Title
Mutational Analysis of Xist and the X-inactivation Center

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Publication Date
2019

Peer reviewed|Thesis/dissertation

Mutational Analysis of Xist and the X-inactivation Center

by
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DISSERTATION
Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Genetics

in the

GRADUATE DIVISION
of the
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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Dedicated to my parents

Acknowledgements

First, I must thank my advisor, Dr. Barbara Panning for supporting my career goals and for her patience with me as I slowly grew into the scientist I am today. To my thesis committee: Kaveh Ashrafi and Dena Dubal, thank you for scientific insight and advice. Thank you to Wendy Ingram, for taking a chance on me when I was an undergraduate at UC Berkeley. I wouldn't be here without her early mentorship.

Thank you to all the members of the Panning lab, past and present, for being great friends and mentors to me. In particular, thanks to Joel Hrit, who believed in me and trusted me to perform the bioinformatic analysis for his paper, an experience that sparked my passion for genomic analysis and changed my career trajectory.

Thank you to the friends who felt like family to me. Thank you for the laughter, the venting over beers, the boardgame nights, the boxing and spin workouts, the dinner parties, the weekend getaways, the concerts, the phone calls, and the words of affirmation. Thank you for always helping me pick up the pieces when things fell apart.

Thank you to my grandparents and aunts and uncles, who sent many care packages, and often reminded me of how proud they were of me.

Thank you to Jeff Hussmann - my biggest cheerleader, my best friend, the life of any party, my programming coach, my go-to concert buddy, my outdoorsy activity partner, and the funniest and smartest man I know.

Thanks to my mother - for unknowingly sparking my interest in genetics, and to my father - for valuing my education above all, even if that meant great sacrifice. I promise to use my academic training, scientific thinking, and acquired grit to fuel a career where I make a positive impact on human health.

Chapter four of this thesis was adapted from a review originally published in Seminars in Cell & Developmental Biology (Goodrich et al. 2016) and was written with contribution from Karen Leung and Barbara Panning.

“We are a way for the universe to know itself.”

- Carl Sagan

Mutational Analysis of *Xist* and the X-inactivation Center

By Leeanne Marie Goodrich

Abstract

In placental mammals, dosage compensation of the sex chromosomes is achieved through inactivation of one X chromosome in female cells. This X chromosome inactivation (XCI) requires tight developmental regulation to ensure all but one X chromosome is silenced.

At the center of this process is *Xist*, a long non-coding RNA. Upon differentiation of a female cell, *Xist* spreads in cis to coat and silence the inactive X chromosome. While upregulation of *Xist*, has been shown to be sufficient for X inactivation to occur, no one has thoroughly investigated whether *Xist* is necessary for the establishment of X chromosome inactivation. In this thesis I provide evidence that *Xist* is not required for dosage compensation of the X chromosome during epiblast-like cell differentiation. This result suggests *Xist*-independent silencing mechanisms for this essential process may be in place.

A 1-2 Mb region of the X chromosome, termed the X-inactivation center (*Xic*) is necessary in two copies for XCI to occur, indicating it is necessary for cells to count the number of X chromosomes present. I delete one copy of the putative 2 Mb *Xic* in male and female mouse embryonic stem cells and present evidence that this deletion is not well tolerated, suggesting that this region requires finer resolution mapping to identify the minimal element required for counting.

Finally, I finish with a review which elaborates on studies that enlighten our understanding of activators and repressors that control XCI. Our findings challenge existing dogmas in the field and provide the foundation for future work focused on uncovering the molecular mechanisms behind *Xist*-independent silencing of the X chromosome.

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Chapter 1

Introduction

Sex chromosomes require dosage compensation

In diploid organisms, one set of chromosomes is inherited from the mother and one set is inherited from the father. This results in a matched set of chromosomes, with the exception of the sex chromosomes, which does not form a matched pair. The sex chromosomes genetically determine the sex of the organism, but they typically differ in their size and content between the sexes.

Various species of animals solve this problem of dosage compensation between the sexes in different ways. For example, *Caenorhabditis elegans* (worms) reduce the gene expression of the XX hermaphrodites on each X chromosome by half (Meyer et al. 2005). *Drosophila melanogaster* (fruit flies) on the other hand, upregulates the gene expression of sole X in males by two-fold (Salz et al. 2011). Placental mammals, including mice and humans, take a different approach by undergoing X chromosome inactivation, where females randomly silence one of their two X chromosomes to give one active (Xa) and one inactive (Xi) chromosome (Lyon 1961). X chromosome inactivation is how female placental mammals balance the number of X-linked gene transcripts between the sexes.

X chromosome inactivation provides a fascinating paradigm by which to study epigenetic regulation - how are two identical chromosomes in the nucleus managed to be treated completely differently? What is the cascade of events that must happen in order for silencing to occur, chromosome wide? How does a female cell sense the number of X chromosomes it has? Questions like these have captivated researchers for over 50 years.

X chromosome inactivation - where and when?

In mice, X inactivation happens in two waves during early development. The first wave of X inactivation is imprinted X inactivation, where the paternal X chromosome (Xp) is always the one to be silenced. In the cells that will give rise to the primitive endoderm and the trophectoderm, imprinted X inactivation of the Xp is maintained (West et al. 1977.). However, in the inner cell mass, which will form the embryo proper, the silent Xp is reactivated, and then these cells undergo a second wave of X inactivation, which is random (Lyon 1961) (Figure 1.1). This means either the maternal or the paternal X chromosome can be silenced. In a mouse, embryonic stem cells can be derived from the inner cell mass of the embryo. Because these mouse embryonic stem cells (mESCs) have not yet undergone X inactivation, female mESCs have two active X chromosomes and therefore provide a model system in which query X chromosome inactivation. X chromosome inactivation is triggered by the destabilization of naive identity and is therefore concomitant with differentiation (Martin et al. 1978). This means researchers can model X chromosome inactivation by differentiating mESCs *ex vivo*.

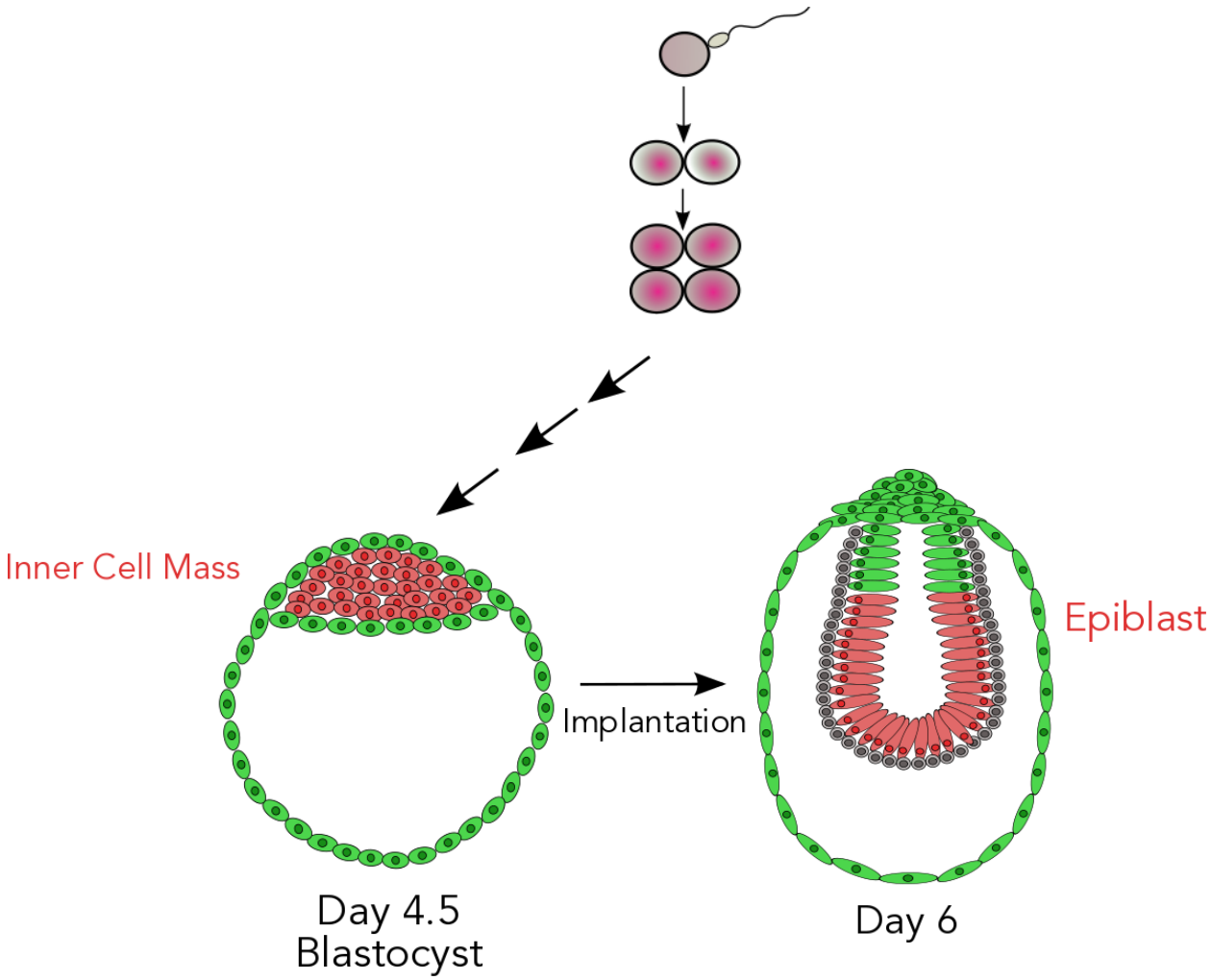

Figure 1.1. X inactivation occurs in early mammalian development. In vivo, random X inactivation occurs when cells in the inner cell mass differentiate into cells that form the epiblast. This occurs early in development, upon implantation of the blastocyst.

Xist is a key regulator of X inactivation

Translocations and truncations have revealed that a genetically defined region of the X chromosome needs to be present in two copies for cells to know that they are female (Brown et al. 1991). This region, called the X inactivation center (*Xic*), is estimated to be about 1-2 MB in length and includes a mix of non-coding RNAs, protein coding genes, and pseudogenes.

Within the *Xic* and at the heart of X inactivation is a ~15kb long non-coding RNA called X inactive specific transcript, or *Xist*. *Xist* is expressed on low levels from both active X chromosomes. When X inactivation gets triggered, *Xist* is massively upregulated on the X chromosome to be inactivated (Xi) and beings to coat the chromosome in *cis* (Brown et al. 1992).

Xist has been shown to be sufficient for silencing in *cis*. If an inducible *Xist* transgene is inserted on an autosome or the sole X in male mESCs, *Xist* will begin to coat and silence surrounding cis sequences (Wutz et al. 2000). While it has been widely assumed *Xist* must be necessary for silencing, a rigorous study testing this hypothesis has yet to be done.

Xist has an antisense transcript, *Tsix*, which is also a non-coding RNA. *Tsix* is expressed on both X chromosomes prior to differentiation, and upon X inactivation gets shut off (Lee et al. 1999). *Tsix* has been known to negatively regulate *Xist* (Monkhorst et al. 2008). Although *Xist* and *Tsix* are both non-coding RNAs, they are both spliced and polyadenylated.

Steps of X inactivation: counting, choice, establishment, and maintenance

The steps that are necessary for random X chromosome inactivation are thought to include: determination of the number of X chromosomes (counting), marking the X chromosomes for differential fates (choice), the initial silencing of the X chromosome designated for inactivation (establishment), and the stable propagation of the silent state to all progeny cells (maintenance) (Gendrel et al. 2014) (Figure 1.2).

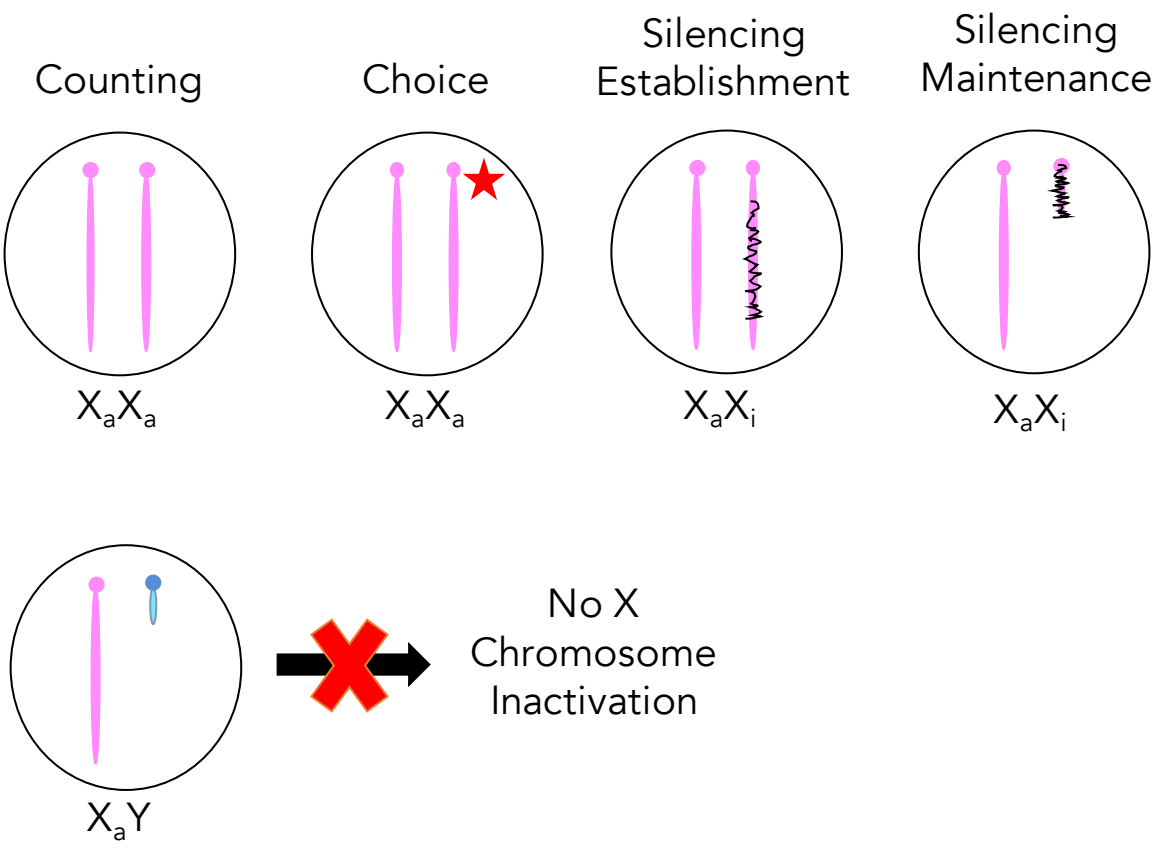

Figure 1.2. Steps of X inactivation. Female cells (top) need to dosage compensation their X chromosomes, and need to count their X chromosomes, randomly choose one X chromosome to be silenced, establishing X inactivation and maintain the inactive X for all subsequent cell divisions. Male cells (bottom) do not need to undergo X inactivation.

Due to a nondisjunction during meiosis, sperm or eggs may have no copies of the X chromosome, or more than one copy of the X chromosome. This can result in embryos with aneuploidy in the sex chromosomes (XO, XX, XXX, XXY). In the case of an XO karyotype, similar to males (XY), the cells do not undergo X inactivation, as doing so would result in silencing the sole X chromosome and subsequent cell death. In cases of more than one X chromosome (XX, XXX, XXY), cells silence all but one X chromosome upon differentiation (Monkhorst et al. 2008). This implies that there exists a mechanism to sense and count the number of X chromosomes a cell has.

Once a cell senses that two X chromosomes are present, one is randomly chosen and designated to be silenced. Heterozygous loss-of-function mutations in either *Xist* or *Tsix* cause non-random XCI, implicating these ncRNAs in choice. If one X chromosome lacks functional *Tsix*, that chromosome will always become the Xi (Lee et al. 1999). Conversely, if one X chromosome lacks functional *Xist*, that chromosome will always become the Xa (Marahrens et al. 1998).

In female cells, once the X chromosomes are counted, and one X chromosome is randomly chosen to be silenced, the initial establishment of X inactivation occurs. *Xist* is the first, and most dramatic marker of initiation as it is transcriptionally upregulated and begins to spread and coat the chromosome in cis. *Xist* recruits epigenetic modifiers such as PRC2, a H3K27 methyltransferase, which deposit silencing marks on the chromatin of the Xi (Plath et al. 2003).

Finally, once X inactivation has been established, the Xi needs to be maintained for all subsequent cell divisions. *Xist* has been shown to be dispensable for the maintenance of the inactive X, since deleting it in differentiated fibroblasts doesn't affect the ability of the cells to preserve silencing (Csankovszki et al. 1999). Presumably this is because once *Xist* has recruited stable marks, the machinery maintains itself.

While these steps are listed in a defined order, it has not been experimentally demonstrated which steps occur first. It is reasonable to speculate that some steps may occur concurrently. These steps provide a framework for better understanding and dissecting the molecular mechanisms behind the complex process of X inactivation.

Summary

The following study is an investigation into how female mouse stem cells might differentiate and dosage compensate in the absence of *Xist*, a key regulator of X chromosome inactivation. In chapter 2, I query X chromosome inactivation in female mESCs lacking functional *Xist*. I show that during differentiation of ESCs into epiblast-like cells, *Xist* knockout mESCs behave similarly to their wild type counterparts with regard to kinetics of X-linked gene silencing as observed by RNA-seq. I also show that of the genes that differ, they might be connected to sister chromatid cohesion, which ties into a previously proposed model for how X chromosomes might sense each other prior to the initiation of X inactivation.

While the identification of the *Xic* provides some insight into counting, this step remains poorly understood on a molecular level. It is not known what kind of information must be present in two copies to allow up regulation of *Xist* on one X chromosome. In chapter 3 I use genome editing to delete one copy of the *Xic* in female mESCs and the sole copy of the *Xic* in male cells. I show that the *Xic* can be deleted transiently in male and female mESCs, but for reasons that are not understood, genomic instability prevents this deletion from stably propagating.

I end with chapter 4, which is adapted from a review summarizing key thoughts to consider in XCI related to activators and repressors. Potential models and hypothesis for how this intriguing complex epigenetic phenomenon could be regulated are proposed.

Chapter Two

Investigating the establishment of X chromosome inactivation in the absence of *Xist*

Introduction

In eutherian mammals, females transcriptionally silence one of their two X chromosomes to equalize dosage between the sexes in a process known as X chromosome inactivation (XCI) (Lyon 1961). This process happens early in development, during implantation of the blastocyst, and is concomitant with differentiation (Avner et al 2001).

At the heart of this process lies a non-coding RNA, *Xist*, which becomes massively upregulated on the X chromosome to be silenced (Brown et al. 1992). *Xist* has been shown to be sufficient for transcriptionally silencing sequences *in cis*. Inducing *Xist* expression from a single transgene on an autosome leads to inactivation of autosomal genes *in cis* (Wutz et al. 2000). *Xist* has been shown to be dispensable for maintenance, as deleting it in differentiated fibroblasts leaves the inactive X unaffected (Csankovszki et al. 1999). *Xist* has been shown to be necessary for imprinted XCI in extra embryonic tissues, but few studies have specifically asked whether *Xist* is necessary for the establishment of random X chromosome inactivation that occurs in the embryo proper.

A previous study created a female mouse line with the first three exons of *Xist* flanked by loxP sites (Yang et al. 2016). Cre recombinase was transcriptionally regulated by a Sox2 promoter, as to bypass the requirement of *Xist* for imprinted XCI. This study demonstrated that female pups lacking *Xist* survive to term, and the *Xist* mutants manage to dampen the expression of X-linked genes. However, some caveats with this experiment include a potential leaky Sox2-Cre promoter, residual expression of

Xist in certain cell tissues, and possible survivorship bias of the cells that escaped perfectly timed Cre recombination. Another study created an *Xist* homozygous mESC cell line and showed *Xist* homozygous mutants are unable to silence a single gene, Huwe1, by RNA fluorescence in situ hybridization (FISH) upon differentiation *ex vivo* but did not investigate any other X-linked transcripts (Schulz et al. 2014).

In this work, we robustly investigate whether *Xist* is necessary during the initial establishment of XCI, using the differentiation of ESCs to Epiblast-like cells as an *ex vivo* model. We, for the first time, query the onset of XCI at single cell resolution in female homozygous *Xist* mutant ESCs. We find similarities in the transcriptome kinetics of X-linked gene expression during differentiation as observed by bulk and single-cell RNA-seq. We also observe some genes behave similarly between mutants and wild type by FISH. Together these results suggest that *Xist* is not necessary for initial dosage compensation during epiblast-like differentiation in mESCs.

Materials and methods

Cell culture

The mESC line LF2 and its derivatives were routinely passaged by standard methods in KO-DMEM, 10% FBS, 2 mM glutamine, 1X non-essential amino acids, 0.1 mM b-mercaptoethanol and recombinant leukemia inhibitory factor.

Generation of mutant mouse embryonic stem cell lines

mESC lines were derived using CRISPR-Cas9 genome editing. A guide RNA to the A repeat or Rex1 UTR region was cloned into the px459-Cas9-2A-Puro plasmid using published protocols (Ran et al., 2013). Templates for homology directed repair were amplified from Gene Blocks (IDT) (Tables S1 and S2). Plasmid and template were co-transfected into LF2 mESCs using FuGENE HD (Promega) according to manufacturer's protocol. After two days cells were selected with puromycin for 48 hours, then allowed to grow in antibiotic-free media. For Rex1-2A-GFP constructs, cells were monitored for GFP fluorescence (indicating homology directed repair) and fluorescent cells were isolated by FACS 1-2 weeks after transfection. For Neo or Hygro insertions, cells were monitored for antibiotic resistance and single cell clones were picked and expanded. All cell lines were propagated from single cells and correct insertion was confirmed by PCR genotyping (supp fig).

EpiLC Differentiation

To convert ESCs to EpiLCs, ESC lines were grown in 2i culture conditions (N2B27 medium consisting 50% DMEM/F12, 50% neurobasal media, 2 mM L-glutamine (GIBCO, #25030), 0.1 mM 2-mercaptoethanol (Sigma, #M7522), N2 supplement (Invitrogen #17502048), B27 supplement without Vitamin A (ThermoFisher #12587010), supplemented with 1 μM GSK3 inhibitor CHIR99021 (Axon Medchem #1386), 1 μM MEK inhibitor PD0325901 (Axon Medchem #2435), and 1000 U/ml LIF (made in house) in Geltrex (ThermoFisher A1413202) coated tissue culture dishes for 4 passages (Buecker et al., 2014; Hayashi et al., 2011).

To achieve EpiLC differentiation, cells were cultured in N2B27 medium supplemented with 10 ng/ml FGF2 (Peprotech 100-18B) and 20 ng/ml Activin A (Peprotech AF-120-14E) in Geltrex (ThermoFisher A1413202)-coated tissue culture dishes for either 3, 4, or 5 days.

FISH

Cells were harvested, resuspended to 500,000 cells/ml, and then cytospun onto glass slides at 800 rpm for 3 minutes. ells were then permeabilized through sequential treatment with ice-cold cytoskeletal extraction buffer (CSK:100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, and 10 mM PIPES buffer, pH 6. 8) for 30 sec, ice-cold CSK buffer containing 0.4% Triton X-100 (Fisher Scientific, #EP151) for 30 sec, followed twice with ice-cold CSK for 30 sec each. Finally, cells were then fixed in 4% paraformaldehyde for 10 minutes and stored at -20C in 70% ethanol until use.

The dsRNA FISH probes were made by randomly priming DNA templates using BioPrime DNA Labeling System (Invitrogen, #18094011). Probes were labeled with Fluorescein-12-dUTP or -UTP (Invitrogen) or Cy3-dCTP or –CTP (GE Healthcare). Probes were stored at -80C until use.

Slides were dehydrated through 5 min incubations in 80%, 90%, and 100% ethanol solutions and air-dried. 2ul of probe was hybridized overnight on the slides in a humid chamber at 37C. The samples were then washed 3X for 5 min at 39C with

2XSSC/50% formamide, 3X for 5 min SSC, and 3X for 5 min with 1X SSC. A 1:250,000 dilution of DAPI (Invitrogen, #D21490) was added to the second 1X SSC wash (final concentration 100ng/mL). Coverslips were then mounted on slides with Prolong Gold (Invitrogen P36930).

Immunofluorescence

For IF experiments, cells were cytospun onto glass slides at 800 rpm for 3 minutes and then fixed in 4% paraformaldehyde for 10 minutes. Fixed slides were washed with PBS + 0.01% Tween-20 (PBST) and stored in 70% ethanol at -20C until use.

Slides were washed twice in PBST for 5 minutes, then blocked in IF blocking buffer (PBS + 5% goat serum, 0.2% fish skin gelatin, 0.2% Tween-20) at room temperature for 1 hour. Primary antibodies were diluted in blocking buffer and incubated on slides at 4C overnight. Primary antibodies used were H3K27me3 (Active Motif #39055) and H3Lys4 (Millipore #07-030) at dilutions of 1:300 and 1:500 respectively. Slides were washed twice in PBST for 5 minutes, then incubated with secondary antibodies diluted in IF blocking buffer. Secondary antibodies used were Alexa488-conjugated goat anti-rabbit IgG (Jackson 711-545-152), Cy3-conjugated goat anti-rabbit IgG (Jackson 715-165-152), and Cy3-conjugated goat anti-mouse IgG (Jackson 715-165-150) at dilutions of 1:500. Slides were washed three times in PBST for 5 minutes with DAPI added to the second wash (final concentration 100ng/mL). Slides were then mounted using prolong gold antifade (Molecular Probes P36930) and imaged.

RNA-seq library prep

At each time point, cells were harvested with Accutase (ThermoFisher #A1110501), pelleted at 500 g x 5 mins, resuspended in 1 ml Trizol, and snap frozen until all samples were harvested.

RNA from all samples was extracted from mESCs using Zymo DirectZol RNA miniprep kit. RNA purity and quantification were assayed with Qubit dsDNA HS Assay Kit (ThermoFisher Q32854) and 500 ng of RNA per sample was used as input for library prep. Libraries were prepared using Lexogen SENSE Total RNA-seq Library Prep Kit.

RNA-seq processing and analysis

Libraries were sequenced on an Illumina Hiseq 4000 with single-end 50 base reads. Reads were aligned to the mouse genome (Ensembl build GRCm38.p6) and gene counts were created using STAR_2.5.3a. Normalization and differential expression analysis was performed with DESeq2v1.20.0. TPMs were generated with Kallisto v0.45.0 and transcript TPMs were collapsed into gene TPMs using a custom script.

Linear regression to determine slope of change in expression over time was performed with scikitlearn. For each sample, the slope was normalized to baseline expression of that gene at Day 0. This prevents genes that are simply more highly expression from exhibiting more variance and therefore artificially steeper slopes and allows us to compare slopes between genes of different levels of baseline expression.

All data was visualized using Matplotlib and seaborn.

10X library prep, sequencing and analysis

Stem cells (Day 0 mESCs, Day 2 EpiLCs, and Day 4 EpiLCs) were harvested with Accutase (ThermoFisher #A1110501) and resuspended at a concentration of 1200 cells /uL 1X PBS with ~ 0.05% FBS. Samples were multiplexed using MULTI-seq labelling and pooling, as described (McGinnis et al. 2019).

Libraries were prepared with chromium single cell 3' reagent kit (v2 chemistry) and were sequenced on an Illumina Hiseq 4000 with paired end 28 bp x 98 bp reads. Data analysis was performed with CellRanger and Scanpy.

Statistical tests

A non-parametric Kolmogorov-Smirnov (KS) test was performed to test whether CDFs of X-linked genes vs autosomes was significantly shifted downwards. In order to only consider genes that were expressed during the ESC to epiLC differentiation, a global cutoff of average TPM > 1 was applied to all samples.

Results

***Xist* homozygous mutant ESCs exhibit normal differentiation**

In order to ask whether *Xist* is necessary during the initial establishment of XCI, we first generated mESCs that lack functional *Xist*. The A repeat is a highly conserved

domain which lies within exon one of *Xist* that is necessary for proper splicing and function of the transcript (Royce-Tolland et al. 2010). We disrupted the A repeat in one or both copies by replacing it with an antibiotic resistance marker. First, we generated heterozygous *Xist* mutant mESCs by replacing one copy of the A repeat with a neomycin resistance cassette. Next, we generated homozygous *Xist* mutants by replacing the other copy of the A repeat with a hygromycin resistance cassette. In both cases, the antibiotic resistance marker was transcriptionally controlled by *Xist*'s antisense transcript, *Tsix*, and allowed for us to select for cells that maintained two X chromosomes (figure 2.1.a). We isolated independently derived clones of the heterozygous and homozygous *Xist* mutants to serve as biological replicates for this study. In addition, we introduced a 2A-GFP into the endogenous Rex1 locus into every line (figure 2.1.b). *Rex1* is a pluripotency transcription factor that is quickly downregulated when mESCs are differentiated into epiblast-like cells (epiLCs), which recapitulates the random XCI that occurs during the transition of the inner cell mass to epiblast.

We observed that the heterozygous and homozygous *Xist* mutant mESCs appeared morphologically similar to their wild type (WT) counterparts (figure 2.1.c). We queried whether the mutant cell lines maintained two X chromosomes by RNA FISH for a gene that escapes XCI, Kdm6a, and found that all three genotypes maintained biallelic expression (figure 2.1.d).

To determine the appropriate window in which to study the Xist homozygous mutant ESCs, we first compared the kinetics of Rex1-2A-GFP silencing and Xist up regulation in WT cells. We performed an epiLC differentiation time course and

measured the percentage of GFP positive cells. In parallel we performed an *Xist* RNA FISH time course, quantifying the percentage of cells that exhibited *Xist* clouds at each time point. We found an inverse relationship between *Xist* upregulation and Rex1-GFP downregulation, with the majority of cells having silenced Rex1-GFP and activated Xist by Day 4 (figure 2.1.e). We next examined whether heterozygous or homozygous mutation of Xist affected silencing of Rex1-GFP and found that the kinetics of silencing were comparable between all three genotypes (figure 2.1.f). *Xist* homozygous mutant mESCs exhibited no *Xist* upregulation by RNA FISH. Wild type, heterozygous *Xist* mutant, and homozygous *Xist* mutant mESCs appeared morphologically similar to each other at the beginning and end of the time course, with GFP images reflecting GFP percentages obtained by FACS (figure 2.1.g). These results suggest that mutation of Xist does not impair differentiation into epiLCs.

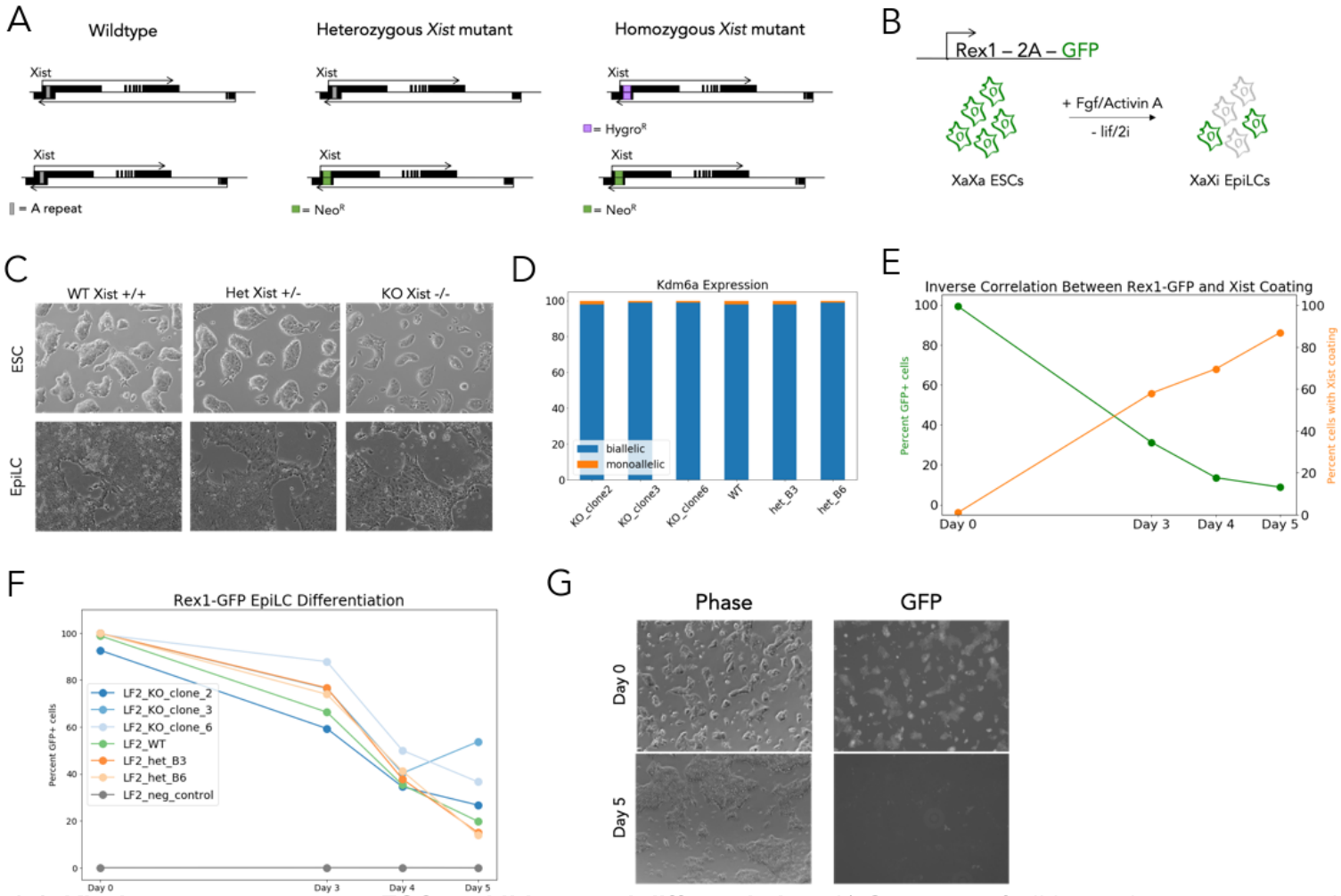

Figure 2.1. *Xist* homozygous mutant ESCs exhibit normal differentiation. A) Genotype of wild type, heterozygous and homozygous *Xist* mutants. B) Rex1-GFP as a reporter for epiLC differentiation. C) Morphology of ESC and epiLCs for all three genotypes. D) Kdm6a, an escapee of XCI, shows biallelic expression. E) Rex1-2A-GFP is inversely correlated with *Xist* coating. F) all cell lines exhibit reduction in GFP during differentiation. G) GFP is reduced as seen by microscopy during epiLC differentiation.

RNA-seq reveals large similarities between kinetics of X-linked gene expression

To determine the effects of Xist deletion on global gene expression during the ESC to epiLC transition, we performed RNA-seq on the three genotypes of mESCs (wild type, two clones of heterozygous *Xist* mutant mESCs, three clones of homozygous *Xist* mutant mESCs) at four time points of epiLC differentiation: Day 0 (undifferentiated), Day 3, Day 4, and Day 5(figure 2.2.a). Normalized expression of transcripts (TPMs) was calculated for each sample.

First, we queried a panel of epiblast specific differentiation genes, including Fgf5, and T and observed a sharp increase in expression between Day 0 and Day 5 in all genotypes (figure 2.2.b). We then queried a panel of pluripotency genes, including Nanog, and Sox2, and found a steep decrease in expression between Day 0 and Day 5 in all genotypes (figure 2.2.b). Xist expression increased sharply over time in the wildtype and heterozygous mutants, but not in the homozygous mutants, as expected (figure 2.2.c). These results provide additional data suggesting heterozygous or homozygous mutation of Xist does not dramatically impair differentiation into epiLCs.

To ask how the global gene expression of each genotype changed over the time course, we performed PCA analysis on the transcriptomes of all samples. This revealed that all genotypes follow similar trajectories through PC space between Day 0 and Day 4 (figure 2.2.d), suggesting that Xist mutants behave similarly to wild type cells during early differentiation. Between Day 4 and Day 5, two of the three *Xist* homozygous mutant clones appear to behave differently in PC space when compared to the wild type and heterozygous mutant trajectories.

Next, in order to query whether our epiLCs were dosage compensating, we looked at the fold change in expression of all X-linked genes. Cumulative distribution plots of fold changes (Day 0 TPM/Day 5 TPM) from RNA-seq revealed statistically significant rightward shift for X-linked genes relative to autosomal genes in all three genotypes (K-S test, $p < 0.05$) (figure 2.2.e, left column). Since all samples experienced the same global cutoff for expression (see methods), the number of genes queried in each CDF is the same, and therefore the p-value reflects the intensity of the shift, with the *Xist* homozygous mutant shift ($p = 1.5e-06$) slightly less than the wild type ($p = 2.3e-09$) or *Xist* heterozygous mutant shift ($p = 3.8e-13$). This shift was not observed when comparing autosomes, such as chromosome 11 which is of similar gene density to chromosome X, to the rest of the autosomes ($p = 0.19$) (figure 2.2.e, right column). Together these results suggest that *Xist* homozygous mutants are capable of dosage compensating genes on the X chromosome, albeit to a smaller degree than their wild type counterparts. This shift could be due to two possible reasons: 1) all X-linked genes are partially dampened in their expression or 2) some X-linked genes are silenced while others aren't.

To address the second possibility, we sought to capture and compare the kinetics of all ~800 appreciably expressed X-linked genes during the epiLC differentiation time course. We applied linear regression to TPMs of each gene over time, followed by normalization of the slopes to allow for comparison between genes (see methods). This regression revealed three archetypes of kinetics (figure 2.2.f). Some genes, such as Prps1, decreased in expression very sharply ($m < - 0.1$). Some genes, such as Hprt, decreased in expression slowly ($m < 0$), and some genes, such as

Huwe1, exhibited a noisy or slight increase in expression (m > 0). In order to holistically compare the kinetics of all X-linked genes between genotypes, we performed hierarchical clustering on the slopes (figure 2.2.g). Most clusters of X-linked genes that exhibited similar slopes held across all genotypes. However, this clustering revealed a subset of X-linked genes that exhibited differences in slope between *Xist* homozygous mutants and genotypes containing at least one functional copy of *Xist*. Performing GO term analysis on this subset of genes revealed a statistically significant (p value here) enrichment for genes involved in sister chromatid cohesion. This clustering also revealed, as expected, closest taxonomic relationships were between independently derived clones each genotype, reinforcing the robustness of this phenotype. Together this data suggests that some X-linked genes decrease in expression in the absence of *Xist*, while other X-linked genes do not.

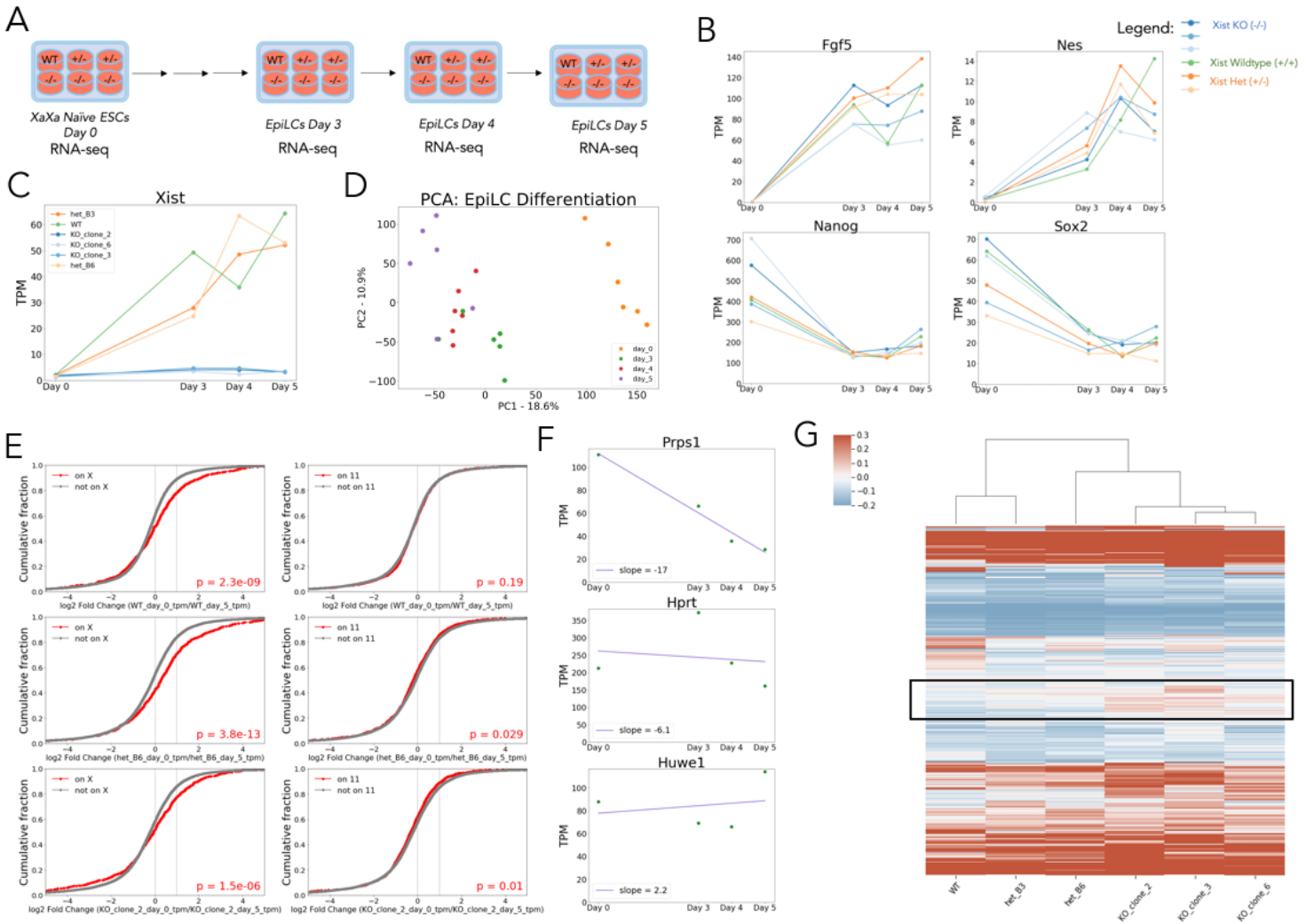

Figure 2.2. RNA-seq reveals large similarities between kinetics of X-linked gene expression. A) Schematic of RNA-seq time course. B) epiblast genes increase and pluripotency genes decrease during differentiation in all genotypes. C) *Xist* is upregulated in WT and hets but not in homozygous mutants. D) PCA shows similar trajectories. E) CDFs show dampening of X-linked genes in all genotypes. F) Three archetypes of X-linked kinetics are observed. G) Clustering reveals large similarities between genotypes, with some differences (black outline).

Single cell RNA-seq reveals similar differentiation trajectories between wild type and *Xist* homozygous mutant ESCs

RNA-seq on a bulk population of cells gives the average expression of a particular transcript. Since we are querying differentiation, a process known to be heterogeneous, bulk transcriptomic methods can blur out the signals of interest - in this case, the initiation of X chromosome inactivation. To obtain a higher resolution picture of how a population of cells moves through the differentiation, applying single cell RNA-seq allows us to identify cells that are actively differentiating and then restrict our analyses to just those subsets.

We performed single cell RNA-seq at multiple time points of epiLC differentiation using the 10X platform in conjunction with MULTI-seq to label and pool samples (McGinnis et al. 2019). Wildtype and *Xist* homozygous mutant cells (KO) were harvested at Day 0 (undifferentiated), Day 2, and Day 4 of epiLC differentiation for single cell RNA-seq. Transcriptomes for each cell were projected into low dimensional space using UMAP dimensionality reduction (figure 2.3.a). We qualitatively observed grouping of cells strongly correlated with each time point, and labelled these “pre-differentiation”, “mid-differentiation” and “late-differentiation” clusters (figure 2.3b). To confirm that these clusters reflected each time point, we applied an unbiased clustering algorithm (leiden) to look for similar groupings (figure 2.3c). Wildtype and *Xist* mutant genotypes showed similar occupancies between clusters (figure 2.3.d). We observed cluster and subclusters that strongly corresponded to the time point of the sample, for both genotypes. Defined clusters corresponding to pre-differentiation, mid-differentiation, and late-differentiation were consistent with upregulated or

downregulated expression of key differentiation and pluripotency markers (figure 2.3.e-f). Of the cells within a defined cluster, the distribution of expression of key markers (Rex1, Fgf5) of interest were qualitatively similar between genotypes (figure 2.3.e, 2.3.g). This suggests that wild type and *Xist* homozygous mutant cells have similar trajectories through epiLC differentiation, consistent with bulk RNA-seq and Rex1-GFP expression demonstrating that *Xist* homozygous mutant cells differentiate without apparent defect. Trends in the distribution of an X-linked gene's expression (Renbp) were also similar between wild type and *Xist* homozygous mutants (figure 2.3.f), suggesting that loss of *Xist* does not dramatically affect the behavior of X-linked genes.

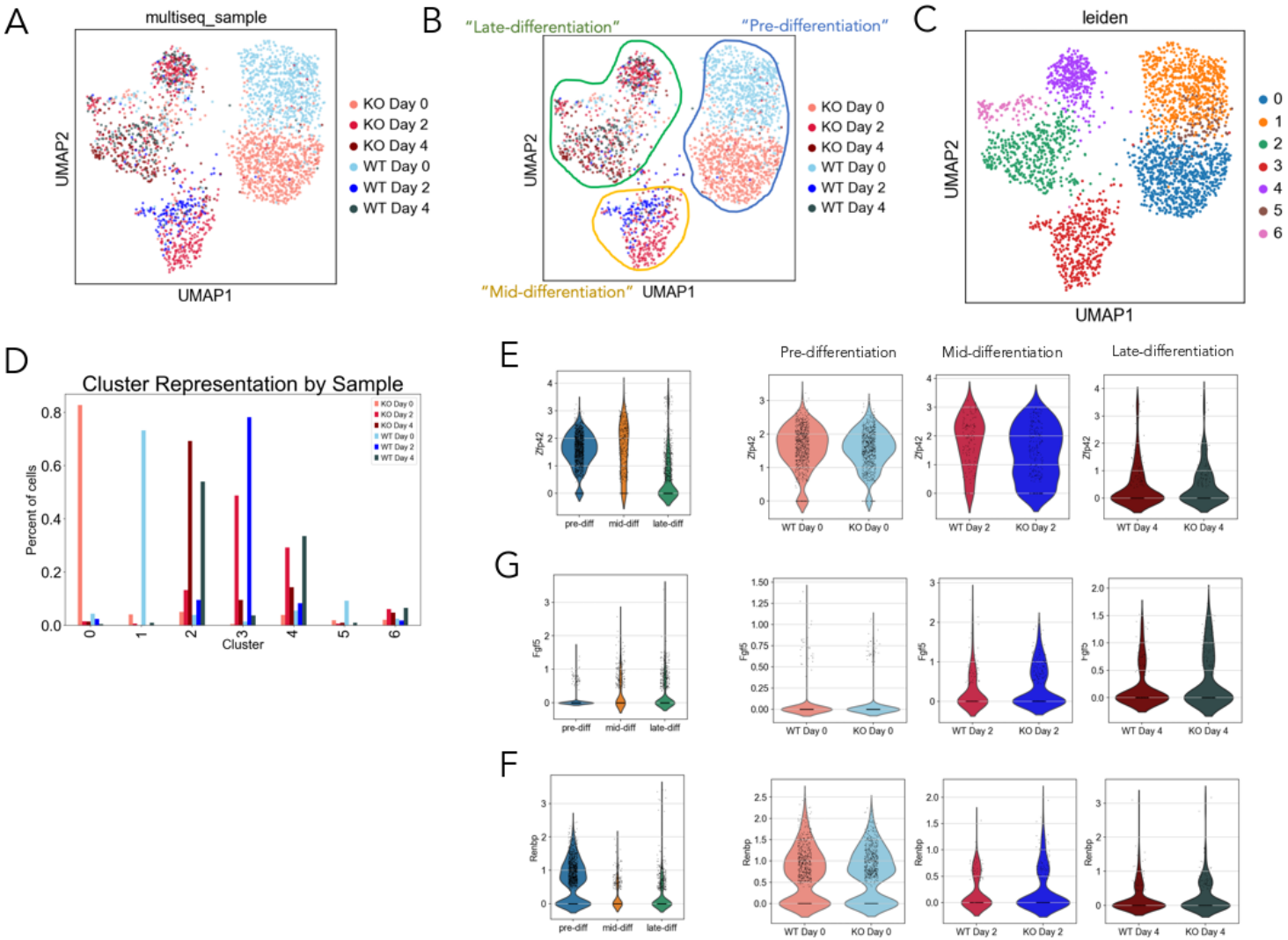

Figure 2.3. Single cell RNA-seq reveals similarities in differentiation trajectory. A) UMAP representation of all samples. B) Labeling of clusters based on time point. C) Unbiased identification of clusters using leiden algorithm is consistent. D) Both genotypes showed similar occupancy of clusters. E-F) Representation of distribution of expression for each of the three defined clusters (left) and how this distribution breaks down by genotype (right).

RNA FISH for a panel of X-linked genes shows variability in silencing between genotypes

Our bulk and single-cell RNA-seq data suggested that a significant number of X-linked genes exhibited similar differentiation-induced changes in expression in wild-type, heterozygous, and homozygous *Xist* mutants. These data suggest that down regulation of some X-linked genes occurs in the absence of Xist. To examine whether this down regulation occurs due to reduced expression on one or both Xs, we employed RNA FISH to detect nascent transcripts from X-linked genes.

We chose genes that were appreciably expressed during this developmental window, and that varied in their location along the X (fig 4a). In each RNA FISH performed, the X-linked gene of interests was paired with Kdm6a, an XCI escapee. Only cells with two pinpoints of expression for Kdm6a were considered. Percent monoallelic (one spot) vs percent biallelic (two spots) calculations for each X-linked gene revealed three archetypes (fig 4b). Some genes showed mostly biallelic expression in the *Xist* homozygous mutants, despite considerable monoallelic expression in the wild type and heterozygous mutants. Some genes showed a lower number of cells with monoallelic expression in the *Xist* homozygous mutants, when compared with the wild type and heterozygous counterparts. Some genes exhibited equal levels of monoallelic expression between all three genotypes. These results suggest there is heterogeneity in whether or not a particular X-linked gene can silence without *Xist*.

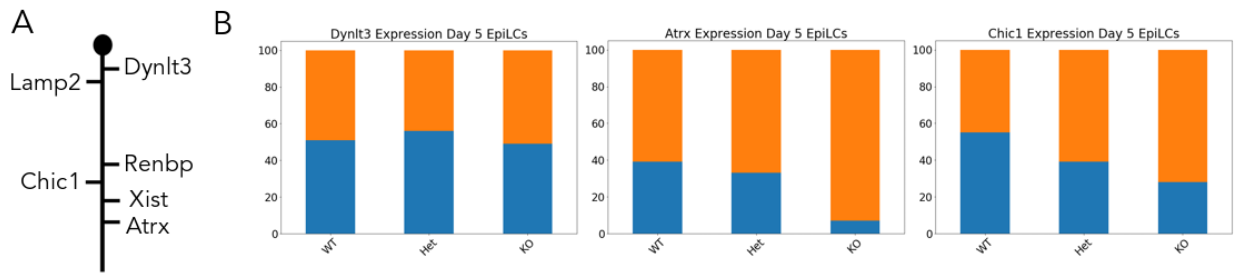

Figure 2.4. FISH for X-linked genes reveals three archetypes. A) Location of X-linked genes probed with RNA FISH. B) Some genes (left) showed silencing off one X in the mutants, some genes (center) failed to show monoallelic expression in *Xist* homozygous mutants, and some genes (right) showed a lower percentage of monoallelic expression in the *Xist* homozygous mutants.

Discussion

X chromosome inactivation is a process essential for life. While *Xist* is a key regulator, potential compensatory mechanisms for this crucial process may exist. To uncover potential *Xist* independent silencing mechanisms, it is important to identify whether *Xist* is necessary for the establishment of X chromosome inactivation in the first place. While work by Lee et al. suggest female mice lacking *Xist* can dosage compensate, the experiments performed do not investigate the initial establishment of X inactivation. Since it is difficult to time the delivery of Cre recombinase during the precise developmental window of X inactivation in the inner cell mass, we cannot say for sure that cells lacked *Xist* during the moment X inactivation was initially established. Additionally, by querying tissues from the pups that survived gestation, by definition, only cells that did not require *Xist* to survive are observed. qPCR for *Xist* in these tissues show varying levels of expression, which might suggest low levels of persistent *Xist* expression can rescue an otherwise inviable cell.

Homozygous *Xist* mutant ESCs show silencing of X-linked genes

The dynamics of what happens to X-linked gene expression at the moment when a female cell lacking *Xist* attempts to initiate silencing is not well studied or understood. In this study, we investigate the establishment of X chromosome inactivation in the absence of *Xist* using mESCs as a tractable *ex vivo* model. We induce X chromosome inactivation by differentiating mESCs lacking functional *Xist* into epiblast-like cells.

Two classes of X-linked genes?

Although some genes on the X chromosome appear to be silenced, some genes appear to be Xist-dependent in their silencing. Our data suggest that there are two classes of genes, ones that can be silenced without *Xist* and genes that require this non-coding RNA for robust silencing.

Our FISH results give a clear readout of monoallelic vs biallelic nascent expression in our mutants, and some genes exhibit monoallelic silencing better than others. This result is consistent with the work by Schulz et al. demonstrating that Huwe1 is not silenced in *Xist*-null mESCs. One possible explanation for our result is that some sections of the X chromosome are silenced, while other sections are dampened in expression, explaining the two classes of X-linked gene silencing observed. FISH for more loci along the X chromosome, along with allele-specific RNA-seq would help with this discrepancy. Additionally, performing these experiments in a cell line with a genetic background that allows for allele specific information on RNA transcripts would help distinguish, chromosome-wide, whether the *Xist* homozygous mutants are dampening their expression from both X chromosomes, or silencing one X chromosome.

Of the ~800 appreciably expressed X-linked genes, we observed variability in reduction of expression, even in wild type. All X-linked genes do not cleanly decrease their RNA transcripts by half. Since we are querying the steady state levels of RNA in the cell at each time point, transcripts that have not yet been degraded may also be captured. Performing NET-seq to only capture nascent RNA transcripts may better reflect changes in transcription during differentiation. However, *ex vivo* differentiation methods take place more gradually than X inactivation *in vivo* - on the order of days instead of hours. Since the typical half-life of RNA in mESCs is ~8 hours, performing NET-seq may not have an appreciable effect.

A reason to investigate 3D structure

Our results hint that genes involved in sister chromatid cohesion are dysregulated in our *Xist* homozygous mutants. Perhaps the Xist-dependent downregulation of cohesion proteins plays a role in the changes in 3D organization of the X chromosome that occurs during silencing. Performing Hi-C or 3C in WT cells and our mutant cells would be reveal any differences in 3D structure.

Our work joins the body of literature suggesting that *Xist*-independent mechanisms for silencing exist (Kalantry et al. 2009, Namekawa et al. 2010). Our study investigates, for the first time, the onset of X inactivation in mESCs lacking *Xist* at single cell transcriptomic resolution. *Xist* homozygous mutants appear to differentiate and silence some X-linked genes as well as wildtype. Perhaps other regulatory ncRNAs in the vicinity are compensating for lack of Xist to help silence some genes. Further work, such as a functional genomic screen for key regulators, will be necessary to uncover the molecular underpinnings of an *Xist*-independent dosage compensation mechanism.

Table 2.1. List of BACs used for FISH probes.

Gene	Probe ID
Kdm6a	RP23-56E7
Lamp2	RP24-173A8
Renbp	RP24-339B4
Dynlt3	RP23-406F22
Chic1	RP24-293G24
Prps1	RP24-255A11

Chapter three

Deletion and analysis of the X inactivation center in male and female mESCs

Introduction

In order to balance X-linked gene dosage between XX females and XY males, female mammals silence one X chromosome in each cell early during embryogenesis. In eutherian embryos, X-inactivation is random in that each X chromosome has an equal probability of being inactivated (Lyon et al. 1961).

Analysis of deletions and translocations involving the X chromosome indicates that a 1-2 Mb region, termed the X-inactivation center (*Xic*), is important for counting, choice, and establishment of XCI (Augui et al. 2011). The *Xic* is necessary in two copies for XCI to occur, indicating it is necessary for counting. In mice, this ~2 Mb region contains protein coding genes, pseudogenes, and regulatory noncoding RNAs, including *Xist* and *Tsix* (Morey et al. 2001).

Xist is up regulated exclusively in female cells. This up regulation requires 2 copies of the *Xic*, which includes *Xist*: when the *Xic* is missing one X chromosome, *Xist* is not up regulated the other wild type X. This observation raises the question of what is encoded in the *Xic* that allows female cells to be competent to up regulate *Xist*. To begin to understand what kind of regulatory elements lie within the *Xic*, the minimal *Xic* must be identified. Translocations and truncations in mice have identified a 1-2Mb region necessary for *Xic* function (Brown et al. 1991), but more focused deletion analysis has not been reported.

Previous studies have attempted to narrow down the *Xic* using transgenes encoding portions of the 1-2 Mb region, centered on *Xist*. One study inserted a 450 kb transgene, which included the sequences surrounding *Xist*, in multiple copies at various

autosomal sites in XY ESCs (Lee et al. 1996). In some integration sites these multi-copy transgenes triggered up regulation of *Xist* from the single X chromosome, albeit in a small proportion of cells. Similar results have been obtained with a multi-copy insertion of 35 kb transgene corresponding to 9 kb upstream and 6 kb downstream of *Xist* (Brockdorff et al. 1998).

While these results suggest that crucial cis-regulatory elements lie within the 450kb or the 35 kb regions, several lines of evidence suggest that this transgenic analysis has not identified the elements necessary for correct up regulation of *Xist*. First, deletion of the 35kb region from one X chromosome in female ESCs does not perturb *Xist* up regulation on the wild type. Thus, this region does not contain the information that is crucial for making cells competent to count X chromosomes and up regulate *Xist*. In addition, while a small number of differentiating multi-copy transgenic ESCs up regulate *Xist* from the endogenous X chromosome, none of these transgene studies report endogenous *Xist* up regulation with a single copy insertion. Together these results indicate that the crucial *Xic* elements reside outside of the region encompassed by the 35kb transgene and that inserting large multi-copy transgenes which encompass *Xist* may not reflect the actual biology behind counting endogenous X chromosomes.

We sought to pursue a more biologically informative approach to identify the minimal *Xic* sequences. We aimed to use CRISPR-Cas9 genome editing (Lin et al. 2014) to systematically delete regions of the 1-2 Mb *Xic* in female mESCs and then query for *Xist* up regulation on the wild type X chromosome.

Materials and methods

Cell culture

The mESC line LF2, E14, and its derivatives were routinely passaged by standard methods in KO-DMEM, 10% FBS, 2 mM glutamine, 1X non-essential amino acids, 0.1 mM b-mercaptoethanol and recombinant leukemia inhibitory factor. The mESC line 2-1 was routinely passaged in the same way, but on top of a layer of irradiated MEFs at a density of one vial (1E6 cells) per 10 cm^2, per passage.

Generation of mutant mouse embryonic stem cell lines

mESC lines were derived using CRISPR-Cas9 genome editing. A guide RNA to the Rlim or OGT's UTR region was cloned into the px459-Cas9-2A-Puro plasmid using published protocols (Ran et al., 2013). Templates for homology directed repair were amplified from Gene Blocks (IDT) (Tables S1 and S2). Plasmid and template were co-transfected into mESCs using FuGENE HD (Promega) according to manufacturer's protocol. After two days cells were selected with puromycin for 48 hours, then allowed to grow in antibiotic-free media. For fluorescent constructs, cells were monitored for fluorescence (indicating homology directed repair) and fluorescent cells were isolated by FACS 1-2 weeks after transfection. To transfect mESCs with Cre recombinase protein, Cre Recombinase Gesicles (Clontech #631449) were added to culture according to manufacturer's protocol. For blasticidin selection, cells were monitored for antibiotic resistance and single cell clones were picked and expanded. Subclones were generated by sorting for single cells with FACS on a BDAriaII machine. All cell lines

were propagated from single cells and correct insertion was confirmed by PCR genotyping.

FISH

Cells were harvested, resuspended to 500,000 cells/ml, and then cytospun onto glass slides at 800 rpm for 3 minutes. Cells were then permeabilized through sequential treatment with ice-cold cytoskeletal extraction buffer (CSK:100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, and 10 mM PIPES buffer, pH 6. 8) for 30 sec, ice-cold CSK buffer containing 0.4% Triton X-100 (Fisher Scientific, #EP151) for 30 sec, followed twice with ice-cold CSK for 30 sec each. Finally, cells were then fixed in 4% paraformaldehyde for 10 minutes and stored at -20C in 70% ethanol until use.

The dsRNA FISH probes were made by randomly priming DNA templates using BioPrime DNA Labeling System (Invitrogen, #18094011). Probes were labeled with Fluorescein-12-dUTP or -UTP (Invitrogen) or Cy3-dCTP or –CTP (GE Healthcare). Probes were stored at -80C until use.

Slides were dehydrated through 5 min incubations in 80%, 90%, and 100% ethanol solutions and air-dried. 2ul of probe was hybridized overnight on the slides in a humid chamber at 37C. The samples were then washed 3X for 5 min at 39C with 2XSSC/50% formamide, 3X for 5 min SSC, and 3X for 5 min with 1X SSC. A 1:250,000 dilution of DAPI (Invitrogen, #D21490) was added to the second 1X SSC wash (final concentration 100ng/mL). Coverslips were then mounted on slides with Prolong Gold (Invitrogen P36930).

Results

HDR mediated recombination rates are very low in female mESCs

Our starting female cell line contains one X chromosome from a 129 mouse background, and the other X chromosome from a Castaneus mouse background. As a result, there is a SNP approximately every 120 base pairs between these two X chromosomes. In order to specifically target Cas9 to the 129 allele that contains the existing loxP site, we designed our guide RNA sequences to include SNPs unique to the 129 X chromosome in the crucial protospacer adjacent motif (PAM) site of the guide RNA. While SNPs in the guide RNA sequence itself can be tolerated and still cleaved at a lower frequency by Cas9, mismatches in the PAM site are not recognized and therefore should not be cleaved (Barrangou et al 2007).

We first chose to target the centromeric end of the *Xic*, and designed guide RNAs for within Rlim, a protein coding gene that is the rough boundary of the centromeric end of the *Xic*. We first chose to use short single stranded donor oligonucleotides (ssODNs) as donor DNA for homology directed repair (HDR) of the Cas9-induced cleavage site. Previous CRISPR method studies had demonstrated short arms of homology were sufficient to serve as templates for HDR in HEK293T cells (Lin et al. 2014). Our ssODN was 200 bp and contained just a loxP site surrounded by homologous sequence to our cleavage site.

We electroporated the 2-1 female mESCs with Cas9, sgRNA, and ssODN. We grew up several 96 well plates of single cell clones from the electroporation, and then used PCR genotyping on the genomic DNA to determine which single cell clones had

our small loxP insertion. Assuming we achieved HDR in only one allele, a PCR product spanning the targeted region should show two bands: one band for the wild type allele, and a slightly larger band for the allele that had successful integration of the loxP. Unfortunately, after several electroporation attempts, we were unable to isolate a single cell clone with integration of our ssODN near our target site, Rlim.

Engineering a strategy to enrich for desired targeting events

We sought to redesign our targeting strategy to be improved in two ways: 1) using fluorescent reporters to enrich for successful HDR and integration of a loxP site and 2) an antibiotic resistance marker to select for cells that successfully excised the region between the two loxP sites (figure 3.1).

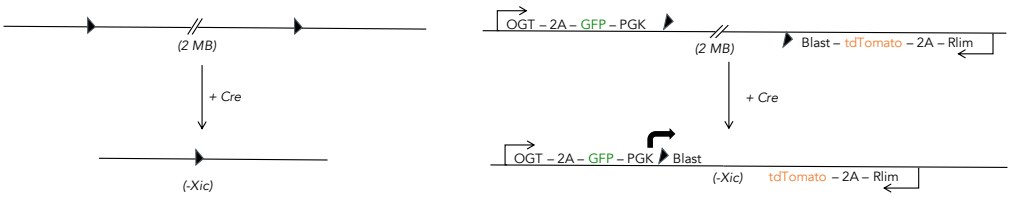

Figure 3.1. Improved strategy for deleting the *Xic*. Our original strategy (left) relied on screening clones by PCR to select for cells that had our desired loxP (black triangle) insertion. Our revised strategy (right) uses fluorescent reporters and antibiotic resistance to enrich and select for cells with the *Xic* deletion.

In order to enrich for cells that have our desired loxP site insertion, we chose to attach the loxP site to a fluorescent reporter. By pairing the loxP with a 2A-tdTomato or 2A-GFP construct, we could then sort for red or green cells. This strategy required us to target protein coding genes that are appreciably expressed in mESCs. We chose to target Rlim as a centromeric boundary and OGT as a telomeric boundary for the *Xic*, because both genes are expressed highly enough to visualize GFP or tdTomato. At this

time we also chose to use a different female mESC line, LF2, because 1) LF2s don't require feeder cells and 2) LF2s anecdotally maintain two X chromosomes better than the 2-1 cell line during culture.

Next, in order to select for cells that successfully recombined out the *Xic* between the two loxP sites, we chose to separate a promoter from the coding region of an antibiotic resistant marker, blasticidin. This way, only when the two distant loxP sites come together and recombine out the *Xic*, will the cell successfully transcribe blasticidin resistance. This engineering is necessary, because the further away two loxP sites are, the less efficient Cre-mediated recombination is. Previous studies have tested the limits of this system and shown that 75% of chromosome 11 could be deleted, albeit with very low efficiency (Zheng et al. 2000).

To generate this cell line, we first generated the OGT-2A-GFP-PGK-loxP cell line by sorting for GFP positive cells post transfection, followed by PCR genotyping and Sanger sequencing to confirm the introduction of our PGK promoter and loxP site. Next we targeted Rlim and inserted the 2A-tdTomato-Blast-loxP cassette, following similar methods.

***Cis* or *trans*? Deleting the sole Xic in male cells provides a critical control**

We successfully generated a female cell line with two loxP sites on the rough boundaries of the putative mouse *Xic*. However, we still did not know the orientation of the two loxP sites. In order to delete one copy of the *Xic*, we needed to have the loxP sites in cis, on the same X chromosome. If the loxP sites were in trans, then Cre-mediated recombination would result in two copies of the *Xic* on one X chromosome, with no copies on the other (Figure 3.2).

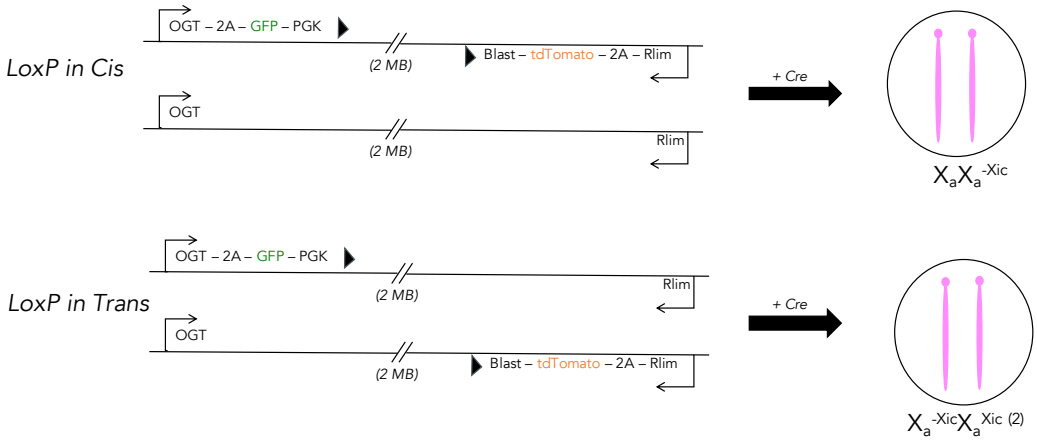

Figure 3.2. Targeting can result in loxP sites in either a cis or trans orientation. Since female cells have two copies of the X chromosome, it was important to consider the outcome of our targeting events. In order to successfully delete one copy of the Xic, loxP sites must be in cis on one X chromosome (top). If loxP sites are in trans (bottom), then we will be unable to excise the Xic upon the introduction of Cre recombinase.

As a control, we chose to generate a male mESC line that had the same insertions at OGT and Rlim to allow for Cre-mediated recombination. Since male cells only have on X chromosome, the orientation of the loxP sites are guaranteed to be in cis, allowing for us to test our Cre in a controlled system. We introduced Cre to our male mESCs with two loxP sites, and then selected with blasticidin to only allow cells that had successfully recombined out the *Xic* to survive.

To test for successful recombination, we designed a set of primers that would give a band only if the surrounding sequences of the two loxP sites were recombined to be close to each other. We ordered a synthesized block of DNA as a positive control for this PCR reaction. PCR genotyping demonstrated successful deletion of the *Xic* in male mESCs (Figure 3.3a). However, Sanger sequencing of this PCR product revealed a small (40 bp) deletion in the expected sequence (Figure 3.3b). This small deletion, with a slight variant in length and location, was observed in an independent Cre

recombination experiment. This deletion was not present in the plasmids used in the transfection, suggesting the cells themselves created this small deletion post transfection.

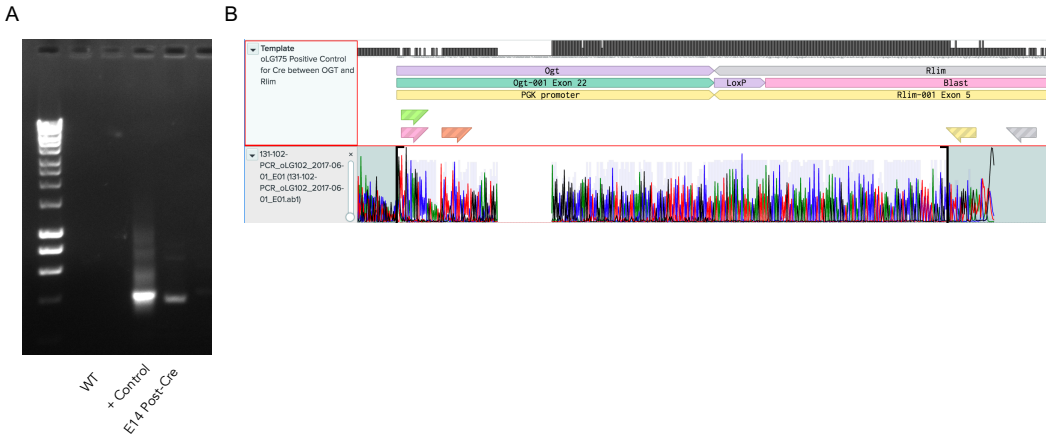

Figure 3.3. Evidence of Xic deletion in male cells. A) PCR genotyping confirms deletion of the sole Xic in male (E14) cells. Expected band size = 823 bp. B) Sanger sequencing of the PCR product shown in panel A reveals a small deletion in the PGK promoter which drives blasticidin resistance.

Deleting one copy of the *Xic* in female mESCs results in genomic instability

Next we took the female cells, which theoretically have a mixture of the two loxP sites in *cis* and in *trans*, and transfected in Cre followed by blasticidin selection for cells that had successful recombination. When we performed PCR genotyping to confirm the recombination, we got a mixture of positive and negative results on our single cell clones, suggesting some of the isolated clones did not have the deletion, despite blasticidin treatment (Figure 3.4a). In order to more robustly generate single cell clones, we then transfected with Cre, followed by single cell plating in a 96-well plate using FACS and very stringent gating. Once those single cells recovered, we then treated with blasticidin.

We continued to follow up on the clones that demonstrated an *Xic* deletion by PCR genotyping by sanger sequencing the PCR product. Again we observed the same small mutation in the PGK promoter, in a slightly different placement than the male cells, supporting the suggestion that this mutation is generated from selective pressure (Figure 3.4b)

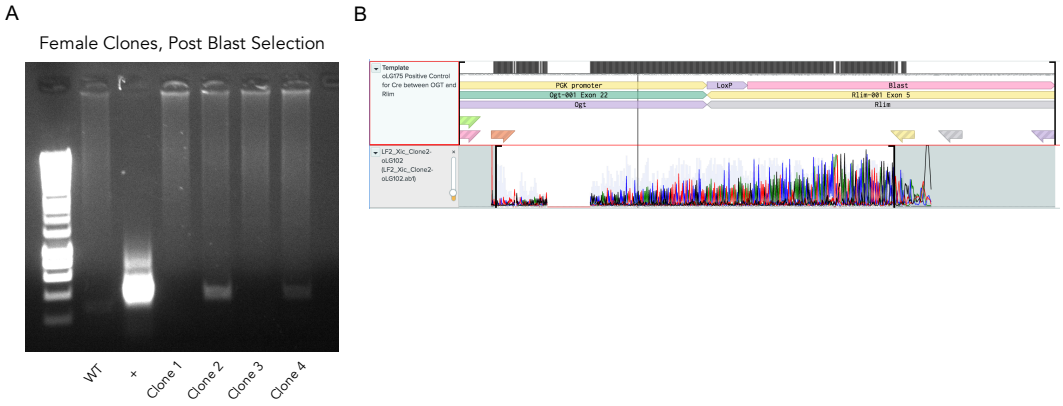

Figure 3.4. Evidence of Xic deletion in female cells. A) PCR genotyping confirms deletion of the *Xic* in female cells. Expected band size = 823 bp. B) Similar to male cells, sanger sequencing of the deletion PCR product reveals a small deletion in the PGK promoter which drives blasticidin resistance.

To test if our female clones were truly missing one copy of the *Xic*, we performed we performed fluorescent in situ hybridization (FISH) for nascent RNA transcripts for *Xist*. Instead of observing one pinpoint of nascent expression, the clones largely demonstrated two pinpoints of expression for Kdm6a, suggesting the cell line still contained two copies of the *Xic*. One subclone had one pinpoint of expression for Xist, but also had one pinpoint of expression for Atrx, a X-linked gene outside of the Xic, suggesting that subclone had lost an X chromosome in culturing (Figure 3.5).

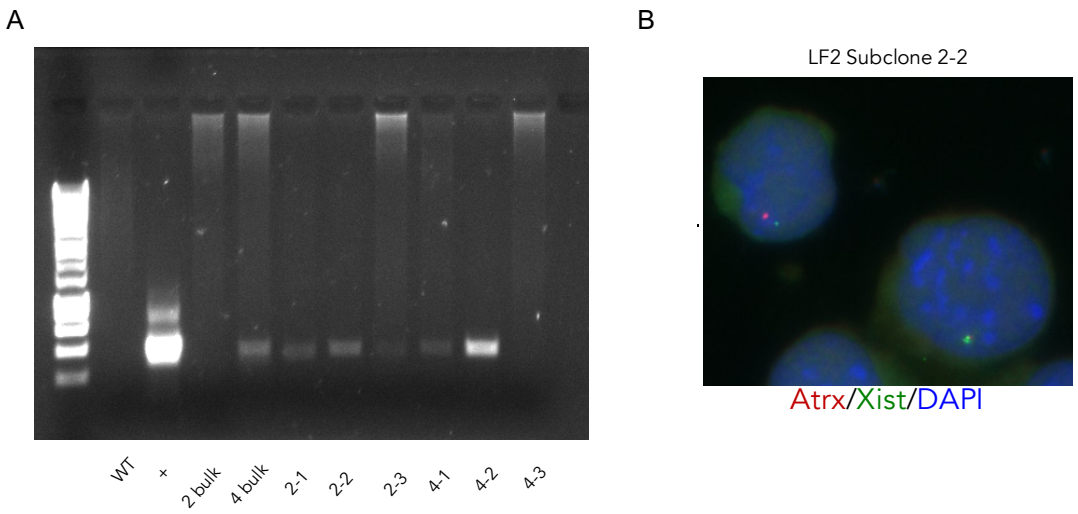

Figure 3.5. Female *Xic* delete subclones. A) PCR genotyping showing variability in Xic deletion band between subclonal populations. B) FISH showing one pinpoint of X-linked gene expression in subclone 2-2, indicating the cell line had lost an X chromosome.

To ensure that this was not due to heterogeneity in the population of wild type and *Xic* delete cells, we performed a second round of blasticidin selection and subcloned the population into 96-well plates by FACS. Reperforming PCR genotyping on the subclones revealed the loss of the PCR product that signified the *Xic* had been deleted. RNA FISH on these subclones also showed two pinpoints of *Xist* expression, suggested that the subclones were likely wild type cells not harboring the *Xic* deletion. Several rounds of reperforming the cre-recombination, followed by blasticidin resistance and subcloning gave the same result. We could transiently observe the deletion PCR product, and even sanger sequence it, but we were unable to get a pure, stable population of mESCs lacking the *Xic*.

Discussion

In summary, we deleted the X inactivation center, a genetic element shown to be necessary in two copies for female cells to count, in both male and female cells. Our engineering of the cell lines cleverly allowed for enrichment and selection of our genotype of interest, saving valuable time and reagents required for screening many plates of clones. However, we were unable to obtain a stable, pure population of female cells that lacked the *Xic*.

With additional time and resources, it is possible that a smaller deletion of the *Xic* could be better tolerated. However, the current design capitalizes on the use of promoters from expressed transcripts as a means of also expressing selectable fluorescent markers. The putative *Xic* has a limited number of transcripts that are expressed highly enough to be compatible with this strategy. One possible way to overcome this is to insert fluorescent markers with their own promoters, but this comes with the cost of random, off target integrations causing false positive fluorescent cells.

Another obstacle to overcome is the starting orientation of the loxP sites. In male cells, this is not an issue, as the loxP sites will always be in cis. In female cells, it becomes critical that cells have the loxP sites in the right orientation prior to transfection with Cre. To overcome this in the future, starting with a cell line that allows allele specific CRISPR targeting is necessary.

With these obstacles in mind, it is important to remember that these edits are performed in live cells that may then produce their own, unexpected, compensatory mutations. A clear example of this was the small deletion in the PGK promoter that was observed in independent experiments. This was unexpected and therefore difficult to

plan for, and necessitates the need to checkpoints along the way, perhaps through a combination of sanger sequencing and whole genome sequencing, to ensure the genome has not undergone unexpected rearrangements.

Ultimately, stable deletion of the *Xic* in female cells would allow for exciting follow up experiments. First, we would differentiate the mESCs in various ways to observe whether or not X chromosome inactivation is adversely affected. Epiblast-like cell differentiation, as described in chapter two, is a straightforward way to do this. Additionally, we could inject the *Xic* delete mESCs into mouse blastocysts and grow them out *ex vivo* to see if they undergo X inactivation during early development. Experiments to query X inactivation include RNA FISH for *Xist*, and immunofluorescence for markers of the inactive X such as H3K27me3 and macroH2A enrichment (Brinkman et al. 2006, Changolkar et al. 2006). If deleting the entire *Xic* had an adverse effect, then we would create successively smaller and smaller deletions to map the minimal element needed in two copies. Identification of minimal element necessary in two copies for *Xist* up regulation is a key first step in understanding the molecular framework that allow ESCs to count their X chromosomes. It would be a groundbreaking step in determining a mechanism for this poorly understood process.

Chapter Four

Activators and repressors: A balancing act for X-inactivation

Abstract

In early female embryos X-chromosome inactivation occurs concomitant with up regulation of the noncoding RNA, *Xist*, on the future inactive X-chromosome. Up regulation of *Xist* and coating of the future inactive X is sufficient to induce silencing. Therefore, unlocking the mechanisms of X-chromosome inactivation requires thorough understanding of the transcriptional regulators, both activators and repressors, which control *Xist*. Mouse pluripotent embryonic stem cells, which have two active X chromosomes, provide a tractable ex vivo model system for studying X-chromosome inactivation, since this process is triggered by differentiation signals in these cultured cells. Yet there are significant discrepancies found between ex vivo analyses in mouse embryonic stem cells and in vivo studies of early embryos. In this review we elaborate on potential models of how *Xist* is up regulated on a single X chromosome in female cells and how ex vivo and in vivo analyses enlighten our understanding of the activators and repressors that control this non-coding RNA gene.

Introduction

The central question in X chromosome inactivation (XCI) is: how do cells with two X chromosomes (Xs) silence only one of their Xs in the correct developmental fashion? In rodent extraembryonic tissues, only the paternally inherited X is silenced (West et al. 1977, West et al. 1978), a process termed imprinted XCI. In the embryo proper of rodents and of other placental mammals XCI is random, and the X inherited from either parent may be silenced (Lyon 1961). Because XCI is random in embryonic tissues,

there is an additional level of complexity-machinery that allows cells to randomly select one X for silencing is integrated into this system. In this review we will describe general models for how XX cells can achieve random silencing of one X, and discuss factors implicated in this process, focusing findings from the mouse system. X chromosome silencing can be divided into two stages (Avner et al. 2001). Establishment of silencing occurs first, when one X transitions from the active to the inactive state. Once silencing is established, the silenced X is stably maintained throughout all subsequent cell divisions. A key player in XCI is the X-linked gene *Xist*, which encodes a non-coding RNA that coats the inactive X (Xi) in cis (Brown et al. 1992). *Xist* is up regulated on the X that will be silenced concomitant with the initial establishment of silencing and continues to coat the Xi during the maintenance of silencing. In addition, *Xist* is sufficient to silence a cis-linked chromosome (X or autosome) during a brief developmental window (Wutz et al. 2000), consistent with a key role in establishment of silencing. Thus, the developmental cues that trigger silencing and the mechanisms that allow random choice of one X for silencing all converge on *Xist*. Identifying the factors that promote *Xist* up regulation on the future inactive X is pivotal to understanding the control of XCI.

Developmental control of XCI

Xist up regulation during random XCI is developmentally controlled (Mak et al. 2004, Okamoto et al. 2004) In the mouse model, activation of *Xist* expression occurs shortly after implantation (Kay et al. 1993, Sheardown et al. 1997). Prior to implantation, the inner cell mass (ICM) of the XX blastocyst, which will give rise to the embryo proper,

is composed of cells with two active Xs (Xas). Implantation, which begins at approximately embryonic day (E) 4.5, triggers a dramatic reorganization of the ICM. Within 24 h, by E5.5, the ICM cells convert to the epiblast, a change to a pseudostratified epithelium accompanied by *Xist* up regulation and establishment of silencing (Sheardown et al. 1997).

A host of dramatic changes occur during implantation (Cha et al. 2015). The exit from the glycoprotein shell of the zona pellucida and establishment of contact with the uterine membrane is characterized by changes in signaling molecules, extracellular matrix contacts, mechanical forces, and oxygen and nutrient availability (Cha et al. 2015). It remains to be determined whether *Xist* up regulation is a consequence of one or more of these changes. Understanding how developmental signaling pathways connect with the transcriptional regulators that control *Xist* is a key question in XCI.

While the peri-implantation developmental window is difficult to access in vivo, pluripotent stem cells isolated from the ICM provide a model system for the study of XCI ex vivo. Pluripotent stem cells resembling cells in the ICM, embryonic stem cells (ESCs), can be isolated and propagated ex vivo under appropriate signaling milieus (Martello et al. 2014). XX ESCs maintain two Xas, mirroring the cells of the ICM and when differentiated in culture *Xist* is up regulated on the prospective Xi. In vivo a complex combination of inputs such as extracellular matrix contacts and nutrient availability may intersect with growth factor signaling to direct *Xist* up regulation. Ex vivo analyses may not fully recapitulate the events that control *Xist* expression. Depending on the differentiation method used, *Xist* up regulation can take several days and is generally highly asynchronous in the ESC system. In contrast, the process appears to

be synchronous and complete within a 12–24 h window in vivo (Gayen et al. 2015). These differences suggest that critical players may be overlooked ex vivo. Factors that are not normally important in vivo may play a role under non-physiological ex vivo signaling conditions. These concerns highlight the importance of testing the effects of candidate regulator mutations in vivo as well as ex vivo. Therefore, whenever possible, we will compare and contrast the mutational analysis of potential *Xist* activators and repressors in culture and in embryos

Xist cis-regulatory elements

In other examples of developmentally controlled up regulation, crucial cis-regulatory elements exhibit a poised chromatin state before they receive the signal that triggers the developmental transition (Bernstein et al 2006, Buecker et al. 2012). This poised state is thought to facilitate rapid changes in gene expression in response to developmental signaling molecules. In vivo, *Xist* exhibits rapid up regulation in concert with a key developmental transition, suggesting that *Xist* cis-regulatory elements in ICM cells may be in a poised state.

Consistent with models for poised expression, *Xist* is transcribed at low levels from all Xas in XY or XX ESCs or ICM cells (Beard et al. 1995, Panning et al. 1996). This expression is two to three orders of magnitude lower than the level seen after *Xist* up regulation, when *Xist* RNA coats the Xi. Crucial to understanding *Xist* expression is identification of its cis regulatory elements. In principle a different set of elements may direct the low-level expression from Xas in ESCs/ICM, the rapid up regulation of *Xist* expression during establishment of silencing in differentiating cells, and the abundant

expression from the Xi during maintenance of silencing in differentiated cells. Chromatin capture methods suggest that *Xist* cis elements in ESCs may lie in an approximately 500 kb topologically associated domain (TAD) (Nora et al. 2012). It remains to be determined whether elements in this TAD play a role in low-level *Xist* expression in ESCs/ICM. In addition, this TAD may contain elements that poise *Xist* for up regulation upon differentiation. While poising is an attractive model for precise developmental control of *Xist*, other models may also explain the rapid and synchronous up regulation of *Xist* on the future Xi upon implantation.

Classes of models

The observation that individuals with supernumerary Xs silence all but one X, lead to the 'n-1 rule (Lyon 1971). This rule postulates that one X per diploid genome remains active, and all additional Xs are silenced (Goto et al. 1998). On the basis of the n-1 rule, models postulating that there is a robust system for ensuring only one X remains active have been proposed. Models vary on (i) whether the mechanism functions before or after *Xist* up regulation and (ii) whether there is an input from the number of Xs.

Models without input from the number of Xs

One class of model posits that all Xs are competent for *Xist* up regulation regardless of the number of Xs in the cell (Monkhorst et al. 2008) (Figure 4.1). In this class of model there are two ways that could ensure that *Xist* is expressed from only one X in XX cells and not at all in XY cells in adult organisms. First, because there is

considerable opportunity for selection against incorrectly dosage compensated cells throughout development, the cells that survive embryogenesis need not reflect the population of cells that initially up regulated *Xist* in the early post-implantation embryo (Takagi et al. 2002). Thus, it is possible that *Xist* up regulation is not restricted to one X in XX cells but may occur more haphazardly. Elimination of incorrectly dosage compensated cells in XX and XY embryos would result in XaXi and XaY adults. Alternatively, feedback mechanisms can be used to ensure only one allele is expressed, as is the case in most other systems of random monoallelic expression (Chess et al. 2012). In XCI a mechanism that detects incorrect dosage compensation in the appropriate developmental window could impact *Xist* expression to ensure production of XaXi or XaY individuals.

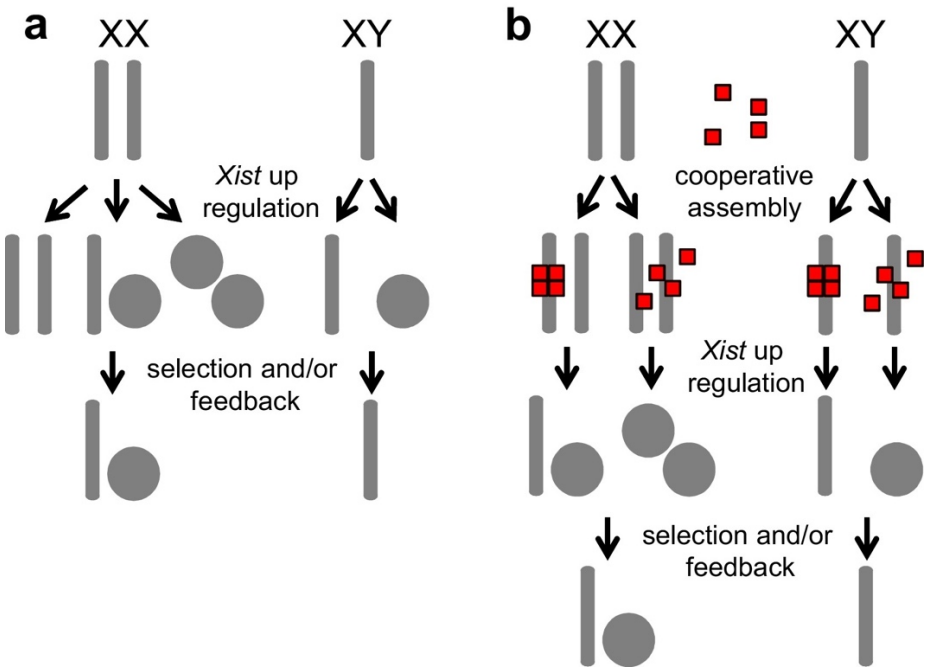

Figure 4.1. Models in which there is no input from the number of Xs. (a) All Xas (gray bars) in XX or XY ESCs/ICM cells (top row) have a small probability of Xist up regulation. As a result the epiblast initially consists of cells (middle row) in which neither, one, or both Xs are silenced in XX individuals and in which the X in XY individuals in active or inactive (Xi indicated by gray circle). Selection or feedback ensures that the final population of cells in the embryo (bottom row) consist of one Xa and one Xi in XX animals and one Xa in XY animals. (b) An autosomal gene product (red squares) is used to designate the Xa. Binding of this product is highly cooperative, such that it can assemble on only one X in XX or XY cells (middle row, left Xs). In some cells cooperative binding may not occur on any Xs within the appropriate time window (second row, right Xs). Xist is up regulated on the unbound Xs and silencing occurs (third row). As in (a), feedback or selection ensures that all somatic cells contain one Xa and one Xi in XX animals and one Xa in XY animals.

Consistent with models invoking selection or feedback, *Xist* up regulation was detected on the single X in a small proportion differentiating XY ESCs and on multiple Xs in diploid XX and tetraploid XXXX ESCs or tetraploid XXYY ESCs (Monkhorst et al. 2008). However, these patterns of *Xist* up regulation are not reported in wild type ESC differentiation experiments from other groups, in which *Xist* up regulation on the single X in XY cells or both Xs in XX cells are not observed with detectable frequency (Panning et al. 1997). It is not clear whether uniquely sensitive *Xist* RNA detection methods or

some aspect of the differentiation protocol explains the differing results. In addition, analysis of early post-implantation embryos did not reveal *Xist* up regulation on both Xs in diploid XX cells (Gayen et al. 2015). These discrepancies highlight the need for an ex vivo differentiation method that employs the same signaling pathways used in vivo.

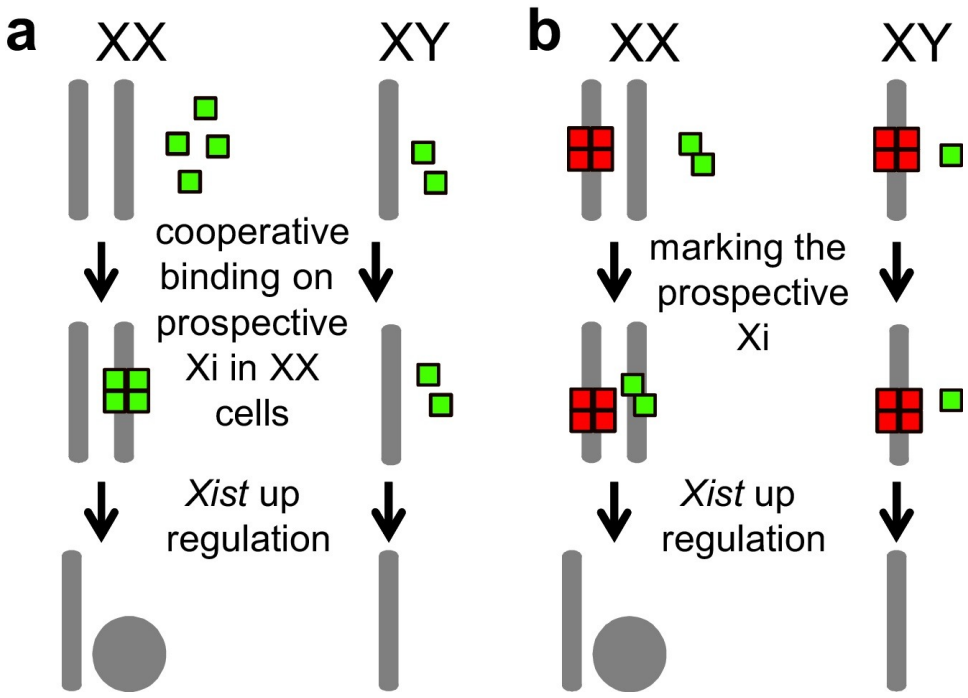

Figure 4.2. Models in which there is input from the number of Xs. (a) An X-linked gene product (green squares) is present at twice the abundance in XX ESCs/ICM than XY ESCs/ICM (top row). Binding of this product is dosage dependent and highly cooperative, such that it can assemble on only one X in XX cells (middle row). Only the X on which this product assembles is competent to up regulate Xist, ensuring only XX cells undergo silencing and that only one X is silenced (bottom row). Note that the dosage-dependent X-linked factor does not necessarily need to bind the X, but it does need to modulate a cooperative binding factor. (b) Two factor model, in which autosomal factor that marks the Xa (red squares), as outlined in Fig. 4.1b, preclude association of the X-linked competence factor (green squares). In both these models, feedback or selection, as in Fig. 4.1, could eliminate incorrectly dosage compensated cells.

A second class of models that does not have input from the number of Xs relies on an autosomally encoded repressor (often referred to as blocking factor) to block *Xist* up regulation on one X per diploid genome (Lyon et al. 1971) (Figure 4.1). In this class of model, *Xist* up regulation could be the default state on all Xs in which *Xist* is not marked for repression. In principle, cooperative assembly could ensure that an *Xist*

repressive complex assembles on only one X per diploid genome (Nicodemi et al. 2007). Such a mechanism could be combined with selection or feedback, as described above, to eliminate any cells in which *Xist* is up regulated on the inappropriate number of Xs.

Delineating the cis-elements necessary for *Xist* silencing on the future Xa would be an important first step in identifying the putative blocking factors. Furthermore, understanding how these factors are controlled by the developmental signaling inputs that lead to *Xist* up regulation on the Xi and repression on the Xa is also key. For example, determining when in development the putative repressive complex assembles on *Xist* would be important for gaining molecular insight.

In principle, an initial mark for *Xist* repression could be placed on one X at any point after fertilization. It could then be propagated, possibly by an independent mechanism, throughout subsequent developmental stages until the information is read out at ICM to epiblast transition. One prediction of a model in which marking occurs early in development would be that non-clonal XO ESC populations should exhibit *Xist* up regulation in a fraction of cells, as some proportion of the founding population should not have the repressive mark. XO ESCs do not exhibit *Xist* up regulation (Maherali et al. 2007), suggesting that if there is a mark placed earlier in development, it requires two Xs to be maintained or read out. In addition, induced pluripotent stem cells derived from mouse embryo fibroblasts have two Xas, and random XCI occurs when these cells are differentiated, indicating that fertilization and transition through cleavage stages is not necessary for selection of the Xa and Xi. Instead, these data suggest that if there is molecular machinery that marks the Xa, it is active in ESCs/ICM or it is poised for

activity in these cells. Developmental signals that direct or accompany the ICM to epiblast transition would then trigger *Xist* up regulation on the unmarked X (if the Xa is marked in the ICM), or would engage the marking machinery (if it is poised in the ICM) and direct *Xist* up regulation on the unmarked X (Figure 4.2).

Models with input from the number of Xs

In contrast to models in which there is no input from the number of Xs, there are several models in which the number of Xs impacts the decision to up regulate *Xist*.

One class of models in which the number of Xs is important in triggering *Xist* up regulation involves XX-specific nuclear organization of X-linked sequences. This class of models posits that the Xs take on special configurations before receipt of or in immediate response to cues that trigger *Xist* up regulation. Two types of configurations have been reported. First, each of the Xs in XX ESCs and ICM cells exhibits a different three-dimensional organization, one correlating with eventual silencing and one correlating with fate as the Xa (Mlynarczyk-Evans et al. 2006). It is possible that these two distributions of X-linked sequences represent transcriptional poising that can be read out in response to the developmental cues that trigger *Xist* up regulation. A second XX-specific organizational feature is the pairing of the Xs, which occurs when XX ESCs are differentiated (Bacher et al. 2006, Barakat et al. 2014). The transient interaction of the Xs is thought to promote *Xist* up regulation on one X, possibly by contributing to asymmetric distribution of factors necessary for *Xist* repression or up regulation. Heterozygous deletion of a region that is sufficient to confer pairing (as an autosomal transgene in male ESCs) (Bacher et al. 2006) did not adversely affect *Xist* up regulation

in XX ESCs (Barakat et al. 2014). In addition, heterokaryon experiments also indicate that this pairing may not be necessary for *Xist* up regulation. In ESC heterokaryons that contain one XY and one XX nucleus within a single cytoplasm, the X of the XY nucleus was just as likely to up regulate *Xist* as either of the Xs in the XX nucleus (Barakat et al. 2014). This result strongly suggests that a diffusible factor produced in ESCs with more than one X plays a role in triggering *Xist* up regulation.

The X contains approximately 1000 genes, and pre-XCI cells with more than one X have the potential to produce twice as much (or more) X-linked gene products as cells with a single X. As a result, pre-XCI cells with one or more than one X are fundamentally different in the ratio of X-linked to autosomal gene products. Other organisms with X dosage compensation systems use this difference to direct production of complexes with activity that depends on abundance of specific X-linked gene products (Cline et al. 1996). These complexes are key for engaging the dosage compensation pathway. In this way the number of Xs determines whether dosage compensation will occur. A similar system may be functioning in mammals. In this scenario, the activity of activators and repressors of *Xist* would be dependent on the X:autosome ratio.

A dosage sensitive X-linked gene product could ensure only cells with more than X are competent to up regulate *Xist* (often referred to as competence factor) (Gartler et al. 1983). In this scenario the appropriate developmental cue triggers *Xist* up regulation exclusively in XX cells and on the allele not marked for repression. In male cells the factors that promote *Xist* up-regulation are not produced (and the X could independently be marked for repression), thereby preventing silencing of the sole X. One major

question raised by this scenario is how is one allele of *Xist* marked for repression and the second allele in XX cells marked for activation? What molecules are involved and is cooperative binding and/or a unique feature of the organization of the X in XX ESCs/ICM used restricted to these factors to one of two otherwise identical Xs in XX cells.

A class of models to restrict *Xist* up regulation to only one X in XX cells postulates that silencing of the X results in a decrease in amounts or activity of a X-linked dosage sensitive *Xist* activator. The decreased abundance of this X-linked factor after silencing of one X precludes up regulation of *Xist* on the other X in XX cells. This class of model relies on rapid turnover of the X-linked *Xist* activator, since its levels must drop sufficiently rapidly to preclude *Xist* up regulation on the remaining X.

Rlim, also known as Rnf12, encodes an X-linked E3 ubiquitin ligase that has been proposed to be a dosage dependent activator of *Xist* expression in random XCI (Jonkers et al. 2009). Autosomal insertion of a Rlim/Rnf12 transgene results in *Xist* up regulation from the single X in XY ESCs and both Xs in XX ESCs. A mutation that eliminates ubiquitin ligase activity abolishes the ectopic *Xist* up regulation induced by the transgene, suggesting that enzymatically active RLIM/RNF12 is necessary to control *Xist*. These results are consistent with a model in which the abundance of RLIM/RNF12 in XX cells is sufficient to facilitate *Xist* up regulation, while that in cells with a single X is not, thus ensuring *Xist* is only activated in cells with more than one X. Since Rlim/Rnf12 is located approximately 500 kb away from *Xist*, Rlim/Rnf12 is silenced rapidly in response *Xist* up regulation on one X (Barakat et al. 2011, Kalantry et al. 2009), with the

resulting decrease in RLIM/RNF12 abundance potentially precluding up regulation on the second X.

While this model is attractive, there are likely additional complexities at play. XX ESCs lacking one copy of Rlim/Rnf12 exhibit *Xist* up regulation on one X upon differentiation, suggesting that the threshold of RLIM required for *Xist* activation is not strictly linked to Rlim/Rnf12 copy number (Jonkers et al. 2009). In XX ESCs lacking both copies of Rlim/Rnf12 no *Xist* up regulation is observed in one study (Barakat et al. 2011). However, independently derived Rlim/Rnf12 null XX ESCs exhibit activation of *Xist* from one X (Shin et al. 2014) and the same group showed RLIM/RNF12 is not appreciably expressed in the ICM of normal embryos and conditional Rlim/Rnf12 null XX embryos lacking RLIM/RNF12 in the ICM show normal XCI (Shin et al. 2014). These differences in the XCI phenotypes of Rlim/Rnf12 mutants in culture and in vivo suggest that additional factors intersect with RLIM/RNF12 in control of *Xist* expression. For example, varying genetic backgrounds or differentiation protocols may impact the signaling pathways and downstream effectors used to trigger *Xist* up regulation ex vivo. As a result the RLIM/RNF12 requirement may be unique to a particular differentiation method or a specific genetic background.

The conflicting results of Rlim/Rnf12 mutants in ESC and mouse studies suggest that recapitulating XCI with ESC's ex vivo does not necessarily reflect the mechanical forces, extracellular matrix contacts, and signaling pathways that the ICM may be receiving in vivo. While studying XCI in vivo is a more developmentally accurate method, having ESCs as a model system is more tractable. The Rlim/Rnf12 results

highlight that the XCI field needs a more developmentally accurate method of differentiating ESCs ex vivo, ideally one that uses the same inputs as in vivo.

Non-coding RNAs as activators and repressors

One feature of all models of XCI is the existence of an X inactivation center (XIC) that is required in cis for inactivation to occur (Brown et al. 1991). The XIC encodes a host of non-coding RNAs, including *Xist*, and in mouse, *Tsix*, which is transcribed antisense to *Xist* on the Xas in ESCs (Lee et al. 1999) and epiblast cells (Gayen et al. 2015). *Xist* and *Tsix* regulate random choice, and mutation of either gene results in non-random XCI. In heterozygous ESCs, Xs carrying *Tsix* mutations become the Xi (Lee et al. 1999), while those carrying *Xist* mutations are fated to be the Xa (Marahrens et al. 1998). An X carrying a mutation of *Xist* and *Tsix* is always the active X (Monkhorst et al. 2008). This epistasis suggests that *Tsix* lies upstream of *Xist* in a pathway designating which X will be the Xa. Thus, understanding how *Tsix* regulates *Xist* is important for elucidating the pathways that control *Xist*. The promoters of *Tsix* and *Xist* e*Xist* in separate topological domains as determined by chromatin capture confirmation (Nora et al. 2012), suggesting that the regulatory elements of the two promoters work independently with little to no physical contact. In this scenario, these two non-coding RNAs would function in cis to regulate each other's expression without necessarily altering the interaction between distant regulatory elements (Navarro et al. 2005).

Because *Tsix* could negatively regulate *Xist*, either at the transcriptional and/or post-transcriptional level, it is an attractive candidate for a factor that could silence *Xist* on the X fated to be the Xa (Sado et al. 2005). It is not absolutely crucial for *Xist*

silencing, as *Tsix* mutant XY ESCs and embryos can down regulate *Xist* (Ohhata et al. 2006) and homozygous *Tsix* mutant ESCs and embryos can also silence *Xist* on one X (Lee et al. 2002). A fraction of *Tsix* mutant XY cells in the epiblast show *Xist* up-regulation (Gayen et al. 2015), suggesting that *Tsix* is necessary for reliable *Xist* silencing and may function as a rheostat. Careful evaluation of heterozygous *Tsix* mutant mice showed that *Xist* is up regulated on the wild-type X and the mutant X in some cells of the epiblast. *Xist* up-regulation exhibits different kinetics on the two Xs in these cells, occurring later on the *Tsix* mutant X (Gayen et al. 2015). The cells expressing both copies of *Xist* are not detected later in development, and increased cell death and decreased proliferation appear to be responsible for eliminating this population. Together these data in male and female embryos suggest that *Tsix* plays a role in silencing *Xist* on Xa, but that the initial choice of which X to silence is not influenced by *Tsix*.

An additional non-coding RNA lies within the *Xist* gene itself. This *Xist* antisense transcript is embedded within the first exon of *Xist* and is co-expressed with *Xist* from the Xi (Sarkar et al. 2015). When this antisense transcript is truncated without disrupting *Xist*, induction of *Xist* is diminished by ~90%, and silencing of the Xi is impaired. Thus, in contrast to *Tsix*, this antisense *Xist* transcript activates *Xist* in cis.

Jpx and *Ftx*, two noncoding RNA encoding genes lie upstream of the *Xist* promoter and inhabit the *Xist* topologically associated domain (Nora et al. 2012). Both *Jpx* and *Ftx* appear to be activators of *Xist*, since individual deletion of these genes results in either less robust *Xist* expression (*Ftx*) or absence of *Xist* RNA (*Jpx*) upon ESC differentiation (Tian et al. 2010, Churceau et al. 2011).While *Jpx* exhibits features

of an essential *Xist* activator in some studies, the effect of deleting *Jpx* may vary with genetic background or exhibit a genetic interaction with *Ftx*, as ESCs with a large deletion that removes *Jpx* and *Ftx* do not exhibit an XCI phenotype and do contribute to chimeras (Barakat et al. 2014). Like *Tsix*, it may be that these non-coding RNAs exhibit a rheostat-like function to ensure that *Xist* up regulation occurs reliably over a variable range of conditions.

Linking *Xist* regulation to pluripotency

The observation that *Xist* up regulation correlates with ESC differentiation lead to the idea that pluripotency transcription factors may repress *Xist* (Deuve et al. 2011). In this model, the differentiation induced decrease in abundance of pluripotency transcription factors relieves *Xist* repression on the future Xi. Support for this model came from genome-wide distribution studies suggesting that *Xist* is regulated by the pluripotency transcription factors OCT4, NANOG, SOX2, and ZFP42 (also known as REX1) (Navarro et al. 20018, Gontan et al. 2012). These factors bind in the vicinity of *Xist* in ESCs and may define potential *Xist* cis-regulatory elements. While deletion of the binding region for OCT4, NANOG, and SOX2 does not affect XCI in ESCs or in embryos (Minkovsky et al. 2013, Nesterova et al. 2011), it is possible that there is redundancy so that loss of regulation by any single cis-element does not yield a phenotype.

The role of pluripotency transcription factors in regulating *Xist* expression may be more complex than initially hypothesized. Like the ICM, the cells of the epiblast are pluripotent (Diwan et al. 1976). In the epiblast and pluripotent stem cells derived from

the epiblast (epiSCs), *Xist* is up regulated on the Xi (Navarro et al. 2010). ESCs and epiSCs rely on a largely overlapping set of pluripotency transcription factors, as do the corresponding ICM and epiblast cells (Brons et al. 2007, Tesar et al. 2007). Thus, *Xist* up regulation is not strictly linked to developmental restriction away from pluripotency or absence of pluripotency transcription factors. It is likely that differences in relative abundance of these factors intersects with alterations mediated by developing signaling pathways, such as post-translational modification of these factors, to trigger the *Xist* up regulation that accompanies the transition from the ICM to epiblast. By altering growth factors or addition of small molecules, ESCs can be differentiated into epiSCs ex vivo, albeit with much slower kinetics than the ICM to epiblast transition in vivo (Li et al. 2013, Rao et al. 2014). Despite this difference, it may benefit the field to explore this more directed differentiation method as an ex vivo complement to studying the effects of mutations of putative *Xist* regulators in mice.

The XCI field is at a particularly exciting time, when factors that regulate *Xist* expression are emerging. Despite these advances, a diverse range of models for how each XX cell may designate one Xa and one Xi remain feasible. These models reveal major themes that are critical when thinking about how two otherwise identical chromosomes (as is the case in inbred mouse strains) can be differentially treated in response to the same developmental signal. Whether XX cells use two-fold differences in abundance of X-linked gene products, whether they take advantage of some unique feature of nuclear organization conferred by the non-coding RNAs of the XIC, what role autosomal gene products play, or whether all these mechanisms function in concert, our

knowledge of *Xist* activators and repressors will be central in understanding this key mystery of XCI.

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