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Review

The Role of *Xist* in X-Chromosome Dosage Compensation

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In each somatic cell of a female mammal one X chromosome is transcriptionally silenced via X-chromosome inactivation (XCI), initiating early in development. Although XCI events are conserved in mouse and human postimplantation development, regulation of X-chromosome dosage in preimplantation development occurs differently. In preimplantation development, mouse embryos undergo imprinted form of XCI, yet humans lack imprinted XCI and instead regulate gene expression of both X chromosomes by dampening transcription. The long non-coding RNA *Xist*/*XIST* is expressed in mouse and human preimplantation and postimplantation development to orchestrate XCI, but its role in dampening is unclear. In this review, we discuss recent advances in our understanding of the role of *Xist* in X chromosome dosage compensation in mouse and human.

Highlights

X-inactive specific transcript (*Xist*) is a long non-coding RNA that remains associated with the X chromosome from which it is expressed.

Xist is unequivocally required for the imprinted form of X-chromosome inactivation (XCI) in mice *in vivo*, but demonstration of its indisputable requirement for random XCI *in vivo* is yet to be shown.

Loss of *Xist* expression in mice *in vivo* and in conventional human pluripotent stem cells correlates with partial reactivation of genes residing on the inactive X chromosome, suggesting an important role of *Xist* in maintenance of the silent state of genes on the inactive X chromosome.

Human preimplantation embryos have a unique X-chromosome dosage compensation state called X-chromosome dampening (XCD), where transcriptional output is tuned down from both X chromosomes. Correlative observations suggest that XCD might be mediated by *XIST*.

X-Chromosome Dosage Compensation

Male (XY) and female (XX) **eutherian** (see Glossary) mammals have equivalent expression levels of most X-chromosome genes despite the presence of an extra X chromosome in females. This X-chromosome dosage compensation is due to the phenomenon of **X-chromosome inactivation (XCI)**, which refers to the transcriptional silencing and **heterochromatinization** of one of the two X chromosomes in females early in embryonic development [1]. Most of our knowledge of XCI is based on mouse studies, where two types of XCI exist: imprinted and random (Figure 1A).

In imprinted XCI, which initiates in all cells of the female mouse four- to eight-cell stage preimplantation embryo, the paternally inherited X chromosome (Xp) undergoes inactivation, while the maternally inherited X chromosome (Xm) remains active [2,3]. As preimplantation development progresses to form the blastocyst, cells of the trophectoderm layer, which give rise to extra-embryonic tissues (e.g., the placenta), maintain their imprinted XCI state. By contrast, epiblast cells of the blastocyst, which give rise to the embryo proper, reactivate the inactive Xp, re-establishing a state with two active X chromosomes. The biallelic X-linked gene expression of epiblast cells is resolved again via XCI, but in this second wave of XCI either the Xp or the Xm is chosen at random for inactivation. Random XCI is maintained in all descendent somatic cells throughout life, resulting in adult mice that are a mosaic of cells expressing either maternal or paternal alleles of X-linked genes [4,5]. A group of X-linked genes express both the maternal and the paternal allele in each cell since these genes **escape** XCI and are thus the exception to the rule (reviewed by [6]). The chromosome-wide inactivation of the X chromosome, in both imprinted and random XCI, appears to always be governed by the **long non-coding RNA (lncRNA)** X-inactive specific transcript (***Xist***), which is encoded in the X-inactivation center (XIC) of the X chromosome [7].

The occurrence of both imprinted and random XCI in the same species, as is the case in mouse, may not be very common. Most mammals studied utilize only one form of XCI for

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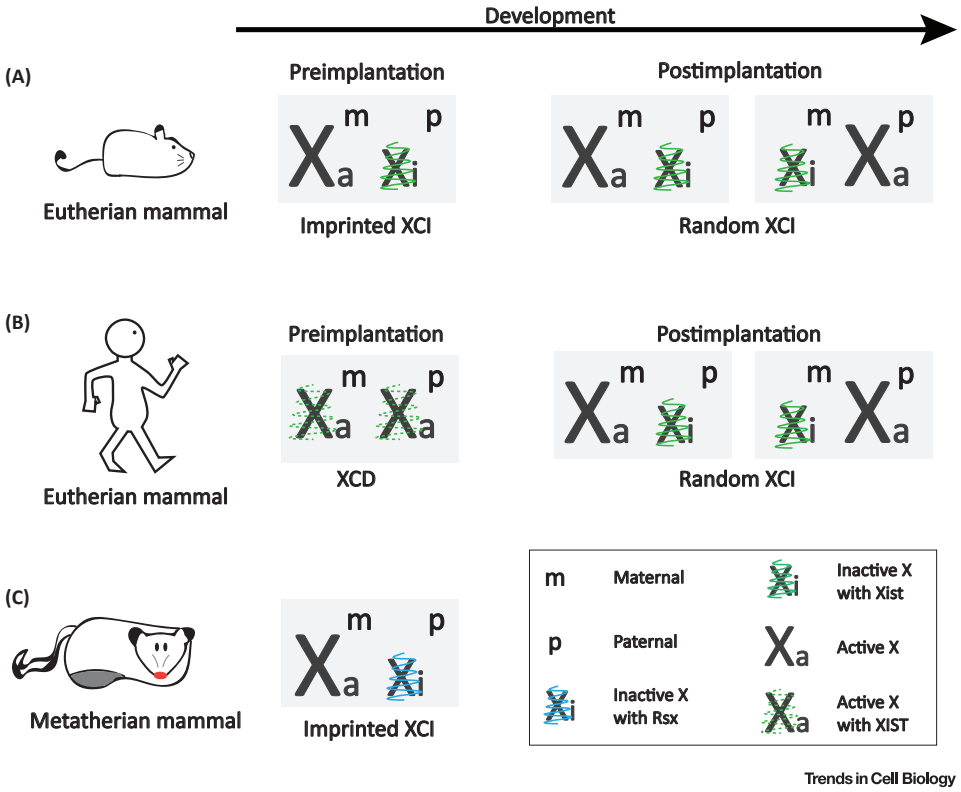

Figure 1. X-Chromosome Inactivation in Different Mammals. The X-chromosome states of eutherian (A and B) and **metatherian** (C) female mammals are shown in embryonic development. (A) Imprinted X-chromosome inactivation (XCI) occurs in mouse preimplantation development, but it is reset in the cells that develop into the embryo to give way to random XCI, resulting in a mosaic adult female mouse. Both imprinted and random XCI in the mouse are regulated by the long non-coding RNA (lncRNA) *Xist*. (B) Humans have evolved away from imprinted XCI as they dosage compensate in preimplantation development by turning down transcription from both X chromosomes via X-chromosome dampening (XCD). Moreover, *XIST* is expressed on both dampened X chromosomes, where its functional role remains to be determined. In postimplantation development, similar to the mouse, human females display random XCI mediated by *XIST*. (C) Metatherians, such as the marsupial opossum (*Monodelphis domestica*), dosage compensate by inactivating the paternally inherited X chromosome using the lncRNA *Rsx*. This imprinted dosage compensation is maintained throughout marsupial development, resulting in a female adult with a transcriptionally inactive paternal X chromosome in all of its cells. *Rsx*, RNA on the silent X; Xa, active X chromosome; Xi, inactive X chromosome; *Xist/XIST*, X-inactive specific transcript; Xm, maternal X chromosome; Xp, paternal X chromosome.

X-chromosome dosage compensation. In marsupials, only imprinted XCI is observed, where the Xp is exclusively chosen for inactivation [8] (Figure 1C). Contrary to this, imprinted XCI does not occur in rabbit, pig [9,10], horse, or human development [11–13] based on analysis of preimplantation blastocysts [9,10,13] or placental tissues [11,12]. In human postimplantation development, both extra-embryonic and embryonic lineages dosage compensate via random XCI [12,14] (Figure 1B). However, in the 1st week of human development, prior to implantation and XCI, the existence of a novel gene-dosage regulation has recently been uncovered [13]. Here, both X chromosomes remain active from the onset of zygotic gene activation until the blastocyst stage [9,13] (Figure 1B). However, transcription from both X chromosomes is tuned down, or dampened, resulting in a net reduction of X-linked gene expression in female blastocyst cells [13]. **X-chromosome dampening** (**XCD**) has not been observed in any other mammal yet, but it has been reported in the nematode *Caenorhabditis elegans* [15], although the underlying mechanism in human and nematode may differ. In the XX hermaphrodite

Glossary

Embryonic stem cells (ESCs): cells derived from the inner cell mass of the blastocyst before it implants in the uterus. ESCs are pluripotent and can self-renew indefinitely.
Erosion: loss of transcriptional silencing and DNA methylation for genes on the inactive X chromosome in cultured primed human pluripotent stem cells. Eroded regions of the inactive X chromosome usually contain more than one gene, and fail to re-inactivate upon exit from pluripotency.
Escape: from XCI happens in both human and mouse development when a small portion of X-linked genes do not undergo transcriptional silencing in early development despite residing on the inactive X-chromosome.
Eutheria: type of mammals whose fetal development requires a placenta (e.g., mouse and human). This excludes pouched mammals (marsupials, e.g., kangaroo) and egg-laying mammals (monotremes, e.g., platypus).
Facultative heterochromatin: a subtype of **heterochromatin** that can also be present as euchromatin (actively transcribed chromatin) in a different context, such as the X chromosome before and after XCI.
Heterochromatin: regions of chromatin with low or no transcriptional activity.
Heterochromatinization: formation of heterochromatin via changes to the epigenome, such as covalent modifications of histones and methylation of DNA, which result in transcriptional inactivity.
Histone hypoacetylation: removal of the acetyl groups from histones, leading to reduced transcriptional activity.
Induced pluripotent stem cells (iPSCs): derived from somatic cells by transcription factor overexpression-induced reprogramming.
Long non-coding RNA (lncRNA): These RNA molecules are more than 200 nucleotides long and do not encode proteins.
Metatheria: also includes marsupials; these mammals give live birth after a rather short gestation time, after which development of the

C. elegans, the 3D conformation of the X chromosomes is remodeled to reduce chromosome-wide gene expression by half to achieve gene-expression balance between XX hermaphrodites and XO males [16]. 3D chromosome conformation also differs between the active and inactive X chromosomes in mammals [17], suggesting that chromosome confirmation remodeling might also be at play in human XCD. However, unlike the mammalian inactive X chromosome (**Xi**), lncRNAs have not been reported to regulate X-chromosome dose in the *C. elegans*. Instead, dampening in the nematode is carried out by the dosage compensation complex, a condensin-containing multi-subunit protein assembly that binds at multiple sites along the X chromosome and leads to chromosome-wide compaction and gene repression [18].

Whether XCD events observed in human preimplantation development and *C. elegans* are related at the molecular level needs further investigation. Moreover, when single-cell RNA-sequencing data (Box 1) of preimplantation human blastocysts are analyzed using different bioinformatics tools and approaches, the dosage compensation observed in human preimplantation embryos has been interpreted as initiation of XCI rather than dampening of both X chromosomes [19,20]. Fortunately, naïve human **embryonic stem cells** (h**ESC**s), which are the *in vitro* counterparts of the pluripotent cells in the human preimplantation embryo, exhibit XCD and thus can be used as a model system to address XCD and its relationship to the initiation of XCI further [21].

In this review, we discuss the experimental evidence examining the role of *Xist* in X-chromosome dosage compensation via imprinted and random XCI in mouse. We also consider *XIST* function in early human development and in human **pluripotent stem cells** (hPSCs), reflecting on potential molecular mechanisms which might regulate context-dependent *XIST* function.

Long Non-coding RNAs Are Key Players in X-Chromosome Regulation

An intriguing fact about X-chromosome dosage regulation in all mammals is the utilization of lncRNAs such as *Jpx*, *Ftx*, *Tsix*, and *XACT,* most of which are located in the XIC of the X chromosome [20,22–28] (Figure 2). When expressed, *Xist* is exclusively associated with the X chromosome from which it is expressed, acting only *in cis* [29]. The lncRNAs *Jpx* and *Ftx* exert their function by acting as activators of *Xist* to fine-tune *Xist* expression and thus indirectly regulate XCI. The *Jpx* lncRNA product acts either *in cis* or *in trans* [23] and binds the *Xist* repressor CTCF, taking away repression of *Xist* transcription [22]. Contrary to this, the *Ftx* transcript itself is not required for *Xist* regulation: it is the act of transcription of the Ftx locus that

Box 1. Methods used for Key Experiments Discussed

Sox2 promoter-driven Cre recombinase: Cre recombinase, which recombines DNA at specific DNA sequences, coined 'loxP sites', is expressed only in cells where the *Sox2* gene is actively transcribed. This system is engineered by cloning the Sox2 promoter and enhancer region upstream of the Cre recombinase gene. When the same cell contains DNA sequences flanked with loxP sites of the same orientation, the intervening sequence will be removed – or deleted.

Tetraploid complementation assay is a method for creating mice where all the cells of the embryo proper are derived from mouse PSCs upon blastocyst injection of these cells. To accomplish this, cells of two-cell embryos are fused experimentally to form a tetraploid preimplantation embryo. While the tetraploid cells of the blastocyst can develop into the extraembryonic tissues required for *in utero* development, they cannot contribute to the embryo proper. Hence, when such tetraploid blastocysts are injected with diploid mouse PSCs, all the tissues of the embryo proper come from these injected diploid cells, creating a non-chimeric mouse.

Single-cell RNA-sequencing is a method of measuring global gene expression from individual cells to determine gene expression patterns unique to individual cells that would otherwise be lost in bulk RNA-sequencing (from a group of cells) due to averaging of the data.

newborn continues in the mother's pouch.

Myelodysplasia: multiple types of cancers of blood progenitor cells in the bone marrow that interfere with normal maturation of blood cells, resulting in reduced platelet, red or white blood cell count.

Naïve pluripotency: pluripotency state of cells of epiblast cells in the preimplantation blastocyst.

Pluripotency: cell state that can give rise to any of the cell types in the body.

Pluripotent stem cells (PSCs): obtained either from preimplantation blastocysts (ESCs) or via reprogramming of somatic cells (iPSCs).

Primed pluripotency: pluripotency state of epiblast cells in the postimplantation embryo.

Reprogramming: forced expression of key transcription factors that remodel the epigenome of differentiated cells (i.e., fibroblasts) to transition these cells into the developmentally early, pluripotent state.

Xa: active X chromosome.

X active coating transcript (XACT): a recently discovered long non-coding RNA on the X chromosome, present in humans but not in mice and expressed only in pluripotent cells from non-silent X chromosomes.

X-chromosome dampening (XCD): the tuning down, but not complete silencing, of genes on the X chromosome. Transcriptional output from a dampened X chromosome is less than that from an active X but more than that from an inactive X-chromosome.

X-chromosome inactivation (XCI): transcriptional silencing of most genes on the X chromosome in female mammalian cells.

Xi: inactive X chromosome with most genes not transcribed due to XCI.

Xi reactivation: of the inactive X-chromosome refers to when all silenced genes become transcribed to resemble the state of the active X chromosome. This is a chromosome-wide event and occurs in both mouse and human development during germ-cell development (cells that give rise to gametes). In mouse development Xi reactivation also

leads to Xist expression *in cis* [25]. Moreover, in mice, the lncRNA *Tsix*, which is transcribed in antisense orientation to Xist, represses Xist expression, thus ensuring Xist induction on the Xi and protecting the **Xa** (active X chromosome) from ectopic silencing by *Xist* [26,27]. *Tsix* is not expressed in human preimplantation development [13], and the roles of *Jpx* and *Ftx* are yet to be examined in humans. A recently discovered lncRNA called ***XACT*** that is unique to hPSCs, but does not reside in the XIC, may aid in maintaining transcriptional activity of the X chromosome from which it is expressed by counteracting *XIST* [20,28]. Interestingly, correlative studies suggest that *XIST* RNA, in addition to XCI, might also mediate the dampening of the transcriptional output of both X chromosomes of female human preimplantation blastocysts [13,21] (discussed later). While *Xist* is unique to placental mammals, marsupials also use a *cis*-acting lncRNA encoded on the X chromosome, termed 'Rsx' (RNA on the silent X), which in many ways appears to act like *Xist* in XCI [8] (Figure 1). Taken together, it is rather interesting that different lncRNAs have evolved to regulate gene expression chromosome-wide *in cis*. This is perhaps due to the unique ability of lncRNAs to bind distant sites on chromatin while still tethered to their transcription loci. *Xist* [30] and other lncRNAs such as *HOTTIP* (HOXA transcript at the distal tip), which is encoded on mouse chromosome 6 and activates genes in its neighborhood [31], reach their target chromatin sites simply by proximity – by being close

occurs in the transition from imprinted to random XCI.
Xist: X-inactive specific transcript is a lncRNA gene on the X chromosome. It does not leave the nucleus and stays associated with the chromosome from which it is transcribed. In mouse, studies have shown that it is required and sufficient for the induction of XCI.

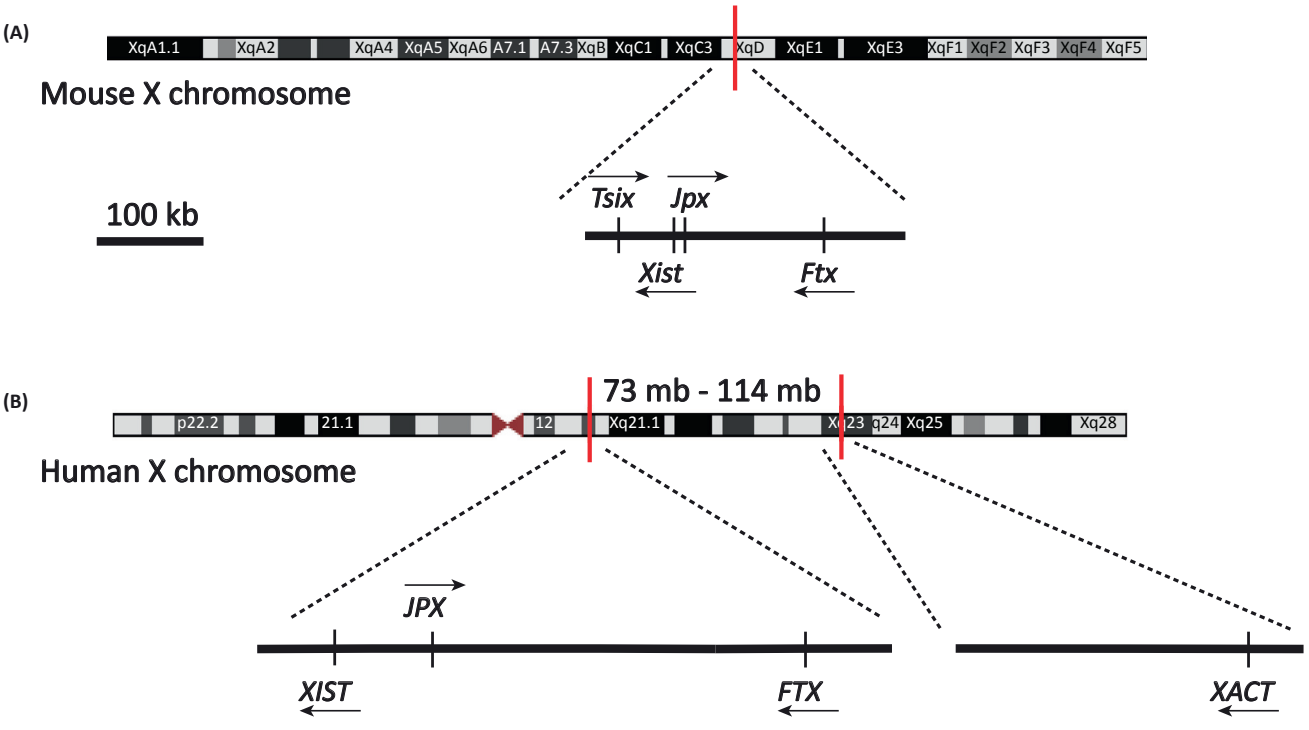

Figure 2. Long Non-coding RNAs (lncRNAs) Involved in X-Chromosome Dosage Regulation in Mouse and Human. The X-inactivation center (XIC) is located on the X chromosome and harbors the master regulator of X-chromosome inactivation (XCI) – the long non-coding RNA (lncRNA) Xist. (A) In the mouse, *Xist* itself is positively regulated by the lncRNAs *Jpx* and *Ftx*, which are also encoded in the XIC, upstream of the *Xist* gene. The lncRNA *Tsix*, which is antisense to *Xist*, has a mutually exclusive expression pattern with *Xist*: it is expressed biallelically from both X chromosomes prior to XCI. *Tsix* 'protects' the active X chromosome in pluripotency from being silenced by *Xist* upon induction of XCI, and is thought to be a repressor of *Xist*. (B) The human *XIST* gene is also encoded in the XIC of the human X chromosome. The lncRNAs *JPX* and *FTX* are also upstream of *XIST* in the human XIC, similar to mouse. The role of *JPX* and *FTX* in regulating *XIST* expression in human is speculated based on mouse studies. Unlike mouse, the human XIC does not contain the *XIST* antisense lncRNA *TSIX*, since *TSIX* expression is not detected in preimplantation blastocysts or human embryonic stem cells. A novel, human-specific lncRNA, X-active coating transcript (*XACT*), however, is encoded about 40-Mb upstream of the human XIC and seems to antagonize *XIST* in naïve pluripotency. Similar to mouse *Tsix,* expression of *XACT* is unique to pluripotent cells and not detected in somatic cells. *Xist/XIST*, X-inactive specific transcript.

to these sites in 3D space due to the folding of chromatin within the nucleus (reviewed and illustrated in [32]). Understanding how X-chromosome dosage is regulated via lncRNAs will thus not only shed light onto X-chromosome biology, but also can serve as a starting point in understanding how lncRNAs localize to and act on chromatin in general.

Xist Is Required for XCI in Mouse

Xist is the best studied lncRNA to date. As the name suggests, *XIST* was discovered due to its association with the inactive X chromosome – X-inactive specific transcript [33–37]. The requirement of *Xist* for XCI was first directly implicated using female mouse ESCs (mESCs) with a mutated Xist gene lacking the first two-thirds of exon 1 [38]. When induced to differentiate, both *in vitro* and *in vivo* (using aggregation chimeras), the X chromosome bearing the mutant-truncated Xist gene was always spared from inactivation while *Xist* was expressed from the wild-type X chromosome, causing non-random silencing [38]. This study demonstrated that Xist is required for choosing the chromosome for inactivation, and suggested, without direct evidence, that *cis* expression of *Xist* is required for XCI.

Since mESCs are derived from the epiblast cells of the blastocyst, the role of *Xist* in preimplantation development, from zygote to blastocyst formation, cannot be studied using these cells. Therefore, to further investigate the role of *Xist* in early development, mESCs were used to generate chimeric mice containing cells with a large deletion of Xist, which were then mated with wild-type mice to generate hemizygote males or heterozygote females [39]. When the mutant Xist was inherited from the mother, both normal female and male pups were born. However, female embryos with a paternally inherited mutant Xist had severe prenatal growth defects and survived until approximately embryonic day 10.5 (E10.5). In these embryos the extraembryonic tissues failed to develop due to the lack of imprinted XCI of the Xp in these tissues [39] (Figure 3). Because of the predetermined choice of the paternal X chromosome for silencing in imprinted XCI, this experiment is the first clear demonstration that XCI, specifically imprinted XCI, cannot be initiated without *Xist*. Note that there is no paternal inheritance of mutant Xist to be studied in males since they inherit a Y chromosome but no X chromosome from their father. The inability of the extraembryonic tissue to support the growth of the embryo in the absence of X-chromosome dosage compensation was investigated further and this failure was attributed to exhaustion of the extra-embryonic ectoderm due to premature cell differentiation [40].

A recent study used single-cell RNA-sequencing to provide high temporal and chromosome-wide resolution of X-linked gene silencing in mouse preimplantation development and its dependence on *Xist* RNA [3]. Comparisons of female wild type to mutant embryos carrying a paternal *Xist* deletion at the 8-, 16-, 32-cell, and blastocyst stages validated the need for *Xist* in initiating imprinted XCI [3], which was also demonstrated recently using RNA-sequencing of single embryos [2]. Such high-resolution data were important to clarify the role of *Xist* in imprinted XCI and to rule out prior arguments for an *Xist*-independent imprinted XCI [41]. The dynamics of silencing during the initiation of imprinted XCI revealed that genes are silenced with different kinetics. Genes silenced at an earlier stage of preimplantation development were those near the XIC (where the Xist gene is located) in 3D space [3,30]. Intriguingly, this recapitulates the finding that at the initiation of random XCI, *Xist* first contacts the sites of the chromosome closest to its site of transcription in 3D, rather than linear space [30]. This correlation between the kinetics of imprinted XCI and the proximity to the Xist locus in 3D space independently supports the notion that imprinted XCI is mediated by *Xist*, and additionally suggests that the mechanism of *Xist* spreading *in cis* during the initiation of imprinted and random XCI is conserved.

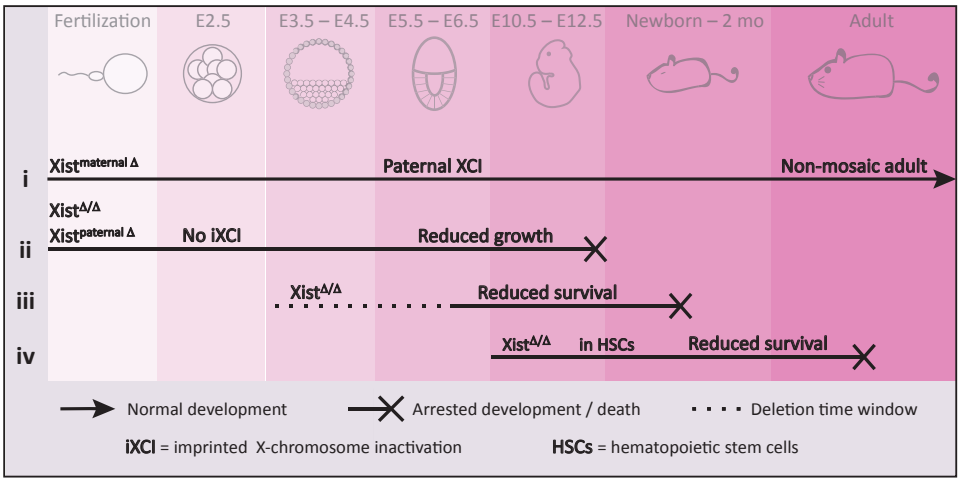

Figure 3. Lack of *Xist* at Various Developmental Time Points Highlights Its Importance in Normal Development. Summary of key studies addressing the role of *Xist* in mouse development from fertilization to birth and into adulthood. (i) When a zygote is formed with a maternally deleted Xist (inherited from the egg), mouse development progresses normally and results in non-mosaic adults where all cells inactivate the paternally inherited X chromosome (since only that X has the only functional *Xist* allele). (ii) However, when *Xist* is deleted from both X chromosomes (from the egg and the sperm), or from only the paternal X chromosome (sperm), extra-embryonic tissues fail to develop in the absence of imprinted X-chromosome inactivation since this process requires paternally inherited *Xist*, and thus mouse development halts 5–7 days postimplantation. (iii) Conditional Xist deletion from both X chromosomes in epiblast cells that give rise to the embryo is often embryonically lethal, and if pups are born, they display partial loss of X-chromosome silencing and do not survive to adulthood. (iv) When Xist is deleted several days postimplantation, specifically in hematopoietic stem cells (HSCs), after the establishment of the Xi, pups are born but succumb to multilineage dysplasia as early as 1.5 months after birth. E, embryonic day; HSCs, hematopoietic stem cells; mo, month; iXCI, imprinted X-chromosome inactivation; *Xist*, X-inactive specific transcript.

Requirement of XCI in the Development of the Mouse Embryo Proper

Although the aforementioned paternal Xist deletion experiment demonstrated that *Xist* is required for imprinted XCI, the early embryonic lethality due to malfunctioning extraembryonic tissues has made addressing the requirement of *Xist* in the embryo itself unfeasible with a germline mutation of Xist. A homozygous Xist knockout is required to address this question, which, when using a germline mutation, affects imprinted XCI as well (Figure 3). However, the maternal germline deletion of Xist allele was valuable to demonstrate the requirement of a functional Xist allele for choosing the X chromosome for XCI *in vivo* [42], extending the prior mESC study [38]. In the meantime, experiments demonstrating the sufficiency of *Xist* RNA for silencing was demonstrated. Particularly, ectopic expression of Xist cDNA from autosomes or the X chromosome [43,44], or activation of the endogenous Xist allele from the single X chromosome in male mESCs with an inducible promoter [30] demonstrated that *Xist* expression is sufficient to cause silencing *in cis.* These gain-of-function experiments also opened the way for dissection of *Xist* RNA for its functional units [43].

Studying the need for *Xist* in random XCI requires a unique approach that specifically deletes Xist in embryonic tissues. A recent study took on the challenge to assess the importance of X-chromosome dosage compensation by random XCI in mouse embryonic development. To silence Xist specifically in the embryo while sparing the extra-embryonic tissue, *Xist* was conditionally deleted in the epiblast lineage [45]. While most mutant mice died *in utero,* surprisingly some mice survived to term, but exhibited growth retardation with reduced body

size, dying within 1 month after birth (Figure 3). Only one mouse, which was a mosaic of XX and XO cells – cells that had lost one of their two X chromosomes – survived to adulthood. These data indicate that *Xist* and random XCI are required for normal embryonic development. However, given the survival to term, the phenotype appeared much weaker than expected and was a surprise – since XCI occurs soon after implantation, the expectation was that *Xist* loss should have led to early embryonic lethality. This can perhaps be explained by another unexpected observation, namely, that X-chromosome dosage compensation was not completely wiped out upon deletion of Xist. The authors reported partial X-chromosome dosage compensation in the absence of *Xist*, concluding that an *Xist*-independent mechanism was responsible [45].

This phenotype of partial X-chromosome dosage compensation in *Xist*-mutant mice was observed due to less-than-expected increase in average expression levels of all genes by RNA-sequencing when compared with wild-type female mice, and due to the presence of mono-allelic gene expression in some, but not all, cells at the single-cell level of a few X-linked genes, assessed by fluorescent *in situ* hybridization [45]. However, understanding the Xist-mutant mouse model system used [45] can perhaps better explain these aforementioned observations. Deletion of Xist in the epiblast lineage was accomplished with the Sox2 promoter-driven Cre recombinase [45] (Box 1). However, the efficiency of Xist excision by the Sox2-driven Cre was not measured in the developmental interval around the induction of random XCI [46], as embryos were only harvested at E8.5 to confirm Xist deletion, a time point at which XCI has already occurred [45]. Originally, Sox2-driven Cre recombinase activity has been shown to occur in blastocyst outgrowths in culture and in all cells of the epiblast in E6.5 embryos *in vivo*, by assessing the removal of a 'stop' cassette in front of a β-galactosidase reporter gene integrated into the ROSA26 locus [47,48]. Together, these findings suggest that the ROSA26 reporter recombined before E6.5 and possibly before XCI would be initiated *in vivo*, indicating that the Sox2-driven Cre recombinase may be ideally suited to delete *Xist* before induction of random XCI. However, since chromatin accessibility may be different for the Xist locus in comparison to ROSA26, a region on mouse chromosome 6 identified because of its high recombination frequency [49], the Cre-mediated excision kinetics may differ for the two loci and thus need to be independently determined for the Xist locus. In addition, the DNA segment flanked by LoxP sites, which need to come together for Cre-mediated recombination, is significantly longer in the Xist deletion construct [45] compared with the ROSA26 β-galactosidase reporter system [47], and therefore potentially less favorable for deletion, again arguing for the need to establish *in vivo* Xist deletion kinetics in this mouse model. Hence, it cannot be ruled out that Xist deletion may have occurred after initiation/completion of random XCI.

Previous studies have shown that deletion of Xist has no dramatic short-term effect on the silent state of genes on the X chromosome when it occurs after the inactive X chromosome is fully established [50,51] (see discussion later). Depending on the proportion of cells undergoing random XCI prior to Xist deletion, the embryos would then survive to term and demonstrate incomplete dosage compensation at the organism level due to the mixture of XaXa and XaXi cells (Xa is the active X chromosome and Xi is the inactive X chromosome). Xist deletion post-XCI establishment would also explain the low number of pups surviving to term, since if not enough cells per embryo undergo X-chromosome dosage compensation prior to Xist deletion, the embryo would not be viable, arguing for the importance of Xist in XCI and embryonic development. Taken together, additional experiments with a temporally precise Xist deletion (and confirmation of Xist deletion before induction of random XCI) are required to dissect whether a novel embryonic X-chromosome dosage compensation mechanism in the absence of Xist or the delayed deletion of Xist relative to the onset of XCI explains the surprisingly weak

consequences of the current Xist deletion in the embryo [45]. For instance, using homozygous Xist knockout mESCs in tetraploid complementation assays [52] (Box 1) could provide wild-type extraembryonic tissues capable of supporting normal development while all cells of the epiblast, derived from mESCs, would lack Xist, ruling out the possibility of random XCI occurring in any fraction of the cells. It would be interesting to see if/when development would fail in this scenario, but our hypothesis is that no viable pups would be obtained from such mice. Regardless, the current data argue that X-chromosome dosage compensation mediated by *Xist* is critical for embryonic development.

The influence of XCI on the developmental potential of female cells has been shown with mESCs, as the double dose of X-linked genes delays the differentiation of these cells due to its stabilizing effect on the naïve pluripotent state [53]. This stabilization is achieved via inhibition of the mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3 (Gsk3) pathways and stimulation of the Akt pathway, and XCI is needed to properly exit **naïve pluripotency** [53]. The delayed exit from **pluripotency** in the presence of two active X chromosomes may also occur *in vivo*, since embryos with a single X chromosome undergo accelerated development [54].

Xist Executes XCI by Recruiting a Diverse Set of Proteins

During initiation of XCI, *Xist* recruits numerous silencing factors to the X chromosome to establish **facultative heterochromatin**, also known as the 'Barr body' [55]. This is accompanied by epigenetic changes including substitution of certain core histones, covalent modifications of histone tails, and promoter CpG methylation (reviewed by [1]). Although some of these *Xist*-induced epigenetic remodeling steps were discovered years ago, most of the proteins binding *Xist* directly and indirectly were identified only recently using mass spectrometry-based approaches and genetic screens [56–60]. While some of the *Xist*-binding proteins influence histone modifications (via the activation of the histone deacetylase HDAC3 through the engagement of SPEN by the 5′ end of *Xist*) [57] or nuclear positioning of the *Xist*-coated Xi (through the binding of lamin B receptor to *Xist*) [61], others induce RNA modification of adenosine methylation (m^6A) on *Xist* to influence its silencing ability [62]. Identification of the *Xist* interactome – that is, the proteins that directly or indirectly bind to *Xist* – has created a newfound appreciation of the multiple roles *Xist* plays in XCI, spanning from orchestrating chromosome-wide silencing, localizing itself to chromatin, altering chromatin state, and remodeling the 3D chromosome architecture, recently reviewed in [55]. While the primary sequence of *Xist* RNA is not well conserved between mouse and human, the gene structure (exons/introns) as well as the presence of key repeat regions [37] are conserved in the two species. Some of these repeat regions are important for *Xist* function since they are the sites where proteins that directly interact with *Xist* bind to [56,57,63]. Therefore, although the mass spectrometry-based unbiased approaches have identified the mouse *Xist* interactome [56–58], it is safe to predict that the human *XIST* interactome will largely overlap with that of mouse [64].

Xist Is Required for Long-Term Maintenance of Random XCI

The Xi with *Xist* expression remains inactive in all somatic progeny of cells. In short-term *in vitro* studies (days to weeks), *Xist* does not seem to play a major role in the maintenance of the silent status of genes in random XCI in somatic cells [44,50]. This appears to also be the case in the maintenance of imprinted XCI in the vole *Microtus levis*, where ablation of *Xist* expression via deletion of its promoter region in trophoblast stem cells, which have already undergone imprinted XCI, does not lead to transcriptional reactivation or loss of repressive chromatin marks of the Xi [65]. However, a longer-term *in vivo* mouse study suggests that the prolonged absence of *Xist* in mice, initiated in the blood lineage using a tissue-specific Cre recombinase,

induces at least partial reactivation of genes on the X chromosome [51]. Notably, the experimentally induced deletion of *Xist* in hematopoietic cells in mice results in poor postnatal survival and development of **myelodysplasia** and various cancers of the blood with 100% penetrance [51] (Figure 3). The inevitable development of cancer in the absence of *Xist* clearly labels *Xist* as a potent tumor suppressor, most likely due to its requirement in the maintenance of gene silencing in somatic cells. In agreement with this *in vivo* mouse study, abnormal reactivation of the Xi has also been reported in human breast cancer cells, although here an extra dose of X-linked genes is either due to Xi **erosion** or loss of an Xi combined with an Xa duplication [66]. However, the connection between *XIST*-dependent maintenance of XCI and cancer formation in humans needs to be further explored. Since the importance of *Xist* in maintaining XCI only became obvious from mouse *in vivo* studies, it is critical to address the role of *XIST* in human cancers with carefully designed experiments.

There are two instances in mouse development that require reactivation of the Xi: once in cells of the inner cell mass of the blastocyst, when imprinted XCI needs to be reversed prior to induction of random XCI, and once more in the development of primordial germ cells, prior to meiosis [67]. In both cases, shutdown of *Xist* expression from the existing Xi precedes the removal of chromosome-wide transcriptional repression [68,69]. **Xi reactivation** is also observed *in vitro*, when female mouse somatic cells such as mouse embryonic fibroblasts are reprogrammed to form induced PSCs (**iPSCs**). Whereas the necessity of *Xist* loss in Xi reactivation is difficult to address in the *in vivo* scenarios described earlier, the *in vitro* **reprogramming** system has allowed detailed studies of this relationship. Using ectopic maintenance of expression or the deletion of Xist in a reprogramming experiment, it was demonstrated that Xist loss is necessary, but not sufficient, for Xi reactivation in iPSC generation, reviewed in more detail in [67]. During reprogramming to iPSCs, Xi reactivation is one of the last steps of reprogramming, requiring DNA demethylation in addition to *Xist* RNA loss [70]. Similarly, in somatic cells, DNA demethylating agents, such as 5-aza-2′-deoxycytidine, induce reactivation of genes of the somatic Xi, albeit in a small proportion of cells, via induction of global DNA demethylation [71,72]. DNA methylation works in synergy with *Xist* RNA and **histone hypoacetylation** [71], as well as the histone 3 lysine 9 (H3K9) trimethylation pathway [72,73] in maintaining the inactive state of the somatic Xi. In fact, it takes the synergism of triple-drug combinations targeting DNA methylation, topoisomerase activity (involved in relieving torsional stress during DNA replication and transcription) combined with knockdown of an *Xist*-interacting protein to obtain dramatic reactivation of the Xi, and even then the reactivation is not for all silenced genes [58]. Complete chromosome-wide reactivation of all silenced X-linked genes in somatic cells has not been reported thus far, highlighting the unbreachable nature of the multiple epigenetic layers protecting the Xi.

Xi Reactivation in Human Pluripotent Stem Cells

Studies of the relationship between *XIST* and Xi reactivation in human cells are not as straightforward as in mouse, mainly because (i) there is no imprinted XCI to be reversed in human preimplantation development [9], (ii) reactivation of the Xi in human primordial germ cells is difficult to study due to the hurdles associated with obtaining appropriate tissue samples and the lack of a human germ cell culture system that recapitulates Xi reactivation, and (iii) reprogramming of human somatic cells under standard conditions does not lead to Xi reactivation [74] as it results in iPSCs that are in a developmentally advanced – primed – pluripotent state [75]. However, when conventional human iPSCs and ESCs in the primed pluripotent state are expanded in culture, *XIST* expression becomes gradually lost, which is accompanied by methylation of the XIST promoter [74,76–78]. The *XIST* loss in these pluripotent cells is usually accompanied by partial reactivation of the Xi, where transcriptional repression of some, but not

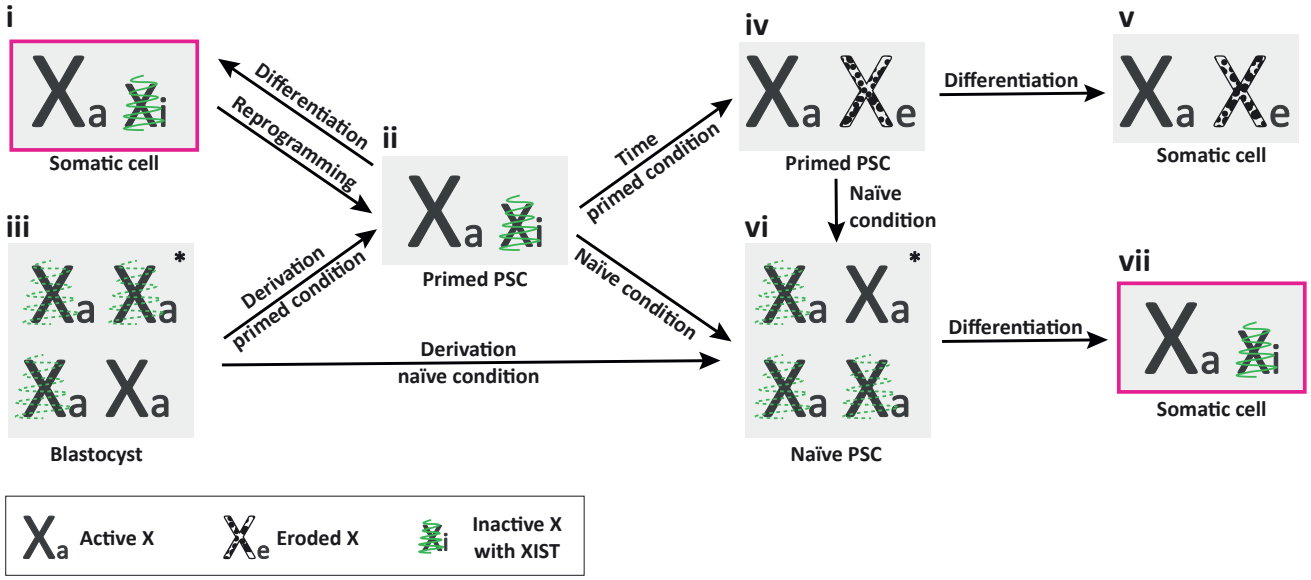

Figure 4. The X-Chromosome State of Naïve and Primed Human Pluripotent Stem Cells. (i) Female human somatic cells have an active and an *XIST*-expressing inactive X chromosome (Xa and Xi). (ii) Reprogramming of these cells to **primed pluripotency** does not change the X-chromosome state. (iii) Similarly, derivation of hPSCs from a preimplantation blastocyst stabilizes the post-XCI state in primed pluripotent culture conditions. (iv) Over time in culture, the Xi loses expression of *XIST* and undergoes epigenetic erosion, resulting in partial reactivation and thus double dose of the X-linked genes that fall in these eroded regions in primed hPSCs. (v) Although these cells can differentiate into somatic lineages, the resulting differentiated cells maintain the eroded X (Xe). Female preimplantation blastocysts have two active X chromosomes and express *XIST*, serving as a unique scenario where *XIST* expression does not cause XCI. (vi) When hESCs are derived under naïve pluripotent culture conditions, or when primed hPSCs are adapted to such naïve conditions, the X-chromosome state of resulting hPSCs resembles that of the preimplantation blastocyst. (vii) Similar to normal development, differentiation of naïve hPSCs results in *XIST*-mediated XCI. The asterisk (*) denotes the state found in the majority of cells. hESC, human embryonic stem cell; hPSCs, human pluripotent stem cells; Xa, active X chromosome; XCI, X-chromosome inactivation; Xe, eroded X chromosome; Xi, inactive X chromosome; *XIST,* X-inactive specific transcript.

all genes, on the Xi goes away, hence the overall inactive state of the Xi erodes, a phenomenon coined 'Xi erosion' [77–80] (Figure 4). Importantly, erosion differs from escape of XCI as the genes undergoing erosion are initially silent on the Xi in early passage hPSCs and become reactivated upon extended passaging of these cells [80], whereas escape is not passage dependent and rather includes genes on the Xi in chromosome regions with reduced *Xist* occupancy [30,81]. The extent of erosion of the Xi (i.e., the number of genes affected by this process) varies between individual hPSC lines; however, *XIST* loss occurs in nearly all hPSC lines studied over time in culture and often leads to Xi erosion [78,80] (Figure 4). Currently, it remains to be tested whether loss of *XIST* expression causes Xi erosion, but the fact that no Xi erosion is observed while *XIST* is expressed in newly derived human iPSC lines [74] suggests such a causative relationship. While *XIST* may have a protective role in preventing Xi erosion, another X-linked lncRNA, *XACT*, has been implicated in driving Xi erosion in primed hPSCs [79]. While the eroded Xi does not interfere with hPSC growth or ability to differentiate, it does modulate these processes [80,82]. Moreover, when primed hPSCs with Xi erosion are differentiated, the reactivated genes on the Xi do not get resilenced, resulting in somatic cells that at least partially lack dosage compensation of X-linked genes [80] (see [83] and [84] for detailed review; Figure 4). Methods of repairing or preventing Xi erosion of female hPSCs are needed for their use in disease modeling [77] and regenerative medicine, particularly when considering X-linked diseases. For instance, iPSCs or iPSC-derived neurons from female patients with Lesch–Nyhan syndrome, a devastating disease affecting neurologic, cognitive, and behavioral functions [85], can be used to model the disease only in the presence of a faithfully silenced Xi.

This is because the disease phenotype is caused by a heterozygous mutation in the X-linked HPRT1 gene, leading to HPRT1 insufficiency in cells where the non-mutant HPRT1 resides on the Xi. When the region of the Xi harboring the HPRT1 gene undergoes erosion, it results in expression of the non-mutant HPRT1 gene product, over-riding HPRT1 insufficiency. Thus, the Lesch–Nyhan diseases phenotypes can no longer be faithfully recapitulated with cultured iPSCs or iPSC-derived neurons in the presence of Xi erosion [77]. In addition, in regenerative medicine such as cell replacement therapies, introducing cells with an Xi erosion into a patient may be treacherous because these cells lack proper dosage compensation of X-linked genes, a phenomenon observed in cancers [66].

The Role of *XIST* in Early Human Development

It is interesting to note that primed hESCs do not reflect the X-chromosome state of the human preimplantation embryos from which they are derived: all cells of a female human blastocyst, including those of the epiblast lineage, have two active X chromosomes and simultaneously express *XIST* [9,13] (Figure 4). The recent discovery of this non-silencing *XIST* in early human development has intrigued many researchers who study X-chromosome dosage compensation, including us. The two immediate questions regarding this unusual X-chromosome state are (i) what role, if any, does *XIST* have, and (ii) what is the molecular mechanism disabling *XIST* from silencing the X chromosome(s). These and many other questions cannot be addressed with *in vitro* studies of conventional (primed) hESCs since their X-chromosome state is different from the cells of the blastocyst from which they are derived, most plausibly due to suboptimal cell culture composition used (reviewed in more detail in [83]). However, recently devised cell culture conditions, which have been formulated to support cells in a naïve (preimplantation) pluripotent state, allow growth of hESCs that better resemble the pluripotent state of cells in the preimplantation blastocyst from which they are derived [86,87]. Most importantly, the X-chromosome state of these naïve hESCs recapitulates many aspects of the human blastocyst, where female cells have two active X chromosome and express *XIST* [20,21]. While most of the cells in a preimplantation female human blastocyst express *XIST* biallelically, this pattern is a minority in naïve hESCs, which exhibit mostly monoallelic *XIST* expression [21]. Hence, naïve hESCs resemble the blastocyst, but not perfectly, as there is still room for improvement in the naïve culture media formulation. The molecular mechanism behind the non-silencing *XIST* is currently not understood, but investigating *XIST*-interacting proteins and *XIST* RNA modifications, which have recently been demonstrated to be crucial for *Xist*'s silencing role in the mouse [55], warrant further investigation. Current naïve culture conditions will allow such studies since naïve hESCs exhibit non-silencing *XIST,* albeit mostly monoallelically [21].

In addition to recapitulating the X state of the preimplantation blastocyst, naïve hPSCs allow *XIST*-mediated induction of XCI upon differentiation [21]. When primed hPSCs with large regions of Xi erosion are adapted to naïve pluripotency and then differentiated, the erosion is, for the first time, reversed and replaced with XCI [21] (Figure 4). Hence, the transition to the naïve state resets the X-chromosome abnormalities of the primed pluripotent state. However, when primed hPSCs are adapted to naïve pluripotency, the memory of the starting Xi does not get lost in the naïve transition, since upon differentiation the starting Xi becomes silenced despite the presence of *de novo* XCI [21]. Therefore, although naïve hPSCs allow studies of *de novo* XCI in humans for the first time, they cannot be used to study choice of XCI since the process is non-random. The epigenetic memory of the starting Xi is unlikely due to DNA methylation, since the naïve state results in robust hypomethylation of DNA [21,88,89], but may be due to the presence of histone modifications. For instance, it is possible that trimethylation of histone 3 lysine 27 (H3K27me3), which was recently shown to regulate *Xist* imprinting in mice [90], marks the inactive or the active X chromosome through the transitions from primed to

naïve pluripotency and eventually differentiation. It is, however, not clear whether naïve hESCs directly derived from the blastocyst or somatic cells directly reprogrammed to the naïve state can undergo random XCI upon differentiation.

Since there is no imprinted XCI in early human development, it has been unclear how X-chromosome dosage is compensated prior to onset of random XCI. Single-cell RNA-sequencing of human preimplantation embryos demonstrates gradual and time-dependent reduction of X-linked gene expression from both X chromosomes in embryonic days 4–7 in development [13]. This gradual dampening of X-linked gene expression correlates with upregulation of *XIST* [13]. XCD has also been observed in naïve *XIST*-expressing hESCs, further suggesting a novel role of *XIST* in human naïve pluripotency [21]. Independent analysis of the sequencing data from the preimplantation blastocyst [13] and naïve hESC [21] studies has instead suggested the presence of XCI instead of XCD in human preimplantation development [19]. If *XIST* is truly initiating XCI in the human blastocyst, given the fact that it is expressed from both X chromosomes in most cells, there must be a critical time point at which point the cell decides to limit *XIST*'s silencing function to a single X chromosome, since silencing both X chromosomes is lethal due to phenotypic nullisomy of most X-chromosome genes [44,91]. Interestingly, blastocyst outgrowth studies demonstrated the presence of an *XIST*-negative transitionary state between the *XIST*-expressing blastocyst cells and the *XIST*-expressing XCI cells [80]. In the transition from XCD to XCI in hPSCs, an *XIST*-negative state is also observed [21]. These data suggest that X-chromosome dosage compensation via XCD does not lead to the initiation of XCI.

If *XIST* is responsible for XCD in naïve pluripotency, it might do so by mediating accumulation of some, but perhaps not all, chromatin modifications that are also responsible for XCI. For instance, H3K27me3 [92] accumulates on the *XIST*-coated active X chromosome in naïve hESCs [21], which might be responsible for dampening of X-linked gene expression. Another hypothesis is that expression of the lncRNA *XACT* may counteract some but not all functions of *XIST*, thereby achieving dampening instead of silencing. Indeed, it has recently been shown that *XACT* prevents accumulation of *Xist* when ectopically expressed on the mouse X chromosome [20], consistent with the idea that *XACT* can limit *XIST*'s activity in naïve hPSCs. Interestingly, in a fraction of cells of rabbit blastocysts, *Xist* gets expressed from both X chromosomes, initiating silencing of both X chromosomes before resolving to monoallelic XCI via unknown mechanisms [9]. It is possible that the human scenario derives from such a mode of initiation of XCI and that XACT has evolved in primates to alleviate the detrimental consequences of inactivating both X chromosomes for too long or in too many cells.

Regardless, naïve hPSCs, for the first time, allow detailed molecular studies of *XIST* and XCD, as well as the transition to XCI as cells exit pluripotency. Moreover, by studying these cells we can now gain insight into early human preimplantation development and understand how it compares to what we already know in the mouse model organism.

Concluding Remarks and Future Directions

The biology of *Xist* unites researchers from multiple disciplines, including but not limited to those studying sex-chromosome dosage regulation, epigenetics (lncRNAs and chromatin remodeling), cell fate changes (reprogramming), cancer biology, disease pathogenesis (X-linked disorders), as well as development. *Xist*'s ability to recruit such a diverse group of researchers has enabled rapid advancement in understanding how *Xist* functions at the molecular level. We now understand that *Xist* acts as a scaffold to bring proteins to their site of action, effectively increasing the concentration of these proteins in a localized manner. Thanks to its very

Outstanding Questions

Requirement of *Xist* in random XCI *in vivo* needs to be addressed with mouse models where deletion of *Xist* can be confirmed prior to induction of XCI.

Molecular mechanisms regulating XCD in human preimplantation embryos and naïve pluripotent stem cells are yet to be discovered.

What is preventing *XIST* from silencing X-linked gene expression in human preimplantation development and naïve pluripotent stem cells?

Our knowledge of *Xist* biology from mouse studies needs to be extended to human *XIST*: what are the protein-binding partners of human *XIST*? What are the steps in the initiation of *XCI*?

Role of *XIST* and *XACT* in human preimplantation development and naïve pluripotent stem cells needs to be examined with deletion studies.

Why do naïve human pluripotent stem cells undergo non-random XCI? What is the epigenetic memory on the X chromosomes in these cells?

An improved culture condition for naïve human pluripotent stem cells that allows higher rate of biallelic *XIST* cells and random XCI upon differentiation needs to be devised.

Are there other eutherians that exhibit XCD in preimplantation development similar to humans?

long-lived outcome and ability to be used in an allele-specific manner, *Xist*'s ability to silence genes can be mined for therapeutic purposes to balance gene expression in trisomic diseases such as Down syndrome [93]. Furthermore, increased understanding of *Xist* can help engineer variants of this lncRNA to silence smaller and specific regions of a chromosome, increasing its therapeutic potential to silence mutant genes in an allele-specific manner.

Xist's unique expression pattern in human preimplantation development (expression without silencing) is a great marker of human naïve pluripotency [21], which can be used to develop new and improved naïve culture conditions in the future. Lastly, from an evolutionary perspective, *Xist* is an interesting lncRNA to study since it carries out similar functions in mouse and human despite lack of sequence conservation, but also seems to have evolved extra functions in a context-dependent manner, which requires further investigation (see Outstanding Questions). Both mouse and human *Xist/XIST* serve as a wonderful model for expanding our knowledge on lncRNA function, while learning about development and dosage regulation.

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