogen synthase kinase 3 beta and extracellular signal-regulated kinase 1/2 in combination with LIF (2i/LIF)—was not enough to support human naive PSCs, and constant expression of the pluripotency transcription factors OCT4, SOX2 and KLF4 was required in combination with 2i/LIF to support naive-like human PSCs (hPSCs). They screened for small molecule inhibitors of additional pathways that could stabilize the naive-like state in the absence of exogenous OCT4, SOX2 and KLF4 expression and formulated the first naive hPSC condition termed NHSM (naive human stem cell medium) [57]. This was followed by the development of several other formulations based on different combinations of small molecule inhibitors and cytokines [58–60]. Each newly devised culture condition resulted in cells with transcriptional profiles different from the human primed PSCs and similar, to various degrees, to the naive PSCs of the human pre-implantation blastocyst, likely reflecting the stabilization of various pluripotency states by each method. To address this systematically, Huang *et al.* [61] used an unbiased approach of comparing the transcription signature of each of these naive *in vitro* states to that of early human pre-implantation development, including oocyte, 1-, 2-, 4-, 8-cell stage embryos, morula and the blastocyst. In this analysis, two of the naive conditions—devised by Takashima *et al.* [58] and Theunissen *et al.* [59]—had the most significant gene expression overlap with the human blastocyst. Moreover, we demonstrated that the X-chromosome state of hESCs in these two culture conditions resembles that of the blastocyst, where *XIST* is expressed and accumulates on active X chromosomes [5,12]. Furthermore, the naive condition devised by Theunissen *et al.* allowed direct derivation of naive hESC lines from pre-implantation blastocysts [59], and the stabilization of the blastocyst X-chromosome state in culture [12]. Hence, we conclude that the X-chromosome state—mainly expression of *XIST* from active X chromosomes—is a reliable way of testing for true naivety of hPSCs that should be employed in assessing new

naive formulations in the future. Importantly, the ability to capture the naive status of *XIST* expression in hPSCs provides a unique system to investigate the inability of *XIST* to silence the X chromosome.

When primed hPSCs harbouring one active and one inactive X chromosome (with or without *XIST* expression from the Xi) are converted to naive pluripotency, the inactive X reactivates first, giving rise to XaXa cells, and only after several passages does *XIST* become expressed from either one or both X chromosomes, although the mono-allelic *XIST* pattern is dominant [12]. Interestingly, XaXa *XIST*-positive naive hESCs exhibited overall dampened X-linked gene expression levels compared with those not expressing *XIST* [12]. Thus, the correlation of XCD and *XIST* observed in human pre-implantation embryos appears to be recapitulated *in vitro* in the transition from primed to naive hESCs. These observations suggest that naive hESCs will also serve as model system for further exploring the novel X-linked gene dosage compensation mechanism of XCD.

In addition to serving as an *in vitro* model of the pre-implantation human embryo, naive culture conditions also provide a means of overcoming the XCI anomalies observed in primed PSCs (discussed in §5). When primed hESCs with either a slight or very high degree of XCI erosion, or even those that are trapped in the *XIST*-negative XaXa state, are adapted to the naive culture condition described by Theunissen *et al*. [59], and then subjected to differentiation, regardless of the starting primed XCI state, all of them result in cells with the proper somatic-like X-chromosome state: with an Xa and an *XIST*-expressing Xi [12]. These findings demonstrate that XCI erosion in primed hPSCs is truly just an anomaly caused by imperfect culture conditions and can be reversed given the right media formulation. Moreover, the ability to induce de novo XCI upon differentiation of naive hESCs (figure 2) [12] now opens opportunities of studying this epigenetic process in the human system for the first time.

7. The novel lncRNA *XACT* and its potential role in regulating human-specific aspects of X-chromosome dosage compensation

The puzzling differences in the way dosage compensation is established in the human compared with the mouse raise the intriguing hypothesis that some regulators of the process may differ between species. *Tsix*, the *Xist* antisense transcript identified in the mouse and described in §3, is one such example, having an important contribution to the regulation of murine XCI and no functional orthologue in the human. More recently, through RNA-sequencing analysis, we identified a novel X-linked lncRNA—*XACT*—which shares with *XIST* the capacity to accumulate on the chromosome from which it is expressed [28]. The appearance of *XACT* seems to be a recent event on the evolutionary scale, which took place in the higher primate branch, suggesting that it might fulfil primate (or human)-specific function [28].

Insights into such function came from the analysis of hPSCs with various X-chromosome states. In fact, expression of *XACT* is restricted to pluripotent cells: *XACT* gets silenced when the cells are induced to differentiate and reactivates upon induction of pluripotency (figure 2) [28]. In primed XaXi^{XIST+} cells, *XACT* is expressed from the active X only, while in *XIST*-negative XaXi cells, *XACT* is accumulating on both X chromosomes (figure 2). While this shift in *XACT* expression profile could simply reflect the partial reactivation of the Xi that characterizes XCI erosion, capturing the transition between the two states suggested an alternative scenario. Indeed, re-expression of *XACT* from the Xi undergoing erosion occurs before loss of *XIST* expression and prior to extended X-chromosome reactivation [47]. *XACT* reactivation from the Xi is thus not a mere consequence of erosion but is instead one of the earliest markers of this phenomenon. Pushing the reasoning further, *XACT* could causally participate in the erosion, by interfering with *XIST* expression or accumulation. In agreement with this hypothesis, when *XACT* was artificially inserted onto one X chromosome in female mESCs, XCI was biased towards the untargeted X chromosome. In other words, forced expression of *XACT* from one X reduced the likelihood of *Xist* accumulating on the very same chromosome, at least in a heterologous system [5].

What about *XACT* in the human embryo? Combining analysis of multiple datasets of single-cell RNA-sequencing and RNA-FISH confirmed that *XACT* is not an artefact of hPSC in culture, and that it is expressed in pre-implantation embryos [5]. Its expression is in fact strongly correlated to that of *XIST* in the early developmental stages (up to early E5), where it accumulates, together with *XIST*, on every X chromosome in both male and female embryos (figure 2). This pattern of active X chromosomes simultaneously decorated by *XIST* and *XACT* is recapitulated to some extent in naive hPSCs derived either in 5iLAF or in t2iL + Gö conditions [5,12,58,59], further reinforcing the idea that these naive conditions indeed bookmark the *in vivo* situation. Intriguingly, in both cases *XIST* RNA was found more dispersed in the nucleus compared with cells in which *XIST* coats the Xi [5,12]. This altered distribution of *XIST* might be linked to its inability to properly silence X chromosome at these stages. As it correlates with the simultaneous presence of *XACT*, it is also tempting to speculate that *XACT* might impair proper *XIST* accumulation in human cells, as it does in the heterologous mouse system described earlier.

8. Other potential mechanisms preventing *XIST*-mediated silencing

XACT is one strong candidate for preventing *XIST* from silencing the X chromosome during human pre-implantation development, but additional, non-mutually exclusive scenarios can be envisioned based on recent advances in studying the mechanism of action of mouse *Xist*. For instance, Patil *et al*. [34] demonstrated that a reversible RNA modification of adenosine residues—N^6-methyladenosine (m^6A)—is enriched on *Xist* and required for its silencing ability. Differences in this or perhaps even other RNA modifications or downstream readers of such modifications in early pre-implantation versus later post-implantation stages of human development might contribute to the functional differences of *XIST*.

RNA antisense purification followed by next-generation sequencing has allowed mapping of chromatin contacts made by mouse *Xist* at the onset of XCI, and combined with chromosome conformation studies, uncovered that *Xist* first contacts distal regions on the X chromosome that

are spatially close to the *Xist* transcription locus [30]. Hence one can postulate that the three-dimensional structure of the X chromosome is important when considering how *Xist* can spread along the X chromatin. Therefore, another speculation is that, due to different three-dimensional folding of the X chromosome in the pre-implantation embryo and/or expression of *XACT*, the chromatin structures might be unfavourable for *XIST* spreading and thus silencing of the X chromosome in naive pluripotency.

Several independent groups recently confirmed known and identified novel proteins that bind to mouse *Xist* RNA at the onset of XCI initiation or on the already established Xi [31–33,62–65]. Functional experiments have demonstrated that some of these *Xist* binding proteins are absolutely required for *Xist*-mediated silencing of the X chromosome. Hence it is plausible that one or more of key *XIST* interacting proteins required for its silencing ability are simply not expressed in the naive context, or that *XIST* is somehow unable to bind to such key protein factors, due to alternative splicing, the presence of competing proteins/RNAs or to chemical modifications.

9. Conclusion

The emerging studies of XCI in the human revealed a quite surprising flexibility in the way dosage compensation is established in various mammalian species [1,2,5,12]. Not only does XCI differ in kinetics and parental origin between human and mouse, but the strategies *per se* by which X-chromosome dosage imbalance is compensated for follow different routes, even if only transiently. XCD is reminiscent of the worm dosage compensation system, but the underlying mechanisms in human are still largely mysterious. We have seen that there are good reasons to believe that *XIST* could also contribute to this process. In this context, *XACT* could act as a switch for *XIST* function, from dampening X-chromosome expression (when *XACT* is present) to fully silencing it (in the absence of *XACT*). Our understanding of human dosage compensation has for long been impaired by the paucity of relevant biological material. Recent developments in the field of human naive pluripotency will help in uncovering molecular mechanisms, although it should be kept in mind that the field in still in its infancy. In this context, and as mentioned in §§6 and 7, we believe that rigorous assessment of the X-chromosome status through monitoring *XIST* and *XACT* expression will be instrumental in assessing true naivety of hPSCs and identifying novel conditions to robustly trigger and, importantly, maintain naive pluripotency.

Authors' contributions. A.S., K.P. and C.R. conceptualized, drafted, edited and proofread the manuscript.

Competing interests. We have no competing interests.

Funding. A.S. is supported by the Ruth L. Kirschstein NRSA F31 Fellowship (GM115122) and the Fowler Fellowship. K.P. is funded by the Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, the UCLA David Geffen School of Medicine, the Jonsson Comprehensive Cancer Center, the NIH (R01 GM115233), and is a Faculty Scholar of the Howard Hughes Medical Institute. Research in the Rougeulle lab is funded by the Agence Nationale pour la Recherche (ANR) and Ligue contre le Cancer.

Acknowledgements. We apologize to researchers who were not directly cited due to limited space.

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