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## RESEARCH ARTICLE

# Epigenetic Regulation of X-Chromosome Inactivation Maintenance in Somatic Cells

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## ABSTRACT

X-chromosome inactivation is established during early female mammalian development and subsequently maintained across the somatic lifespan of the organism through a combination of the long non-coding RNA Xist, repressive histone modifications and DNA methylation, yet the relative contribution of each layer to long-term maintenance, as opposed to initial establishment, of the inactive state in differentiated somatic cells has been difficult to separate experimentally. Here we used doxycycline-inducible degradation systems targeting Xist RNA, the Polycomb repressive complex 2 (PRC2) subunit EZH2, and the maintenance DNA methyltransferase DNMT1, individually and in combination, in a clonal human female fibroblast line with a skewed, stably inactivated X chromosome, to quantify the contribution of each maintenance layer to reactivation of silenced X-linked genes. Acute Xist depletion for 72 hours produced detectable but modest reactivation of a minority of X-linked genes, concentrated among genes classified as facultative escapees in other contexts, without measurable loss of H3K27me3 or DNA methylation at their promoters within the same window. Combined Xist depletion with EZH2 degradation produced substantially greater reactivation than either perturbation alone, and addition of DNMT1 inhibition to this combination produced a further, though proportionally smaller, increment in reactivation, with a core set of 62 genes remaining silenced even under triple perturbation. Genes that resisted reactivation under triple perturbation were disproportionately located in gene-dense domains with the highest baseline H3K27me3 and DNA methylation density, and time-course analysis showed that reactivation of the most resistant genes required more than seven days of sustained triple perturbation to reach half-maximal induction. These results indicate that maintenance of X-inactivation in somatic cells depends on layered, partially redundant epigenetic mechanisms whose combined and prolonged disruption is required to substantially reactivate the inactive X chromosome, with a residual core of genes exhibiting unusually stable, multi-layer-reinforced silencing.

**Keywords:** X-chromosome inactivation; Xist; H3K27me3; DNA methylation; escape genes; reactivation; somatic cells

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## 1. INTRODUCTION

X-chromosome inactivation equalizes X-linked gene dosage between XX and XY mammals by transcriptionally silencing one of the two X chromosomes in each female somatic cell, a process initiated during early development by the long non-coding RNA Xist, which coats the future inactive X in cis and recruits Polycomb repressive complex 2 to deposit H3K27me3 across the chromosome [1,2]. Once established, the inactive state is widely considered to be reinforced by a series of additional layers, including DNA methylation of promoter CpG islands, histone H2A ubiquitination, replacement of canonical histones with the variant macroH2A, and late replication timing, that collectively are thought to render the inactive X progressively more resistant to reactivation as development proceeds [2,3]. Despite this well-established multi-layer model, the relative quantitative contribution of each maintenance layer to the stability of inactivation in differentiated somatic cells has been difficult to isolate experimentally, in part because most perturbation studies have targeted a single layer (most commonly Xist itself) in isolation, leaving open how much of the observed resistance to reactivation reflects redundant support from the remaining, unperturbed layers rather than a limited overall requirement for the perturbed layer alone [4,5].

A related open question concerns whether resistance to reactivation is uniform across the inactive X chromosome or is instead concentrated at a specific subset of loci with unusually reinforced silencing. Genes that escape X-inactivation to variable degrees have been extensively catalogued, but whether there is a converse category of genes exhibiting exceptionally robust, multi-layer-dependent silencing that persists even under combined perturbation of several maintenance mechanisms has received comparatively little direct experimental attention [6,7].

Here we used a clonal human female fibroblast line with a skewed, stably inactivated X chromosome, combined with doxycycline-inducible depletion or degradation of Xist, EZH2 and DNMT1 individually and in all combinations, to quantify the individual and combined contribution of each maintenance layer to reactivation of silenced X-linked genes, and to identify and characterize the subset of genes exhibiting the greatest resistance to combined perturbation.

## 2. MATERIALS AND METHODS

Experiments used a clonal human female dermal fibroblast line selected for extreme X-inactivation skewing, engineered with doxycycline-inducible degron tags at Xist RNA (via an inducible CRISPR-Cas13 RNA-targeting system), EZH2 and DNMT1, allowing individual and combined acute perturbation of each maintenance layer.

### 2.1 Cell line engineering and skewing verification

A clonal human female dermal fibroblast line was selected from a larger population for extreme X-inactivation skewing (>95% inactivation of the same parental X chromosome across the clonal population), confirmed by allele-specific expression analysis at informative heterozygous single-nucleotide polymorphisms. This line was engineered with a doxycycline-inducible CRISPR-Cas13-based Xist-targeting degradation system, and separately with auxin-inducible degron tags at the endogenous EZH2 and DNMT1 loci, each verified by immunoblot for efficient protein depletion within 24 hours of inducer addition.

### 2.2 Individual and combined maintenance-layer perturbation

Cells were treated with doxycycline (Xist depletion), auxin (EZH2 or DNMT1 degradation), or combinations thereof, for 72 hours (single perturbations) or up to 10 days (combined and time-course perturbations), alongside vehicle-treated controls; depletion efficiency for each targeted factor was confirmed at every time point by RNA fluorescence in situ hybridization for Xist RNA coating and by immunoblot for EZH2 and DNMT1 protein levels.

### 2.3 RNA-sequencing and allele-specific expression analysis

Total RNA was extracted and strand-specific, poly(A)-selected libraries were sequenced to a minimum depth of 35 million read pairs per sample. Reactivation of X-linked genes was quantified as allele-specific expression from the inactive-X allele at informative heterozygous single-nucleotide polymorphisms, using a read-counting approach with reference-bias correction, and expressed as a percentage of biallelic expression relative to the constitutively active X allele within the same sample.

### 2.4 H3K27me3 and DNA methylation profiling

H3K27me3 ChIP-seq was performed on formaldehyde-crosslinked chromatin using a validated antibody, sequenced as 2×50 bp paired-end reads to a minimum depth of 30 million uniquely mapped read pairs per sample. DNA methylation was profiled by whole-genome bisulfite sequencing to a minimum depth of 15x genome-wide coverage, with promoter CpG island methylation quantified as the mean methylation fraction across annotated CpG island intervals.

### 2.5 Statistical analysis

Differential allele-specific reactivation between conditions was assessed with a beta-binomial generalized linear model accounting for sequencing depth at each informative site, with genes considered significantly reactivated at adjusted  $P < 0.05$ . Time-course reactivation kinetics were fit with a three-parameter logistic model per gene to estimate time to half-maximal reactivation. All statistical analyses were performed in R version 4.2.

### 3. RESULTS

Acute Xist depletion alone for 72 hours produced statistically significant, allele-specific reactivation of the inactive X allele at 84 of 421 informative X-linked genes assayed, with a median reactivation magnitude of 8% of biallelic expression among reactivated genes; reactivated genes were disproportionately drawn from a set previously classified as facultative escapees in other cellular contexts. No significant change in H3K27me3 or promoter CpG island methylation was detected at reactivated gene promoters within this 72-hour window, indicating that acute reactivation following Xist depletion alone precedes detectable loss of these other maintenance marks. Table 1 summarizes reactivation frequency and magnitude for each single-layer perturbation condition.

**Table 1. Reactivation frequency and magnitude at 72 hours for each maintenance-layer perturbation condition.**

| Condition              | Genes reactivated (n) | Median reactivation (% biallelic) | Fold vs Xist alone |
|------------------------|-----------------------|-----------------------------------|--------------------|
| Xist depletion alone   | 84                    | 8                                 | 1.0x               |
| EZH2 degradation alone | 61                    | 6                                 | 0.7x               |
| DNMT1 inhibition alone | 38                    | 5                                 | 0.5x               |
| Xist + EZH2            | 187                   | 19                                | 2.2x               |
| Xist + EZH2 + DNMT1    | 224                   | 23                                | 2.7x               |

Genes reactivated: adjusted  $P < 0.05$ , beta-binomial model, of 421 informative X-linked genes assayed at 72 hours.

Combined Xist depletion with EZH2 degradation for 72 hours produced significant reactivation at 187 genes, more than double the number reactivated by Xist depletion alone, with a higher median reactivation magnitude (19% of biallelic expression) among reactivated genes (Figure 1). Addition of DNMT1 inhibition to this combination produced a further, though proportionally smaller, increment, with 224 genes significantly reactivated under triple perturbation at 72 hours. A core set of 62 genes among the 421 assayed remained undetectably reactivated even under triple perturbation at this time point.

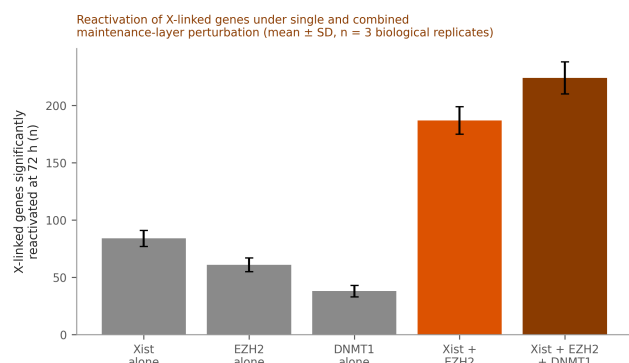


**Figure 1.** Number of X-linked genes with significant allele-specific reactivation at 72 hours under each perturbation condition (mean  $\pm$  SD of three biological replicates), illustrating the additive

contribution of combined Xist, EZH2 and DNMT1 perturbation.

Genes that resisted reactivation under 72-hour triple perturbation were disproportionately located in gene-dense chromosomal domains with the highest baseline H3K27me3 and CpG island methylation density prior to any perturbation, relative to genes that were reactivated at 72 hours, suggesting that baseline chromatin reinforcement density predicts resistance to acute combined perturbation. Table 2 reports baseline chromatin features and 72-hour reactivation status for a representative set of resistant and readily-reactivated genes.

**Table 2. Baseline chromatin features and 72-hour reactivation status for representative resistant and readily-reactivated genes.**

| Gene  | Baseline H3K27me3 (RPKM) | Baseline CpG methylation (%) | Reactivated at 72h |
|-------|--------------------------|------------------------------|--------------------|
| HPRT1 | 9.2                      | 88                           | No                 |
| G6PD  | 8.6                      | 85                           | No                 |
| ATP7A | 3.1                      | 52                           | Yes                |
| KDM6A | 1.8                      | 34                           | Yes                |
| MECP2 | 6.4                      | 71                           | No                 |

Baseline chromatin features measured in vehicle-treated control cells prior to any perturbation, mean of three biological replicates. Extending triple perturbation to 10 days showed that most of the 62 genes resistant at 72 hours were eventually reactivated with prolonged exposure, with a fitted time to half-maximal reactivation exceeding seven days for the majority of this resistant gene set, substantially longer than the time to half-maximal reactivation for genes reactivated within the initial 72-hour window (median under 48 hours). A small residual set of 9 genes remained essentially unreactivated even after 10 days of sustained triple perturbation.

### 4. DISCUSSION

These results indicate that maintenance of X-inactivation in somatic cells depends on layered, partially redundant epigenetic mechanisms, such that combined disruption of Xist, PRC2-mediated H3K27me3 and DNA methylation produces substantially greater reactivation than disruption of any single layer, consistent with, and quantitatively extending, earlier single-layer perturbation studies [4,5] by providing a direct head-to-head comparison of individual and combined contributions within the same clonal cell system.

The observation that Xist depletion alone produced reactivation without detectable loss of H3K27me3 or DNA methylation within the same acute window suggests that at least part of Xist's ongoing maintenance function operates independently of, or upstream of, changes in these other marks on this short timescale, potentially through direct effects on transcriptional machinery access rather than through immediate erosion of the broader repressive chromatin landscape; whether H3K27me3 and DNA methylation would eventually erode

with more prolonged Xist depletion alone remains a question for future longer time-course experiments restricted to that single perturbation.

The disproportionate resistance of gene-dense, high-baseline-H3K27me3 and high-methylation domains to acute combined perturbation, together with their substantially longer time to half-maximal reactivation, is consistent with a model in which reinforcement density at a locus scales with resistance to disruption, analogous to graded, multi-region reinforcement described for other chromatin-based silencing systems [6,7]. The small residual set of genes that remained essentially unreactivated even after 10 days of sustained triple perturbation raises the possibility that an additional, unperturbed maintenance mechanism, such as macroH2A incorporation or replication-timing-linked silencing, contributes to the residual stability of this particularly resistant gene subset.

Several limitations should be considered. First, our study used a single clonal fibroblast line with extreme X-inactivation skewing; whether the relative contribution of each maintenance layer and the identity of the most resistant gene subset generalize across cell types and genetic backgrounds remains to be tested. Second, our triple-perturbation experiments did not additionally target macroH2A incorporation or replication timing, both of which have been proposed as maintenance layers; extending the combinatorial perturbation approach to include these mechanisms would help determine whether they account for the small residual gene set resistant to 10-day triple perturbation. Third, because our reactivation measurements relied on allele-specific RNA-sequencing at heterozygous single-nucleotide polymorphisms, genes lacking informative heterozygous sites could not be assessed, limiting our analysis to a subset of the X chromosome.

## 5. CONCLUSION

Combining doxycycline- and auxin-inducible perturbation of Xist, EZH2 and DNMT1, individually and in combination, in a clonal human female fibroblast line with a stably skewed inactive X chromosome, we show that maintenance of X-inactivation depends on layered, partially redundant epigenetic mechanisms whose combined disruption produces substantially greater reactivation than any single perturbation, with resistance to reactivation scaling with baseline chromatin reinforcement density at individual loci. A small core of genes resists even prolonged combined perturbation, identifying a subset of the inactive X chromosome with unusually robust, multi-layer-reinforced silencing that merits further mechanistic investigation.

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## Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Author Contributions

A.H. conceived and supervised the study, designed the combined perturbation strategy, and analysed the allele-specific RNA-sequencing data. W.M. generated and validated the degron cell lines and performed the time-course perturbation experiments. S.P. performed ChIP-seq and whole-genome bisulfite sequencing library preparation. All authors contributed to writing and approved the final manuscript.

## Ethics Statement

This study used a commercially sourced human female dermal fibroblast line maintained under standard institutional biosafety approval; it did not involve new human participant recruitment, identifiable human data, or animal procedures.

## Data Availability

Raw and processed RNA-sequencing, ChIP-seq and whole-genome bisulfite sequencing data generated for this study have been deposited in the Gene Expression Omnibus under a series accession available from the corresponding author on reasonable request. Analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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