X Inactivation and Escape: Epigenetic and Structural Features

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X inactivation represents a complex multi-layer epigenetic mechanism that profoundly modifies chromatin composition and structure of one X chromosome in females. The heterochromatic inactive X chromosome adopts a unique 3D bipartite structure and a location close to the nuclear periphery or the nucleolus. X-linked lncRNA loci and their transcripts play important roles in the recruitment of proteins that catalyze chromatin and DNA modifications for silencing, as well as in the control of chromatin condensation and location of the inactive X chromosome. A subset of genes escapes X inactivation, raising questions about mechanisms that preserve their expression despite being embedded within heterochromatin. Escape gene expression differs between males and females, which can lead to physiological sex differences. We review recent studies that emphasize challenges in understanding the role of lncRNAs in the control of epigenetic modifications, structural features and nuclear positioning of the inactive X chromosome. Second, we highlight new findings about the distribution of genes that escape X inactivation based on single cell studies, and discuss the roles of escape genes in eliciting sex differences in health and disease.

Keywords: X chromosome, X inactivation, 3D-structure, LncRNAs, escape gene, epigenetics, dosage

INTRODUCTION

Evolution of the mammalian sex chromosomes from a pair of autosomes resulted in the emergence of distinct heteromorphic chromosomes that govern sex determination (Graves, 2016). The Y chromosome contains few genes (~70) and is present only in males, while the X chromosome contains many genes (~900–1500) and is present in two copies in females and one copy in males. This contributes to gene dosage imbalance between X-linked and autosomal genes and between the sexes (Disteche, 2016). To relieve these imbalances two mechanisms of dosage compensation evolved: X upregulation of expressed genes in males and females, and X inactivation or silencing of one X chromosome in females (Deng et al., 2014).

Here we focus on X chromosome inactivation (XCI), a mechanism that results in silencing of a randomly chosen X chromosome in early female embryogenesis (Lyon, 1961). XCI is characterized by a cascade of molecular events beginning shortly after embryo implantation, and is faithfully maintained throughout somatic cells in an organism, providing a robust model to study epigenetic and structural changes associated with gene silencing (Galupa and Heard, 2018). This complex process starts with the cis-coating of the future inactive X chromosome (Xi) by the long-non-coding RNA (lncRNA) Xist (Borsani et al., 1991; Brockdorff et al., 1991; Brown et al., 1991). Layers of chromatin and DNA modifications catalyzed by proteins initially recruited by Xist RNA are then put in place over several days during early development for stable transcriptional...
silencing of each gene on the Xi (Froberg et al., 2013; Mira-Bontenbal and Gribnau, 2016). These modifications are associated with profound changes in the 3D structure and location of the Xi, both processes depending on X-linked lncRNA loci. The Xi adopts a bipartite structure consisting of two superdomains of chromatin condensation separated by the lncRNA locus Dzx4, and the Xi visits the nucleolus, a process facilitated by the lncRNA Firre (Zhang et al., 2007; Rao et al., 2014; Deng et al., 2015; Minajigi et al., 2015; Yang et al., 2015; Giorgetti et al., 2016; Fang et al., 2019).

Despite the multiple layers of gene repression that stabilize XCI, a subset of developmentally critical genes remains expressed, albeit at a lower level, from the Xi (Carrel and Willard, 2005; Berletch et al., 2011). These escape genes adopt chromatin signatures and structural features more akin to those found in regions of active transcription (Balaton and Brown, 2016). Such genes can have higher expression in females, leading to sex differences in normal physiology and in susceptibility to disease. Abnormal escape gene dosage contributes to a milieu of deleterious phenotypes including infertility, intellectual disability, immune diseases, and cancer (Disteche, 2016; Balaton et al., 2018).

This review focuses first on mechanisms that govern X chromosome structure and nuclear location in relation to XCI, with a specific emphasis on the role of X-linked lncRNAs in these processes. We next discuss mechanisms that allow a select subset of genes to escape silencing in the context of the repressed Xi environment and how single-cell RNA sequencing has been used to identify novel escape genes. Lastly, we review new data on the role of escape gene dosage in sex differences in health and disease.

**LONG NON-CODING RNAs CONTROL EPIGENETIC AND STRUCTURAL FEATURES OF THE INACTIVE X CHROMOSOME**

The importance of lncRNAs in controlling nuclear structure and gene expression has become increasingly clear (Engreitz et al., 2016). Here we consider three X-linked lncRNAs, Xist, Dzx4, and Firre, which have been implicated in various aspects of the onset and maintenance of XCI.

**Xist**

Recent reviews have considered the role of Xist in great detail (Mira-Bontenbal and Gribnau, 2016; Galupa and Heard, 2018). Thus, we will focus our discussion on issues related to structural changes on the Xi. One of those is a localized chromatin conformation change at the X inactivation center (XIC), which is essential for the correct initiation of XCI. The XIC harbors both the Xist locus and its antisense transcription unit Tsix, together with multiple other loci that regulate Xist (Froberg et al., 2013; Galupa and Heard, 2018). The Xist and Tsix promoters lie in separate but adjacent regions of local chromatin interactions called topologically associated domains (TADs) (Nora et al., 2012). Interestingly, swapping the Xist/Tsix transcriptional unit and placing their promoters in each other’s TADs leads to a switch in their expression dynamics, indicating the topological partitioning of the XIC is critical for proper initiation of XCI (van Bemmel et al., 2019).

Once XCI is initiated, chromosome-wide structural changes give rise to the condensed Barr body coated by Xist RNA (Figure 1). Imaging studies show the rapid formation of a nuclear compartment devoid of transcriptional machinery and euchromatic marks, in which X-linked genes, initially located at the periphery of the Xist RNA cloud, adopt a more internal position when silenced (Chaumeil et al., 2006; Clemson et al., 2006). Subsequent chromatin conformation analyses by Hi-C demonstrate that Xist is essential for the formation of the unique Xi bipartite structure further discussed below (see section “Dzx4”) (Minajigi et al., 2015; Giorgetti et al., 2016). Most of the Xi shows attenuation of local TADs and of large A/B compartments of active and inactive chromatin, normally evident on autosomes and the active X chromosome (Lieberman-Aiden et al., 2009; Dixon et al., 2012; Minajigi et al., 2015; Giorgetti et al., 2016). Xist RNA pull-down studies have identified two RNA-binding proteins implicated in liquid-to-solid phase transition, FUS and hnRNP2, suggesting the possibility that phase separation facilitates heterochromatin formation and Xi silencing (Calabrese et al., 2012; Patel et al., 2015; Ryan et al., 2018). Indeed, most proteins within the Xist interactome are predicted to be prone to phase separation. By high-resolution RNA-FISH about one hundred Xist foci can be identified on the Xi, these foci having a comparable shape, size, and morphology to other phase-separated condensates such as paraspeckles and stress granules (Cerase et al., 2019).

Xist RNA-mediated gene silencing is accomplished through recruitment of proteins that establish epigenetic and structural modifications on the Xi (Figure 1A; Chu et al., 2015; McHugh et al., 2015; Minajigi et al., 2015; Moindrot et al., 2015; Monfort et al., 2015). Originally, spreading of Xist RNA and its protein complexes was thought to be linear, but new studies have revealed complex interactions between epigenetic and genomic features, such as genomic distance from the Xist locus, gene density, and proximity to long interspersed nuclear elements (LINE) that act as waystations to enhance Xist RNA coating throughout the Xi 3D space (Sousa et al., 2019). Sequence analysis of Xist RNA reveals that a significant proportion of the primary RNA sequence is comprised of blocks of local tandem repeats (named A-F) with different functions in XCI. The utmost importance of the A-repeat in X silencing but not in Xist RNA coating was recognized early on, and has been further strengthened by demonstrating that it recruits the transcriptional repressor SPEN (Wutz et al., 2002; Zhao et al., 2008; Nesterova et al., 2019). The B-repeat together with a short part of the C-repeat are crucial for spreading of Xist RNA and for attracting the polycomb silencing complexes PRC1 and PRC2 (Pintacuda et al., 2017; Nesterova et al., 2019). Conversely, ablating PRC1 or PRC2 impairs Xist spreading, supporting interactive roles for this mega-protein-RNA complex (Colognori et al., 2019).

A new study has now clarified the precise order of appearance of histone modifications relative to X silencing (Zylicz et al., 2019). Importantly, loss of histone acetylation, in particular
FIGURE 1 | Long non-coding RNAs control epigenetic and structural characteristics of the Xi. (A) XCI begins with the expression of Xist located in the XIC (black). Xist RNA spreads (squiggly black line) along the X chromosome and recruits several protein complexes (see text). Major histone modifications take place, including histone deacetylation by HDAC3, followed by ubiquitination of H2AK119 mediated by the PRC1 complex, methylation of H3K27 mediated by the PRC2 complex, and finally incorporation of histone macroH2A. Gene A represents a gene that becomes inactivated (red) while gene B is an escape gene (green) that remains unchanged. This schematic focuses on histone modifications and does not show recruitment of other proteins or other repressive epigenetic modifications such as DNA methylation. Shown below the schematic are examples of female mouse nuclei showing an Xist cloud (green) after RNA-FISH, and enrichment of histone modifications (pink) by immunostaining of H2AK119Ubi, H3K27me3, and macroH2A. Nuclei are counterstained by Hoechst 33342. (B) The genomic location of Firre, Dxz4 and Xist is indicated on a schematic of the mouse X chromosome along a Hi-C contact map of the Xi in mouse Patski cells. Two superdomains of frequent contacts are separated by the Dxz4 region. The color scale shows normalized contact counts (adapted from a published figure (Bonora et al., 2018) in Nature Communications, under Springer Nature Publishing License: http://creativecommons.org/licenses/by/4.0/. (C) The Xi preferred locations are near the nucleolus or the nuclear periphery, as shown in examples of mouse fibroblast nuclei after RNA-FISH for Xist (green) to locate the Xi and immunostaining for nucleophosmin (red) to locate the nucleolus. Nuclei are counterstained by Hoechst 33342.
H3K27ac, is clearly one of the very first events following Xist RNA accumulation during XCI initiation (Figure 1A). Indeed, the histone deacetylase HDAC3 is pre-loaded at putative enhancers and is vital for efficient silencing of most genes on the Xi. Ubiquitination of histone H2K119 is then initiated by the nuclear matrix-PRC1 protein complex (hnRNPK-PCGF3/5-PRC1), signaling subsequent recruitment of other PRC1 complexes and of PRC2 (Figures 1A,B; Pintacuda et al., 2017). Tri-methylation of histone H3K27 is mediated by PRC2 complexes and of PRC2 (Figures 1A,B; PCGF3/5-PRC1), signaling subsequent recruitment of other putative enhancers and is vital for efficient silencing of most genes on the Xi. Ubiquitination of histone H2K119 is then initiated by the nuclear matrix-PRC1 protein complex (hnRNPK-PCGF3/5-PRC1), signaling subsequent recruitment of other PRC1 complexes and of PRC2 (Figures 1A,B; Pintacuda et al., 2017). Tri-methylation of histone H3K27 is mediated by PRC2 and appears slightly later, even after gene silencing (Zylicz et al., 2017). The anchoring of megabase size chromatin loops at Dxz4 causes the appearance of a line (or flame) emanating from Dxz4 in the contact map (Figure 1B). Whether contacts between Dxz4 and other X-linked loci rapidly fluctuate in individual cells remain to be determined. Surprisingly, deletion of Dxz4 causes only minor reactivation of X-linked genes and few changes in escape gene expression (Giorgetti et al., 2016; Bonora et al., 2018). A recent study reported no changes in gene expression at all, suggesting inconsistencies between cell lines (Froberg et al., 2018). Furthermore, mice with a deletion of Dxz4 on the Xi show no apparent phenotype (Andergassen et al., 2019). Thus, the bipartite structure of the Xi has no clear function in gene regulation of the Xi at this point. However, conservation of the locus and of the bipartite structure between human and mouse suggest preservation of function.

**Firre**

Firre is another X-linked lncRNA locus that influences the epigenetic features and 3D structure of the Xi. We and others have shown that Firre is transcribed only from the active X chromosome (Calabrese et al., 2012; Froberg et al., 2018; Andergassen et al., 2019; Fang et al., 2019). Multiple isoforms of the Firre transcripts including lncRNAs and circular RNAs have been reported (Izougu et al., 2018). Like Dxz4, the Firre region harbors many local repeats including the R0 repeat that recruits the chromatin organizers CTCF and YY1, as well as RAD21, a component of the cohesin ring complex (Yang et al., 2015; Hacisuleyman et al., 2016). The Firre locus recruits the nuclear matrix protein hnRNPU and interacts with many genomic regions, which might explain why depletion of Firre RNA causes widespread autosomal gene dysregulation (Hacisuleyman et al., 2014; Andergassen et al., 2019; Fang et al., 2019; Lewandowski et al., 2019). In regards to XCI, depletion of Firre RNA in differentiated fibroblasts does not disrupt Xist coating or gene silencing; however, a loss of H3K27me3 is observed on the Xi (Yang et al., 2015; Fang et al., 2019). In contrast, embryonic stem cells depleted of Firre prior to differentiation show no changes in H3K27me3 (Froberg et al., 2018). Together, these results support a role for Firre RNA in maintenance but not initiation of H3K27me3 enrichment on the Xi in differentiated cells. Firre RNA is also important for maintenance of Xi location within the nucleus. Indeed, depletion of Firre in differentiated cells causes a decrease in perinucleolar and nuclear periphery association of the Xi (Yang et al., 2015; Fang et al., 2019). The perinucleolar and periphery compartments are often associated
with heterochromatin and nucleolar location of the Xi has been proposed to be important for faithful replication of its epigenetic state (Figure 1C; Zhang et al., 2007; de Wit and van Steensel, 2009). Association of the Xi with the lamin B receptor (LBR) recruited by Xist RNA (Chen C.K. et al., 2016). However, both the active and inactive X chromosomes are preferentially located near the nuclear periphery, suggesting that factors unrelated to XCI may control positioning (Bischoff et al., 1993). Surprisingly, tethering of one or both XIC alleles to the nuclear lamina via a TetR-EGFP-LaminB1 fusion protein does not disrupt XCI initiation, which would imply that XIC pairing and a visit to the nucleolus are not essential for XCI initiation (Pollex and Heard, 2019).

Deletion of Firre on the Xi does not disrupt the bipartite organization of the Xi, but causes localized changes in contact distribution, which may reflect disruption of the long-range contacts between Firre and Ddx4 thought to secure Xi compaction (Darrow et al., 2016; Barutcu et al., 2018; Bonora et al., 2018; Froberg et al., 2018; Fang et al., 2019). Long-range contacts between loci may help isolate the Xi in a specific compartment or phase of the nucleus either near the nuclear periphery or the nucleolus. The lncRNA loci Xist, Firre, and Ddx4 could play a concerted role in condensation and isolation of the Xi in a specific phase, which would ensure differential regulation of the two X chromosomes. Few studies have directly tested such a hypothesis due to the challenges in experimental design (Heard et al., 2004).

DETECTION OF GENES THAT ESCAPE X INACTIVATION BY SINGLE-CELL ANALYSES

Despite being located in a constitutively repressed environment, a select subset of genes has evolved mechanisms to avoid silencing and thereby remain expressed from the Xi. A number of genes escape XCI in an individual-, tissue-, and cell type-specific manner, which can cause sex differences in gene expression (Berletch et al., 2015; Cotton et al., 2015; Tukiainen et al., 2017; Balaton et al., 2018). In mouse, 3–7% of X-linked genes escape transcriptional silencing, while that number increases to 20–30% in human (Berletch et al., 2015; Balaton and Brown, 2016). Comparisons between species show that a core set of genes escapes XCI in most cells and tissues in mammals, while other genes vary between cell types, tissues and species. New approaches by single-cell RNA sequencing (scRNA-seq) combined with SNP (single nucleotide polymorphism) analyses of allelic gene expression provides data on thousands of individual cells of varying types and thus promises a more complete picture of the variation in escape status between cell types (Figure 2A). Assigning escape status to a given gene formally requires finding biallelic reads in single cells or in a tissue with completely skewed XCI. A main advantage of single cell approaches is that tissues with random XCI can be analyzed, but allelic dropout can cause problems for genes with low expression (Reinius and Sandberg, 2015). A limited scRNA-seq analysis of about 1,000 cells representing two human cell types has uncovered genes (e.g., FH1 and ATP6AP2) with incomplete XCI in a subset of cells, and also confirmed heterogeneity of XCI for TIMP1 (Tukiainen et al., 2017). Another allelic scRNA-seq in human fibroblasts shows little overlap with other studies in terms of identified escape genes, highlighting the difficulty of building a consensus (Wainer Katsir and Linial, 2019). Variability in escape from XCI between individuals has been confirmed in a single-cell allelic expression analysis from five individuals (Garieri et al., 2018).

Developmental scRNA-seq studies have followed XCI progression during embryogenesis. A study of human preimplantation embryos reports that bi-allelic X-linked gene expression may persist until the blastocyst stage, together with dampening of both alleles (Petropoulos et al., 2016). However, re-analysis of these and additional data has provided a more detailed panorama of random XCI from human oocyte to blastocyst, demonstrating progressive establishment of mono-allelic X-linked gene expression and of X upregulation to maintain balance of expression throughout the genome (Moreira de Mello et al., 2017; Zhou et al., 2019). Unfortunately, very little information on escape genes is included in these studies, despite findings of extensive sex differences in overall gene expression. In mouse, XCI dynamics during embryonic stem cell differentiation show a gradual decrease in expression of escape genes, consistent with partial spreading of silencing (Chen G. et al., 2016).

STRUCTURAL AND EPIGENETIC FEATURES OF ESCAPE GENES

Expectedly, genes expressed from the Xi lack epigenetic signatures characteristic of inactivated genes and appear to be located away from repressive genomic elements. A recent study using allele-specific PRO-seq and predictive machine learning shows that primary determinants of escape from XCI include distance from Xist and density of LINE elements (Sousa et al., 2019). Interestingly, escape genes often cluster in domains, a common finding in human, while mouse escape genes are often isolated (Tsuchiya et al., 2004; Prothero et al., 2009; Berletch et al., 2011). In general, escape regions lack repressive histone marks such as H3K27me3, and are enriched in active histone marks such as acetylation, and in transcription elongation marks including RNA PolII S2P and H3K36me3 (Figure 1A; Distech and Berletch, 2015; Sousa et al., 2019). DNA hypomethylation of CpG islands is a reliable predictor of escape status, which has been successfully used to identify escape genes in an array of human tissues where allelic analyses are difficult due to few SNPs and/or absence of XCI skewing (Cotton et al., 2015; Duncan et al., 2018). Surprisingly, escape genes in brain and liver adopt specific DNA methylation signatures that include enrichment in non-CG hypermethylation (mCH) throughout their gene body, which may help maintain an open chromatin structure (Keown et al., 2017; Duncan et al., 2018).

Escape genes tend to reside toward the outside of the compacted inactivated interior of the Xi (Figure 2B; Chaumeil et al., 2006; Heard and Bickmore, 2007; Splinter et al., 2011; Deng et al., 2015; Bonora and Disteche, 2017). However, it remains to be determined exactly how the 3D structure of the Xi...
influences the propensity for escape from XCI. One factor may be repeat E of Xist, which is required for localization of ASH2L, a component of the histone methyltransferase that methylates H3K4 for increased expression (Yue et al., 2017). Other factors may be involved, for example, the lncRNAs often found near escape genes (Reinius et al., 2010). Escape genes often co-localize with clusters of CTCF binding and with TADs, suggesting local Xi compartmentalization (Figure 2C; Giorgetti et al., 2016; Bonora et al., 2018). However, deletion of Ddx4 and loss of the bipartite structure of the Xi causes little or no disruption in escape gene expression (Giorgetti et al., 2016; Bonora et al., 2018; Froberg et al., 2018).

It is increasingly clear that intrinsic genetic escape elements act in cis to facilitate expression from the Xi (Balaton et al., 2018). We and others have proposed that spreading of silencing into the escape region or vice versa spreading of gene activity into a silenced gene may be prevented by insulator elements such as CTCF or YY1, but these elements may not be sufficient and mechanisms may differ between escape genes (Filippova et al., 2005; Li and Carrel, 2008;
For example, having two X chromosomes increases the risk suggesting that sex bias in X-linked gene expression play a role. Regulation and an increase in the capacity to blunt the effects of having two X chromosomes leads to improved blood pressure mice of either gonadal phenotype (Davis et al., 2019). In addition, that XX mice with either ovaries or testes lived longer than XY hormones versus sex chromosome complement, which showed (FCG), a mouse model capable of differentiating the effects of sex. This was demonstrated by using the four core genotype two X chromosomes prolongs lifespan, independent of gonadal X-linked gene expression in normal healthy individuals. One et al., 2008). In fact, few traits have been linked to sex bias in role in phenotypic sex differences has not been clarified (Xu et al., 2007). Interestingly, a BAC insert at the silenced Hprt locus in mouse retains escape status in vivo, both through the onset of XCI and the life of the mouse (Peet et al., 2018). It will be interesting to identify the apparently conserved insulator elements involved in this process and to test their role in shaping regions of escape, for example by isolating them into separate chromatin loops or phases within the nucleus.

**ROLE OF X-LINKED GENES IN SEX DIFFERENCES AND IN DISEASE**

One main consequence of escape from XCI is differential gene expression between males and females (Mele et al., 2015). A recent study based on thousands of transcriptomes spanning 29 human tissues provides a detailed survey of sex-biased gene expression in humans and demonstrates that expression of escape genes is usually female-biased (Tukiainen et al., 2017). However, a subset of escape genes located in the pseudautosomal region shared between the X and Y chromosomes is male-biased, probably due to lower expression in females due to spreading of silencing on the Xi (Tukiainen et al., 2017). The biological implications of these sex bias remain largely unexplored. While it is clear that certain escape genes, e.g., Kdm6a, and its Y-paralog Uty are expressed in different parts of the mouse brain, their role in phenotypic sex differences has not been clarified (Xu et al., 2008). In fact, few traits have been linked to sex bias in X-linked gene expression in normal healthy individuals. One example is longevity, with recent evidence suggesting that having two X chromosomes prolongs lifespan, independent of gonadal sex. This was demonstrated by using the four core genotype (FCG), a mouse model capable of differentiating the effects of hormones versus sex chromosome complement, which showed that XX mice with either ovaries or testes lived longer than XY mice of either gonadal phenotype (Davis et al., 2019). In addition, having two X chromosomes leads to improved blood pressure regulation and an increase in the capacity to blunt the effects of brain injuries (Pessoa et al., 2015; McCullough et al., 2016).

In terms of disease susceptibility there is ample evidence suggesting that sex bias in X-linked gene expression play a role. For example, having two X chromosomes increases the risk of developing autoimmunity [reviewed in Syrett and Anguera (2019)]. This phenomenon may be a result of unusual XCI patterns in immune cells, which could leave certain X-linked genes involved in immune response susceptible to reactivation (Wang et al., 2016; Syrett et al., 2019). This is supported by studies demonstrating that the Toll-like receptor seven gene, which escapes XCI in human lymphocytes, causes systemic lupus erythematosus when overexpressed in mouse models (Deane et al., 2007; Souyris et al., 2018). The X chromosome harbors a high number of genes important in brain function, and dosage of some escape genes has been implicated in neurological phenotypes such as seizures and Autism Spectrum Disorder (Shoubridge et al., 2019). For example, loss of function mutations in the escape gene, IQSEC2, contribute to the manifestation of phenotypes that include moderate to severe intellectual disability (Shoubridge et al., 2019). Another example is KDM5C, an escape gene that encodes a histone demethylase and regulates neuronal development and function (Iwase et al., 2016; Scandaglia et al., 2017; Kim et al., 2018). Mutations in KDM5C cause intellectual disability in males and females, illustrating the dosage sensitivity of this gene (Brookes et al., 2015).

Variability in expression of escape genes may contribute to sex differences in predisposition to certain cancers (Arnold and Dieterle, 2018). Many types of cancers are sex-biased in nature and some of those skewed toward a male prevalence may be explained by mutations in X-linked escape genes called EXITS (Escape from X Inactivation Tumor Suppressor) (Table 1; Dunford et al., 2017). One example is bladder cancer, with incident rates ranging from three to five times higher in men than women (Edgren et al., 2012). Recent studies in FCG mice show that XX mice with bladder cancer survive at increased rates compared to XY mice regardless of gonadal sex, suggesting that X-linked gene dosage is an intrinsic determinant of survival (Kaneko and Li, 2018). Interestingly, the female-biased escape gene KDM6A is a strong tumor suppressor that acts through demethylation-dependent and -independent mechanisms to reduce bladder cancer cell proliferation (Kaneko and Li, 2018).

Disorders of sex chromosome number such as Turner syndrome (45,X) and Klinefelter syndrome (47,XXY) directly implicate escape genes in abnormal phenotypes, since such genes would be haplo-insufficient and overexpressed, respectively, in these conditions. One of the hallmark of Turner syndrome is

| Gene* | Escapes status† | Cancer type | Male to female ratio | References |
|-------|-----------------|-------------|----------------------|------------|
| KDM6A | Ubiquitous      | Bladder     | 3:5:1                | Kaneko and Li, 2018 |
|       |                 | Lymphoma    | 1.6:1:4:1            | Morton et al., 2006; Horesh and Horowitz, 2014 |
|       |                 | Glioblastoma| 2:1                  | Sun et al., 2015 |
|       |                 | T-cell acute lymphocytic leukemia | 3:1 | Goldberg et al., 2003; Van der Meulen et al., 2015 |
| KDM5C | Ubiquitous      | Clear cell kidney | 2:1 | Ricketts and Linehan, 2015 |
| ATRX  | Variable        | Glioblastoma| 2:1                  | Sun et al., 2015 |
| DDX3X | Ubiquitous      | Medulloblastoma| 2:1 | Sun et al., 2015; Valentin-Vega et al., 2016 |
|       |                 | T-cell acute lymphocytic leukemia | 3:1 | Goldberg et al., 2003; Valentin-Vega et al., 2016 |

*Gene list based on (Dunford et al., 2017).
†Escape status as reported in Patrat et al. (2009) and Cotton et al. (2013, 2015).
premature ovarian failure and infertility. The X chromosome is three-fold more enriched for genes expressed in female reproductive organs when compared to autosomes, indicating a role in female fertility (Liu, 2019). Defining the contribution of specific X-linked genes in abnormal Turner phenotypes is a work in progress. For example, the escape genes KDM6A and TIP1 are hypothesized to be involved in premature ovarian failure and aortic aneurism formation, respectively (Trolle et al., 2016; Viuff et al., 2019). Recent screens of X-linked copy number variation (CNV) in cohorts of healthy women and those with primary ovarian insufficiency (POI) show a high prevalence of deletions encompassing escape genes as well as IncRNAs (Yatsenko et al., 2019). Of course, escape genes would not be the only X-linked genes involved in Turner infertility, since all X-linked genes become reactivated in female primordial germ cells, a process mediated by PR-domain containing protein 14 (PRDM14) for removal of H3K27me3 on the Xi (Mallol et al., 2019). The infertility observed in Klinefelter individuals could potentially also result from abnormal escape gene dosage, but another large factor would be abnormal meiotic pairing of the sex chromosomes. Interestingly, any abnormal X chromosome numbers (XXY, XXX or X) cause a general disruption of DNA methylation patterns at autosomal genes, demonstrating widespread epigenetic effects of X aneuploidy (Trolle et al., 2016; Skakkebaek et al., 2018). Furthermore, the number of X and/or Y chromosomes influences spatial chromosome conformation, particularly of the active X chromosome, but the role of this structural change is not elucidated (Jowhar et al., 2018). Together, these findings implicate improper X-linked gene dosage as a causative factor in disease phenotypes ranging from brain function, cancer susceptibility, to impaired fertility.

**PERSPECTIVE**

While a great deal has been learned about the various IncRNAs and proteins that control structural and epigenetic features of the Xi and its silencing, their exact modes of action remain to be further studied. In terms of the 3D structure of the Xi it will be of great interest to define factors involved in nuclear compartmentalization and phase separation. Additional experiments are needed in order to link the specific 3D structure and nuclear location of the Xi with its distinct epigenetic landscape. Moreover, little is known about contacts between each X chromosome with the rest of the genome in female cells and tissues, and about such contacts with the heterochromatic Y chromosome in male cells and tissues. Tissue-specific differences in the arrangement of chromosomes within the nucleus are poorly understood, and few functional studies have examined the consequences of manipulating chromosomal location. The epigenetic controls of escape from XCI also warrant further functional studies. Unfortunately, current analyses toward identifying escape genes in specific cell types and tissues are limited due to the relatively small number of informative polymorphisms in human. However, single-cell analyses are progressing at a fast rate, with some methods allowing analyses in thousands of cells in tissues to establish maps of gene expression or accessibility in a whole organism, as shown for example in a recently published atlas of mouse tissues/cell types (Cusanovich et al., 2018). While there is compelling evidence of sex differences in susceptibility to disease, understanding the role of individual sex-linked genes will require careful manipulation of their dosage.

**AUTHOR CONTRIBUTIONS**

JB, HF, and CD outlined and wrote the review. All authors approved the final manuscript.

**FUNDING**

The authorship of this review was supported by grants GM131745 from the National Institutes of General Medicine and DK107979 from the National Institutes of Health Common Fund 4D Nucleome Program.

**ACKNOWLEDGMENTS**

We thank Gala Filippova, Xinxian Deng, and Di Kim Nguyen for critical reading of the manuscript and for providing constructive editorial comments. We also thank Wenxu Ma and Giancarlo Bonora for originally making the Hi-C contact maps and 3D model for the figures (see references in figure legends).

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Fang et al. X Inactivation and Escape
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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