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The Polycomb group protein Eed protects the inactive X-chromosome from differentiation-induced reactivation**Sundeep Kalantry¹, Kyle C. Mills¹, Della Yee¹, Arie P. Otte², Barbara Panning³, and Terry Magnuson^{1,*}**¹ Department of Genetics and the Carolina Center for the Genome Sciences, University of North Carolina, Chapel Hill, North Carolina 27599-7264, USA.² Swammerdam Institute for Life Sciences, University of Amsterdam, 1098 SM Amsterdam, The Netherlands.³ Department of Biochemistry and Biophysics, University of California San Francisco, San Francisco, CA 94143, USA.**Abstract**

The Polycomb group (PcG) encodes an evolutionarily conserved set of chromatin-modifying proteins that are thought to maintain cellular transcriptional memory by stably silencing gene expression¹. In mouse embryos mutated for the PcG protein Eed, X-chromosome inactivation (XCI) is not stably maintained in extra-embryonic tissues². Eed is a component of a histone-methyltransferase complex that is thought to contribute to stable silencing in undifferentiated cells due to its enrichment on the inactive X-chromosome (Xi) in cells of the early mouse embryo and in stem cells of the extra-embryonic trophoblast lineage^{3–8}. Here we demonstrate that the Xi in *Eed*^{-/-} trophoblast stem (TS) cells and in cells of the trophoblast-derived extra-embryonic ectoderm in *Eed*^{-/-} embryos remains transcriptionally silent, despite lacking the PcG-mediated histone modifications that normally characterize the facultative heterochromatin of the Xi. While undifferentiated *Eed*^{-/-} TS cells maintained XCI, reactivation of the Xi occurred when these cells were differentiated. These results indicate that PcG complexes are not necessary to maintain transcriptional silencing of the Xi in undifferentiated stem cells. Instead, PcG proteins appear to propagate cellular memory by preventing transcriptional activation of facultative heterochromatin during differentiation.

KeywordsPolycomb group complex; X-inactivation; chromatin; Eed; *Xist* RNA; trophoblast stem (TS) cells; primitive endoderm

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Results

Polycomb Group (PcG) proteins are employed to maintain transcriptional repression of developmentally important genes through many rounds of cell division¹. PcG proteins contribute to X-chromosome inactivation (XCI)², the transcriptional silencing of one of the two X-chromosomes in female mammals to equalize X-linked gene dosage with XY males⁹. XCI is a paradigm of cellular transcriptional memory, as once silencing occurs in cells of the early embryo it is stably maintained through cell division in descendant cells. In mouse embryos mutant for *Eed*, which encodes a PcG protein that is part of the Polycomb repressive complex 2 (PRC2), the inactive X-chromosome (Xi) is reactivated in extra-embryonic tissues, suggesting that PRC2 is necessary to maintain transcriptional silencing of the Xi². *Eed* and other PRC2 proteins are enriched on the Xi in progenitor cells of the extra-embryonic trophoblast lineage^{4–8}. These cells do not exhibit reactivation of the Xi in *Eed*^{17Rn5-3354SB} (*Eed*^{-/-}) mutants². In differentiated extra-embryonic trophoblast cells of the parietal yolk sac that do show reactivation of the Xi in *Eed*^{-/-} embryos, however, *Eed* is no longer enriched on the Xi in corresponding wild-type cells^{2, 4, 5}. This raises the question as to how transient enrichment of *Eed* on the Xi in progenitor cells of extra-embryonic tissues contributes to stable transcriptional silencing of the Xi in differentiated cells derived from these progenitors.

A role for PRC2 in silencing of the Xi in progenitor trophoblast cells may have been masked in *Eed*^{-/-} embryos by maternally-loaded *Eed* protein^{2, 4–8, 10}. To determine whether PRC2 is required for stable transcriptional silencing of the Xi in the extra-embryonic progenitor cells, we derived *Eed*^{-/-} trophoblast stem (TS) cells. TS cells, which originate from the extra-embryonic trophoblast, are subject to imprinted XCI and exhibit exclusive inactivation of the paternal X-chromosome (Xp)^{3, 11}. Undifferentiated wild-type (WT) TS cells display constitutive enrichment of *Eed* and other PRC2 proteins on the silent Xp^{3–5, 11}. Our TS cell lines carry an Xp-linked green fluorescent protein (*GFP*) transgene that is subject to imprinted XCI in extra-embryonic cells^{2, 4, 12}. As the *Eed*^{-/-} embryos used to generate the mutant TS cell lines initiate imprinted XCI normally, GFP fluorescence provides a convenient readout of transcriptional reactivation. Analysis of expression by FISH of the X-linked genes *Hprt*, *Mecp2*, and *Pgk1* indicates that the X-*GFP* transgene faithfully recapitulates reactivation of endogenous genes on the Xp in *Eed*^{-/-} female TS cells (Supplementary Fig. 1). The mutant TS cells also stably maintain their karyotype as well as two X-chromosomes (Supplementary Fig. 2).

GFP fluorescence was absent in all cells in WT TS cell cultures, indicating that these cells maintained proper XCI of the Xp (Fig. 1a). While most cells in *Eed*^{-/-} TS cell cultures did not show GFP expression, cells located on the periphery of mutant colonies, where cells are more likely to be differentiating, expressed Xp-*GFP*, indicating defective XCI in these cells. Examination of cultured mutant TS cells for the distribution of *Cdx2*, a homeodomain protein expressed in undifferentiated but not in differentiated trophoblast cells¹³, showed that cells harboring an active Xp are largely devoid of *Cdx2* staining (Fig. 1b). Differentiating trophoblast cells in *Eed*^{-/-} blastocyst outgrowths also displayed Xp activity (Fig. 1d). Together these results indicate that Xp-reactivated cells are in fact differentiating trophoblast cells. *Eed*^{-/-} female TS cells differentiated into precursors of the terminally

differentiated giant cells, but not giant cells themselves (Fig. 1c; Supplementary Fig. 3), which is also observed in mutant embryos². *Eed*^{-/-} male TS colonies, however, were able to differentiate into giant cells (Fig. 1c). This female-specific absence of giant cells in *Eed*^{-/-} differentiated TS cell cultures is consistent with an XCI defect. The reactivation of the Xp in female mutant trophoblasts may lead to death of or a block in further differentiation of precursors of the terminally differentiated giant cells. In agreement, cells with a reactivated X-chromosome do not proliferate further (Supplementary Fig. 3). These results suggest that TS cells, which normally show enrichment of Eed on the Xp, do not require Eed to maintain transcriptional silencing of the Xp, unless they are induced to differentiate.

The PRC2 complex mediates tri-methylation of histone H3 lysine 27 (H3-3mK27), and PRC2-mediated H3-K27 methylation can recruit a second PcG complex, Polycomb repressive complex 1 (PRC1)¹⁴. PRC1 and PRC2 complexes are recruited to distinct as well as shared target loci¹⁵, and it has been suggested that while H3-3mK27 may contribute to PRC1 recruitment to the Xi, it is not the sole determinant¹⁶. To determine the consequences of Eed depletion on PcG protein localization and function in TS cells, we analyzed the distribution PRC2 and PRC1 proteins, and the histone modifications mediated by these complexes in *Eed*^{-/-} TS cells. We first examined whether H3-3mK27 and other components of the PRC2 complex, Ezh2, Su(z)1214, localize to the Xi in *Eed*^{-/-} TS cells. While all three co-localized with Eed on the Xi in WT TS cells, they were absent from the Xi in all *Eed*^{-/-} TS cells (Fig. 2a). Moreover, *Eed*^{-/-} TS cells were completely devoid of H3-3mK27 staining. Eed is therefore required for PRC2 complex formation on the Xi and global H3-3mK27 methylation in undifferentiated TS cells. We next tested for the presence of the murine PRC1 proteins Cbx2 and Phc2 on the Xi in both WT and *Eed*^{-/-} female TS cells. Whereas these PRC1 components localized to the Xi in WT TS cells, they were absent in *Eed*^{-/-} TS cells (Fig. 2a). A PRC1 protein-containing complex targets the ubiquitylation of lysine 119 on Histone H2A (Ub-H2A)^{16, 17}. Using an anti-Ub-H2A antibody, we detected this modification on the Xi in WT but not in *Eed*^{-/-} female TS cells (Fig. 2a). Thus, the Xp in all *Eed*^{-/-} TS cells lacks all downstream modifications dependent on PRC2, including those mediated by PRC1. Therefore PRC2 activity is necessary to recruit PRC1 to the Xi and neither complex is necessary for transcriptional silencing of the imprinted Xi in TS cells.

XCI is maintained by multiple chromatin modifications¹⁸, which may compensate for the absence of Eed and associated epigenetic modifications to ensure silencing of the Xp in undifferentiated cells. We assayed the distribution of non-Eed dependent chromatin modifications of the Xi, to determine whether these modifications may contribute to transcriptional silencing of the Xp in *Eed*^{-/-} TS cells. The mono-methylation of lysine 20 of histone H4 (H4-1mK20), an epigenetic modification mediated by Pr-Set7 histone methyltransferase, is an early mark of XCI¹⁹, was enriched on the Xi in WT TS cells and absent in all *Eed*^{-/-} TS cells (Fig. 2a). The histone H2A variant macroH2A, which is visible on the Xp as early as at the 8-cell stage²⁰, also failed to accumulate on the Xi in the *Eed*^{-/-} TS cells (Fig. 2a). Therefore Eed is necessary for the enrichment of macroH2A and H4-1mK20 on the silent Xp in TS cells, and neither of these chromatin modifications is necessary to stably maintain XCI.

The X-linked non-protein coding *X (inactive)-specific transcript (Xist)* RNA coats the Xi to initiate transcriptional silencing^{7, 21}. Silencing occurs before many chromatin modifications are detectably enriched on the Xi^{7, 21}, suggesting the possibility that *Xist* RNA alone may be sufficient for transcriptional repression in stem cells derived from the early embryo. We assayed the distribution of *Xist* RNA in *Eed*^{-/-} TS colonies to determine whether this unusual RNA plays a role in maintaining silencing of the Xp in the absence of PRC2-mediated histone modifications, macroH2A, and H4-1mK20. *Xist* RNA exhibited a distribution consistent with coating of the Xp in WT TS cells. In contrast, *Xist* RNA did not coat the Xi and showed only a small localized pinpoint of accumulation in all XX *Eed*^{-/-} TS cells (Fig. 3a; Fig. 4b). RT-PCR analysis of *Xist* expression confirmed that steady-state levels of the *Xist* RNA were decreased in *Eed*^{-/-} TS cells compared to WT TS cells (Fig. 3c, d). Thus, *Xist* RNA coating of the Xp in *Eed*^{-/-} TS cells is not necessary to maintain transcriptional silencing. The absence of enrichment of macroH2A and H4-1mK20 on the Xp in *Eed*^{-/-} TS cells may be a consequence of the lack of *Xist* RNA coating, as these modifications are dependent on *Xist* RNA coating during random X-inactivation in the embryo proper^{4, 5, 7, 8, 19, 20, 22}. Trophoblast (TB) cells in *Eed*^{-/-} blastocyst outgrowths also lacked *Xist* RNA accumulation (Fig. 3b). Consistent with a lack of random XCI defects in the embryo proper in *Eed*^{-/-} females², *Xist* RNA did accumulate on the Xi in the inner cell mass (ICM)-derived cells in *Eed*^{-/-} blastocysts (Fig. 3b).

Failure of *Xist* accumulation on the silent Xp in both *Eed*^{-/-} TS and TB cells suggests that PRC2 either directly or indirectly influences *Xist* RNA coating. *Tsix*, encoding antisense transcripts originating from the *Xist* locus, is required to suppress *Xist* transcription from the maternal X-chromosome in extra-embryonic cells⁹. *Tsix* transcription was not detected in either WT or *Eed*^{-/-} TS cells (Fig. 3c). Lack of *Xist* RNA accumulation, therefore, is not due to induction of *Tsix* transcription in *Eed*^{-/-} TS cells. The defect in *Xist* RNA coating in mutant cells may be due either to decreased transcription or to decreased stability of the transcript. Coating of the Xi has been shown to be mediated by stabilization of the *Xist* RNA; the rate of transcription remains unchanged^{23, 24}. Lower levels of *Xist* RNA in *Eed*^{-/-} TS cells may therefore reflect destabilization of the transcript in the absence of Eed and other factors enriched on the Xi.

Our data indicate that the Xp in *Eed*^{-/-} TS cells has lost many epigenetic features of Xi chromatin. We next assayed whether the Xp acquires characteristics of transcriptionally active chromatin in the absence of PRC2 and all tested marks of Xi-chromatin. Histone H3-dimethyl lysine 4 (H3-2mK4) is correlated with actively transcribed chromatin, and is depleted from the Xi²⁵. In WT TS cells, the *Xist* RNA-coated inactive-Xp is conspicuously devoid of H3-2mK4 (Fig. 4a). The Xp in a majority of *Eed*^{-/-} TS cells, as denoted by the pinpoint nascent *Xist* RNA expression, also lacks H3-2mK4 (Fig. 4a). A subset of the *Eed*^{-/-} TS cells harbor an Xp with overlapping H3-2mK4 staining. This fraction coincides with the percentage of cells that have an active Xp (Fig. 4b). FISH detection of nascent Xp-*GFP* RNA demonstrates that 95% of the cells that have reactivated their Xp show overlapping H3-2mK4 staining (Fig. 4c, d). We obtained similar results with two other marks of active chromatin that are normally absent from the Xi, acetylated histone H3 and histone H4^{26, 27} (not shown). These studies suggest that the Xp remains inactive despite the absence of

silencing marks because it has not acquired epigenetic marks associated with active chromatin. It is feasible that in the absence of Eed as yet undiscovered factor(s) may mediate silencing of the imprinted Xp in undifferentiated cells. These putative factors, however, do not compensate for the loss of Eed during differentiation. When Eed is absent, differentiation induces reactivation of the Xp, suggesting that chromatin reconfiguration during differentiation can alter the epigenetic modifications on the Xp and thus promote transcriptional activation.

In addition to the trophoctoderm, a second extra-embryonic lineage, the primitive endoderm (PE) lineage, also undergoes imprinted XCI¹⁸. We assayed whether Eed plays a role in maintaining the silent Xp in this lineage, by examining WT and *Eed*^{-/-} female embryos harboring the Xp-*GFP* transgene by confocal microscopy. Embryonic day 6.2 (E6.2) and E7.0 mutant embryos showed extra-embryonic Xp-activity only in differentiating TB cells present in the ectoplacental cone (Fig. 5a; not shown). Cells of the extra-embryonic ectoderm, a derivative of the trophoctoderm that is a source of TS cells and which differentiates TB cells¹¹, or the PE-derived visceral endoderm (VE) did not display reactivation of the Xp in *Eed*^{-/-} embryos (Fig. 5a; not shown). We next derived three different wild-type cell lines typical of the PE and its derivatives (Supplemental Fig. 5), to determine whether PRC2 proteins were enriched on the silent Xp in the PE lineage. These cells carry an inactive Xp (Supplemental Fig. 4). IF and FISH analysis of these cell lines demonstrated *Xist* RNA coating but a lack of enrichment of Eed, Ezh2, and H3-3mK27 (Fig. 5b; not shown). Isolated VE from WT E6.5 embryos similarly showed an absence of Xi-enrichment of the PRC2 complex, despite *Xist* RNA coating (Fig. 5c; not shown). Together these results suggest that PRC2 does not contribute to XCI in differentiated cells of the PE lineage.

Eed is reported to accumulate on the Xi in PE cells when they form and differentiate during peri-implantation stages⁸. The presence of maternally-loaded Eed protein at that period of development may prevent the Xp from becoming reactivated in PE-derivatives of *Eed*^{-/-} embryos^{4, 5, 7, 8, 10}. Once the PE is differentiated, the Xi-epigenetic machinery may no longer be required to prevent reactivation of the silenced X-chromosome, thus making the PRC2 complex dispensable.

Our results show that Eed is not required to maintain silencing of the Xp in undifferentiated TS cells, despite its enrichment on the Xp in these cells. Indeed, there was no dramatic difference in the differentiation potential and expression profiles of undifferentiated WT and *Eed*^{-/-} TS cells. Thus the global absence of PRC2 and H3-3mK27 did not affect stem cell identity, as might be expected if Eed was necessary to stably propagate the transcriptional profile in undifferentiated TS cells. These results suggest that PcG proteins and the histone modifications they mediate are not necessary to maintain cellular transcriptional memory in undifferentiated TS cells. Instead lack of PRC2 and H3-3mK27 affects differentiated descendants of undifferentiated TS cells, as Eed is necessary to prevent the reactivation of the silent Xp during TS cell differentiation. Eed is also not required to maintain XCI in the trophoctoderm-derived undifferentiated extra-embryonic ectoderm cells or in differentiated derivatives of the PE. This suggests that cells that are stably maintaining their differentiation state and thus their transcriptional profile, i.e., undifferentiated or fully differentiated cells,

may not need PcG proteins to propagate transcriptional silencing. The reactivated Xp in differentiated cells harbors marks of active chromatin. Thus PRC2 and the associated histone modifications on the Xi may function to block differentiation-induced alterations in chromatin structure that promote transcriptional activation, rather than to stabilize the heterochromatin *per se* in undifferentiated cells. PcGs therefore may maintain cellular memory by preventing transcriptional activation during differentiation, when cells are undergoing dynamic changes in gene activity.

PRC2 enrichment on the Xp is transient, and is lost when trophoblast cells differentiate *in vivo* and *in vitro*^{4, 5}. PRC2 also accumulates on the Xi transiently when random XCI is initiated in the embryo proper and in differentiating embryonic stem cells^{4, 5}. This has led to the proposal that PRC2, via the histone H3-3mK27 modification it catalyzes, contributes to the initial silencing of the Xi, perhaps by stabilizing the Xi chromatin⁴. Our results suggest the possibility that PRC2 may function to prevent differentiation-induced Xi-reactivation during random XCI in embryonic lineages as well as during imprinted XCI in extra-embryonic lineages and in the early mouse embryo. Other chromatin modifications, such as histone H3-K4 hypomethylation and H3-K9 hypoacetylation, are tightly coordinated with the initial transcriptional silencing of the Xp and precede enrichment of PRC2 on the Xp during imprinted XCI in the pre-implantation embryo^{7, 8, 21}, consistent with a role for PRC2 after initiation of XCI. In *Drosophila*, the Eed homolog ESC is also expressed transiently and only required during early development for maintenance of gene silencing later^{28, 29}. Thus, the mechanism of maintenance of cellular memory by PcGs, by preventing reactivation of genes during differentiation, may also be conserved through evolution.

Methods

Mouse Strains

The *Eed*^{17Rn5-3354SB} line of mice originated in a mutagenesis screen³⁰, and they have been maintained in heterozygosity and genotyped as previously described^{2, 10}. The X^{GFP} is from the D4/XEGFP transgenic line¹².

Trophoblast Stem (TS) Cell and Primitive Endoderm-derived (Endo) Cell Lines

TS cells were derived and cultured as described¹¹. Male fibroblast feeder cells were used as feeders to culture female TS cells, to distinguish the two in immunofluorescence (IF) and fluorescence in situ hybridization (FISH) stainings for detection of the inactive X-chromosome. Multiple samples of both wild-type and *Eed*^{-/-} TS cells were analyzed. The cells were cultured for 8–18 passages and 6–28 passages, respectively. TS cells were differentiated by culturing in TS medium lacking Fgf4 and heparin¹¹ for 3–5 days. Primitive endoderm-derived cell lines (Endo) were isolated by culturing blastocysts as per TS cell derivation protocol¹¹. Some cultured blastocysts after dissociation/first passage yielded smaller, rounder, and refractory cells morphologically distinct from TS cells and were subcultured, giving rise to Endo cell lines. Once established, these cell lines were cultured in TS media without fibroblast feeders, Fgf4 and heparin.

Visceral Endoderm

Visceral endoderm layer of E6.5 embryos was dissociated in 0.25% trypsin by using a mouth pipette and fine-drawn capillary. Isolated cell clumps were neutralized in 100 μ L of DMEM and 10% fetal calf serum containing 10u/mL of RNAsin RNase inhibitor (Promega). The cells were then cytopspun (Shandon Cytospin 3), at 800 rpm for 5 min, onto Superfrost slides (Fisher). After air drying for 3–5 mins, the cells were processed for IF and FISH.

Immunofluorescence (IF)

TS and Endo cells were cultured on 22X22 cm gelatinized glass coverslips. Cultured or cytopspun cells were permeabilized by sequential 30 sec incubations in ice-cold CSB buffer (100 mM NaCl, 300mM sucrose, 3mM MgCl₂ in 10 mM PIPES, pH 6.8), CSB buffer with 0.5% Triton X-100, and CSB buffer again. The cells were then fixed for 10 min in 4% paraformaldehyde in PBS. Fixed cells were washed three times in PBS/0.2% Tween-20. At this point, the samples were either stored at 4°C in PBS/0.2% Tween-20 or 70% ethanol, or processed further. Cells were incubated in blocking buffer (5% normal goat serum, 0.2% Tween-20, 0.2% fish skin gelatin, in PBS) for 30 mins at 37°C, in a humid chamber. Cells were next incubated in with primary antibodies, diluted in blocking buffer, for 1–2 hrs at 37°C in a humid chamber. After washing 3X with PBS/0.2% Tween-20, the cells were incubated with secondary antibodies for 1 hour at 37°C in a humid chamber, followed by 3X PBS/0.2% Tween-20 washes. The cells were mounted in Vectashield mounting media (Vector Labs) containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Detailed information about the antibodies used is provided in Supplementary Information.

Fluorescence In Situ Hybridization (FISH)

RNA FISH was carried out essentially as described²³. In experiments where IF and FISH were performed on the same sample, FISH followed IF staining with the exception that tRNA (Invitrogen) and RNAsin RNase inhibitor (Promega) were added to the blocking buffer at all steps. After IF staining, cells were post-fixed in 2% paraformaldehyde in PBS for 10 min, followed by sequential incubations in 70% (5 min), 85%, 95%, and 100% ethanol (2 min each). Cells were then air dried for 10 min, followed by RNA FISH. *Xist* RNA accumulation was detected by a Cy3-dCTP (Amersham) labelled probe generated by nick-translation of an *Xist* exon 6 template DNA. Nascent *Xist* and *GFP* RNA transcripts following IF were detected by denaturing air-dried cells in 70% formamide/2X SSC at 80°C for 3–5 mins, immediately followed by quenching in ice-cold 2X SSC for 5 min. After dehydration by incubation in 70% (pre-chilled at –20°C), 85%, 95%, and 100% ethanol, cells were hybridized with a strand-specific Cy3-UTP (Amersham) labelled *Xist* FISH riboprobe using the *Xist* exon 6 template or a random-primed Cy3-dCTP labelled GFP probe using a pEGFP-C3 (Clontech) plasmid template. RNase treatment of control samples prior to FISH resulted in loss of *Xist* and *GFP* pinpoint signal.

Reverse Transcription-PCR (RT-PCR)

Total RNA was purified using Trizol reagent (Invitrogen). 2.5 μ g of DNase-treated total RNA was reverse transcribed at 42°C with 50 ng of random primers and SuperScript II

reverse transcriptase (Gibco). After reverse transcription, the first-strand product was diluted 1:1 prior to PCR. Information on the primers used for PCR is provided in Supplementary Information.

Blastocyst Culture and Staining

Blastocysts were dissected at E3.5 and cultured for 6–7 days on gelatinized tissue-culture grade glass slides (Erie Scientific, Cat. # 10-7A) in 70% feeder conditioned TS cell culture media with FGF4 and heparin11. FISH was performed as described for TS cells.

Microscopy

Details are included in Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

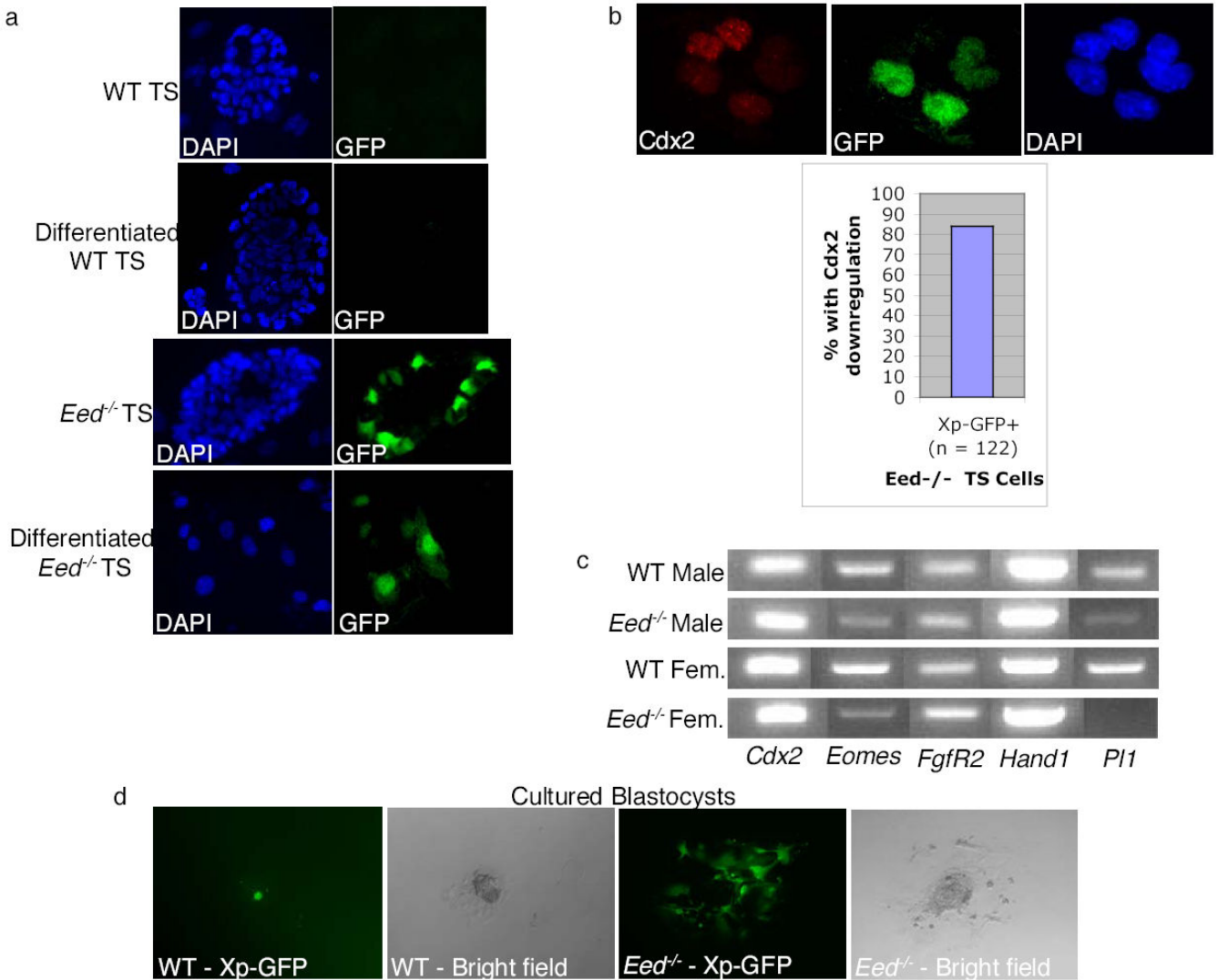
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**Figure 1.**

The paternal X-chromosome (Xp) is active only in differentiating *Eed*^{-/-} trophoblast stem (TS) cells. An Xp-linked GFP transgene is used as a reporter of X-linked gene activity and nuclei are stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). (a) Wild-type (WT) TS cells undergo imprinted XCI of the Xp, as indicated by a complete lack of Xp-GFP expression in all cells, including after differentiation. *Eed*^{-/-} TS colonies contain cells with an active Xp located preferentially at the periphery of the colonies, where differentiated cells are found. (b) Immunofluorescence (IF) detection of Cdx2, a marker of undifferentiated trophoblast cells, in cultured *Eed*^{-/-} TS cells. Cdx2 is downregulated in mutant cells harboring an active Xp, as indicated by GFP expression, indicating that these cells are differentiated. (c) RT-PCR analysis of male and female WT and *Eed*^{-/-} TS cells for markers of undifferentiated and differentiated trophoblast cells. *Cdx2*, *Eomes* (*Eomesodermin*), and *Fgf receptor 2* (*FgfR2*), all markers of undifferentiated trophoblast cells, are expressed in all four cell lines examined. *Hand1*, a marker of intermediate, non-giant differentiated trophoblast cells is also expressed in all cell lines. *Pl1* (*Placental lactogen 1*), a marker of

trophoblast giant cells, is absent only in female *Eed*^{-/-} TS cells. Thus, *Eed*^{-/-} female, but not male, TS cells are blocked from terminal differentiation into giant cells, consistent with reactivation of the Xp during initial differentiation of the female mutant TS cells resulting in a block to their further differentiation. **(d)** Trophoblast (TB) cells located on the periphery of WT blastocyst outgrowths lack Xp-activity, as indicated by Xp-*GFP* expression, while TB cells in *Eed*^{-/-} female blastocyst outgrowths harbor an active Xp.

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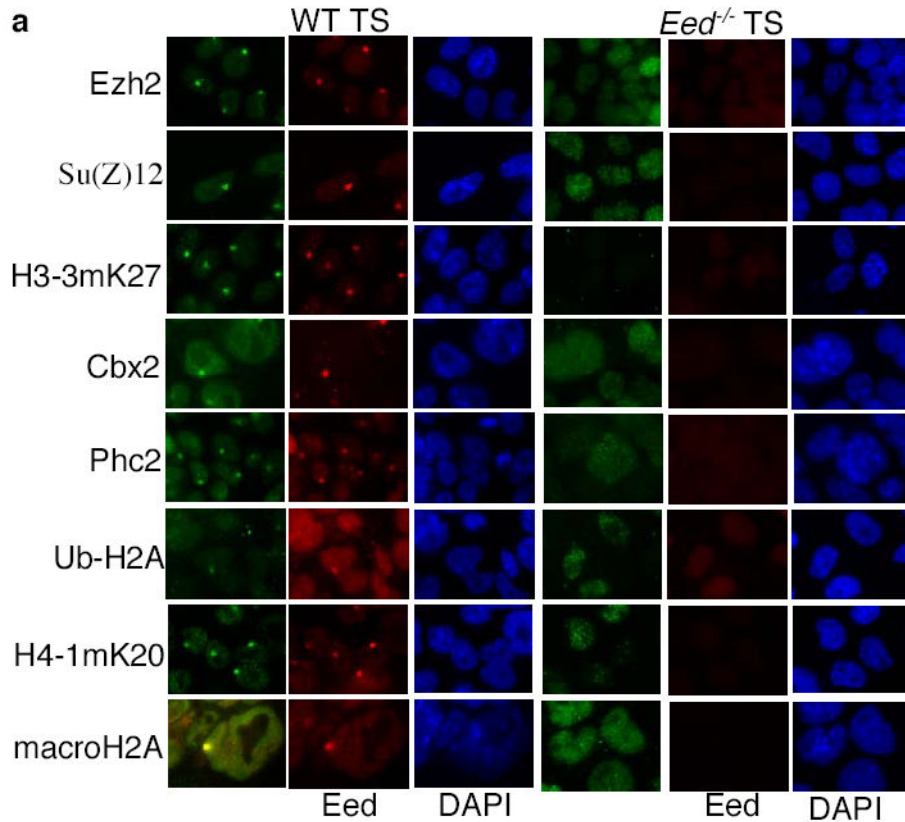
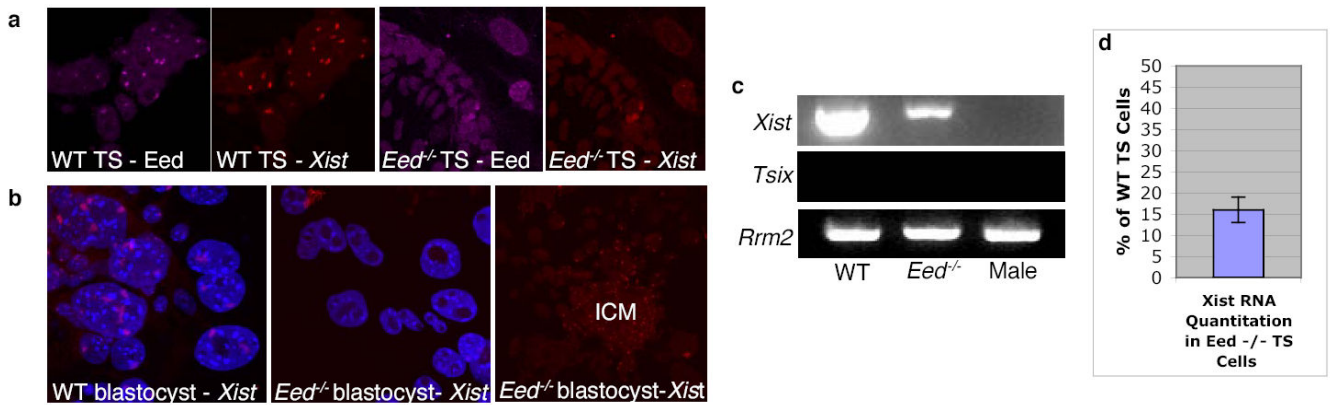


Figure 2.

All features of the Xi-heterochromatin are absent in *Eed*^{-/-} female TS cells. (a) IF detection of the Polycomb repressive complex 2 (PRC2) proteins Ezh2, Su(z)12, the PRC2-mediated histone modification tri-methyl lysine 27 of histone H3 (H3-3mK27), the PRC1 proteins Cbx2 and Phc2, PRC1-like mediated histone modification ubiquitylated-H2A (Ub-H2A), Pr-Set7 mediated histone modification mono-methyl lysine 20 of histone H4 (H4-1mK20), and the histone variant macroH2A. Left three rows, WT female TS cells; right three rows, *Eed*^{-/-} female TS cells. Middle panels in both WT and *Eed*^{-/-} TS cell columns are Eed immunostains; right panels are nuclei stained with DAPI. All proteins or epigenetic marks that colocalize with Eed on the inactive-X in WT TS cells, and are absent in all *Eed*^{-/-} TS cells.

**Figure 3.**

Xist RNA fails to coat the Xp in all *Eed*^{-/-} TS cells. **(a)** IF-FISH detection of Eed (purple) and *Xist* RNA (red) in wild-type (WT) and *Eed*^{-/-} TS cells. In WT TS cells, *Xist* (red) and Eed (purple) colocalize on the Xi in the nucleus (blue); *Eed*^{-/-} TS cells lack *Xist* RNA coating of the Xi. **(b)** Trophoblast (TB) cells in cultured *Eed*^{-/-} blastocysts also show lack of *Xist* RNA accumulation onto the Xi. WT blastocyst outgrowths harbor TB giant cells characterized by larger nuclei with endoreduplicated genomes and multiple inactive-Xs, as marked by multiple *Xist* foci. *Eed*^{-/-} female embryos do not differentiate TB giant cells, due to X-inactivation defect in diploid TB cells². Inner cell mass-derived cells in *Eed*^{-/-} blastocysts, however, do display *Xist* RNA accumulation onto the Xi. **(c)** RT-PCR analysis of *Xist* and *Tsix* RNAs in WT and *Eed*^{-/-} TS cells. *Xist* is expressed in *Eed*^{-/-} TS cells, but its steady-state levels are decreased compared to WT TS cells. *Tsix* is not detectable in both WT and *Eed*^{-/-} TS cells. **(d)** Real-time RT-PCR quantitation of *Xist* RNA in *Eed*^{-/-} TS cells relative to WT TS cells. Male mouse embryonic fibroblast cells serve as control not expressing *Xist* RNA.

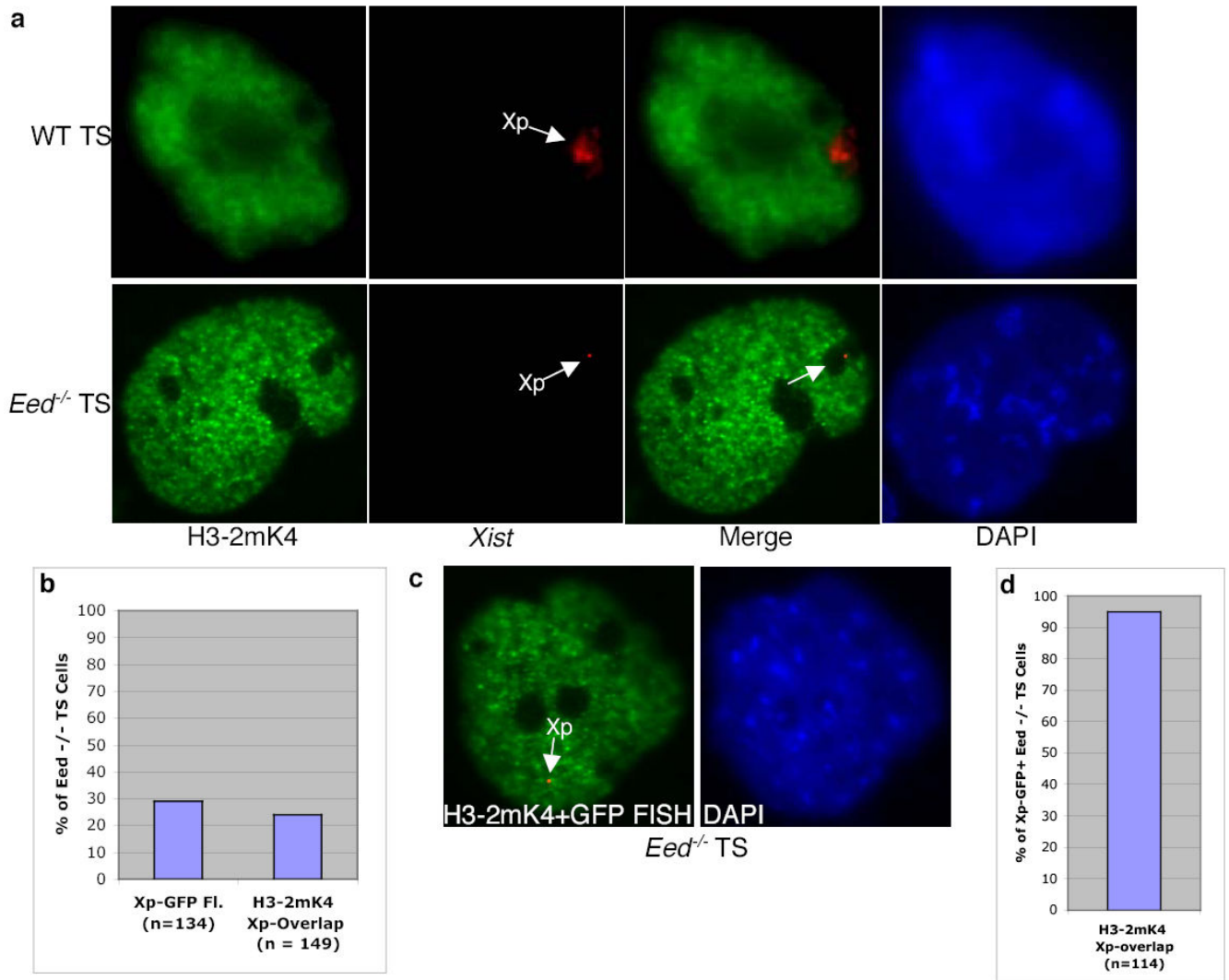
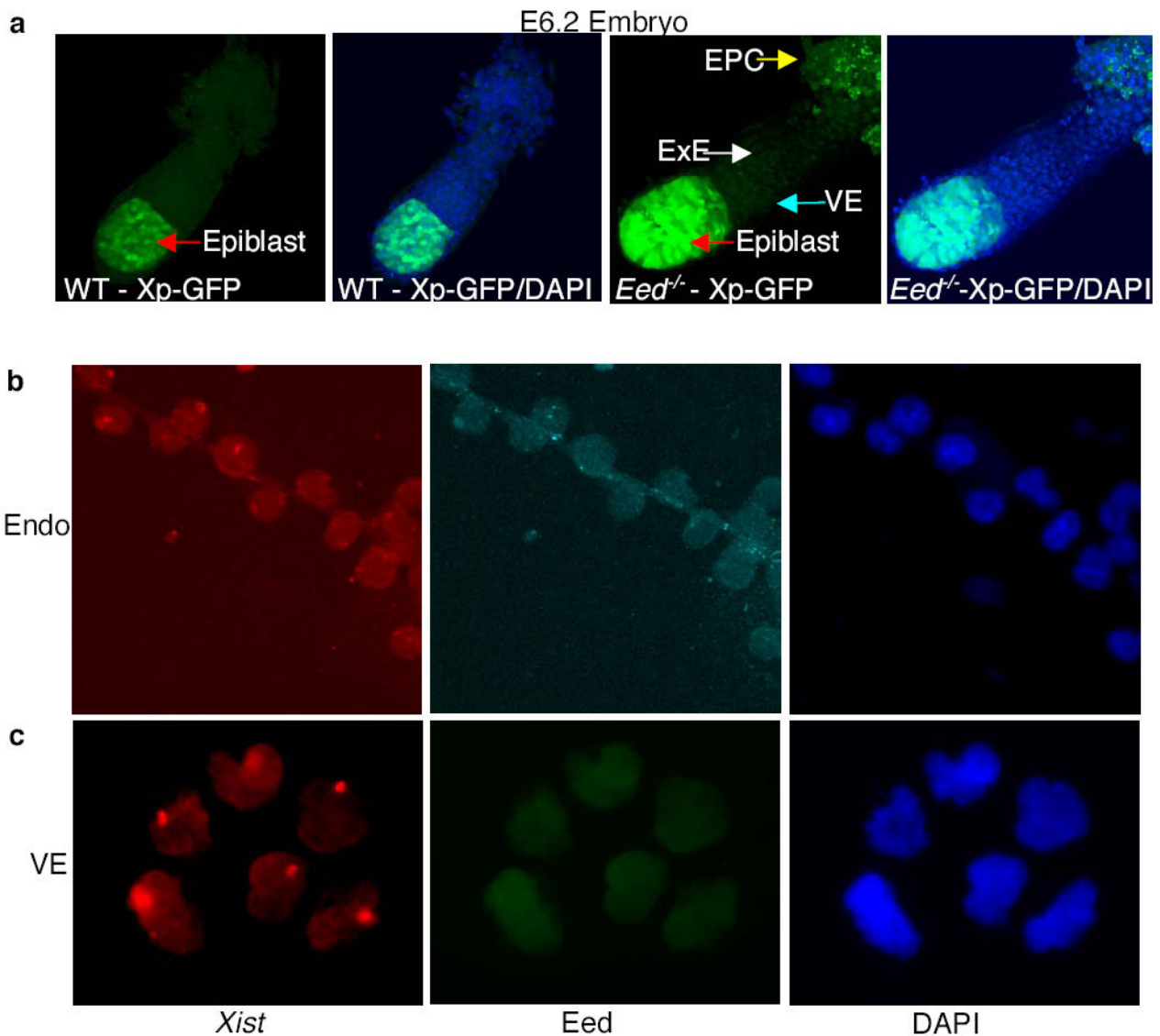


Figure 4.

Absence of an epigenetic hallmark of active chromatin, histone H3-di-methyl lysine 4 (H3-2mK4), from the paternal X-chromosome (Xp) in *Eed*^{-/-} TS cells. **(a)** IF detection of H3-2mK4 (green) and FISH detection of *Xist* RNA (red) and *Xist* merged with H3-2mK4 in a representative WT female TS cell nucleus. The Xi, as marked by *Xist* RNA accumulation, is devoid of H3-2mK4 in all WT TS cells. *Xist* RNA does not coat the Xi in *Eed*^{-/-} female TS cells but is detected as a pinpoint. In most mutant TS cells, the pinpoint *Xist* RNA signal falls within a hole devoid of H3-2mK4. Nuclei are stained blue with DAPI. **(b)** Similar percentages of cells have an active Xp, as assayed by Xp-GFP expression, and an Xp that overlaps with H3-2mK4 staining, suggesting that trophoblast cells with an active Xp also harbor H3-2mK4 on that chromosome. **(c)** H3-2mK4 IF and FISH detection of the Xp-GFP RNA in *Eed*^{-/-} female TS cells. **(d)** 95% of Xp-GFP expressing *Eed*^{-/-} TS cells also have H3-2mK4 staining of the Xp. Only differentiating *Eed*^{-/-} TS cells reactivate the Xp (Fig. 1) and these cells no longer exclude marks correlated with transcriptional activity.

**Figure 5.**

Lack of XCI defects in the trophoctoderm-derived undifferentiated extra-embryonic ectoderm and the differentiated derivatives of the primitive endoderm in *Eed*^{-/-} embryos. (a) WT embryos do not display any Xp-activity in the extra-embryonic tissues of the embryo, due to imprinted XCI of the Xp in these cells. The epiblast (embryo proper, red arrow) undergoes random XCI, resulting in a mosaicism of X-chromosome activity; in some cells the maternal-X is active and in some cells the Xp is active, as indicated by Xp-GFP expression. E6.2 *Eed*^{-/-} female embryos reactivate the Xp only in differentiating trophoblast (TB) cells in the ectoplacental cone (EPC; the proximal end of the embryo, yellow arrow). Undifferentiated extra-embryonic ectoderm (ExE, white arrow), a source of trophoblast stem (TS) cells and precursors of differentiated trophoblast cells, and visceral endoderm layer (VE, light blue arrow) are devoid of Xp-activity in *Eed*^{-/-} embryos. (b) Absence of PRC2 enrichment on the inactive-Xp in female primitive endoderm-derived (Endo) cell lines. (c)

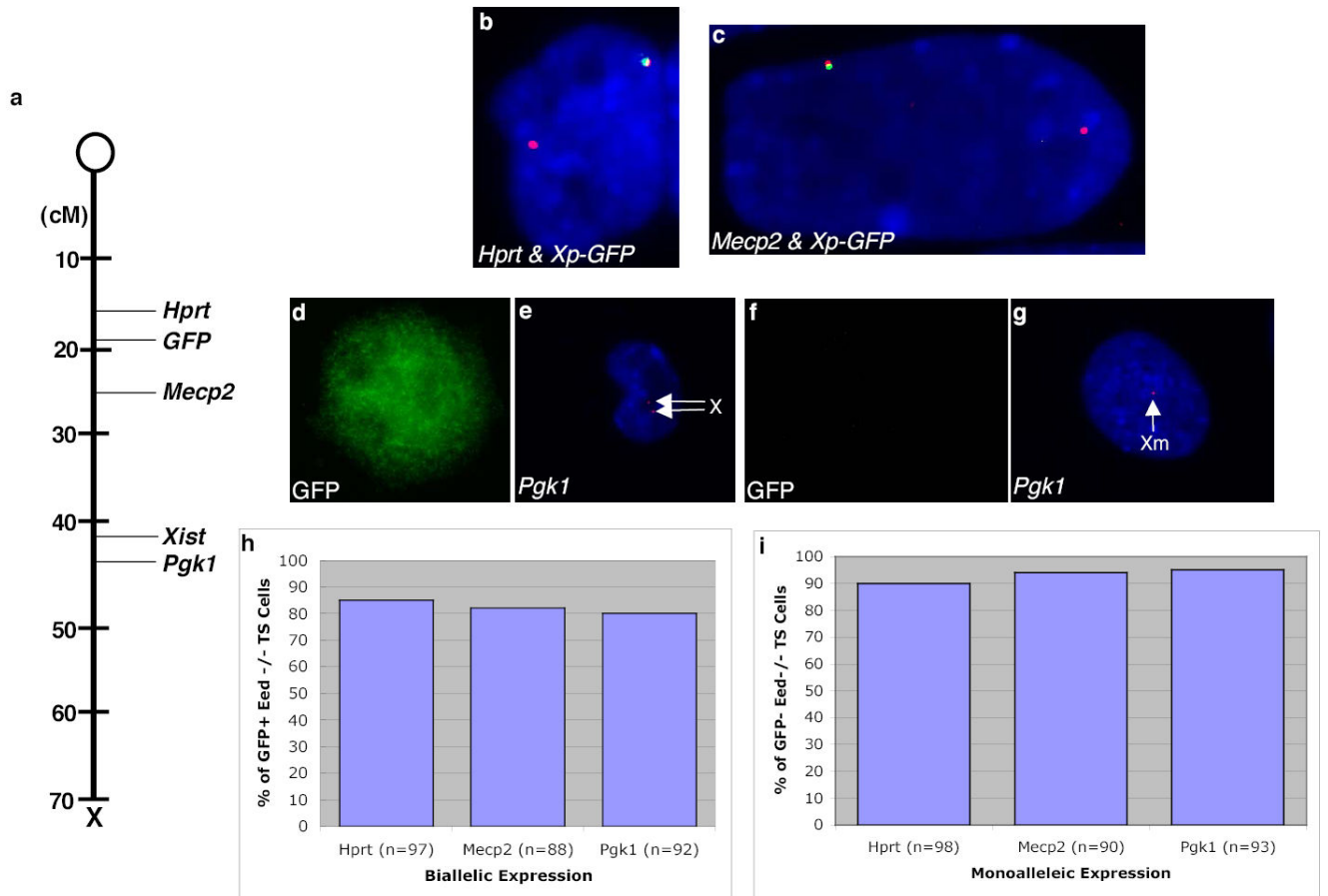
Visceral endoderm cells isolated from E6.5 mouse embryos also accumulate *Xist* RNA but not Eed on the inactive-X (Xi). Nuclei are stained with DAPI.

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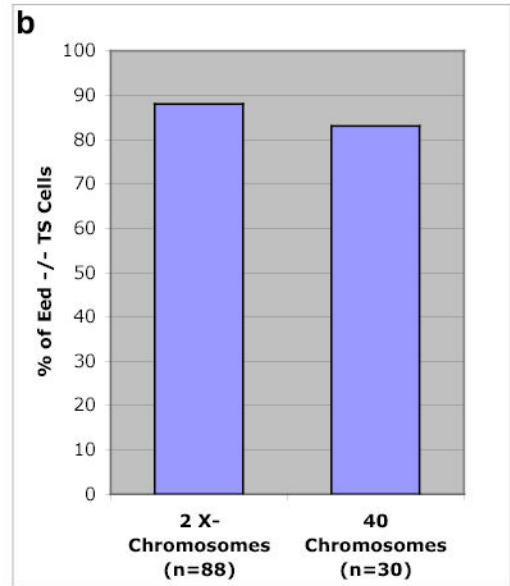
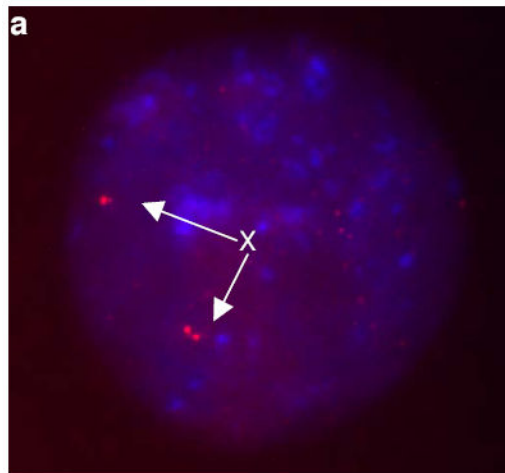
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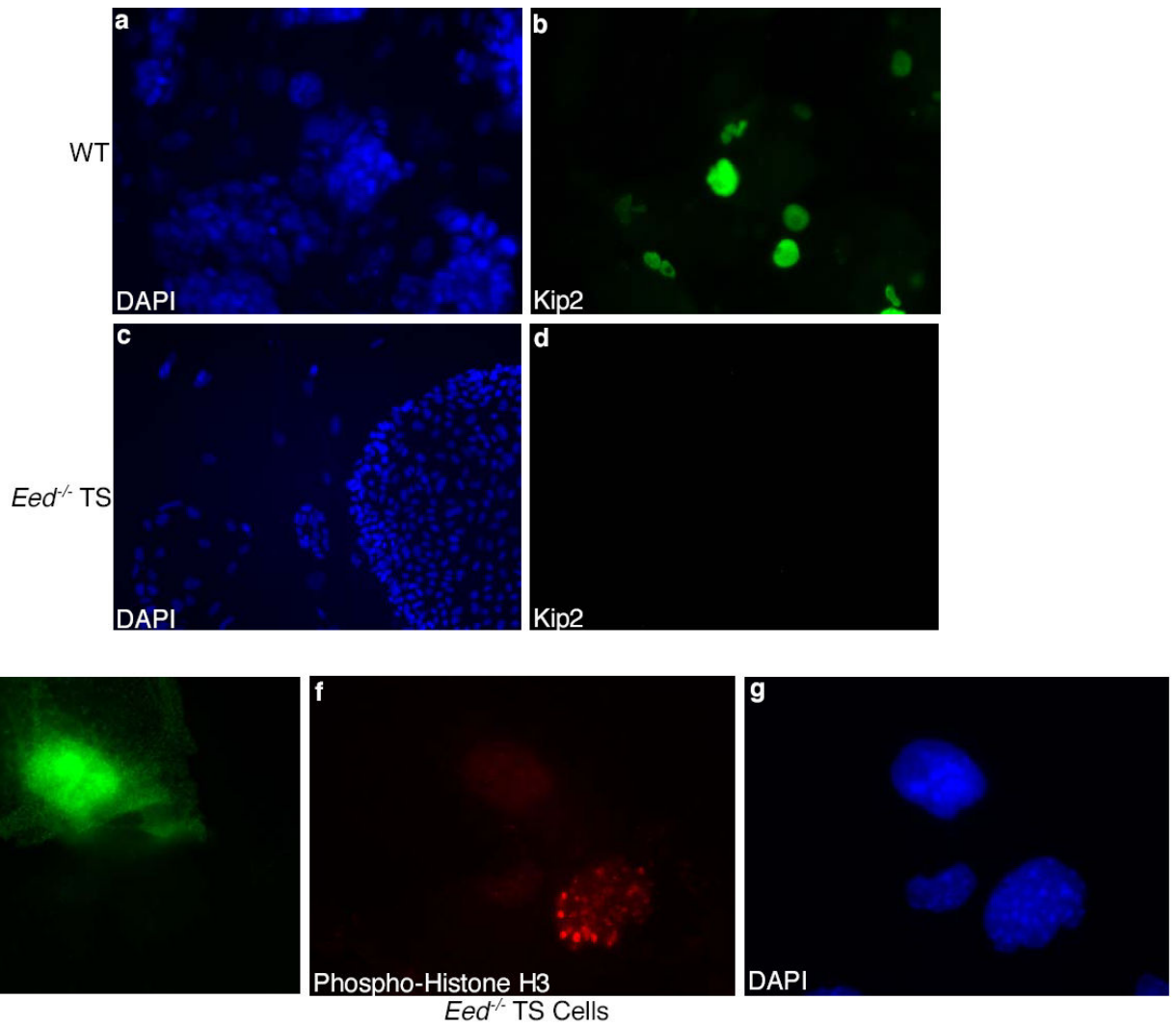
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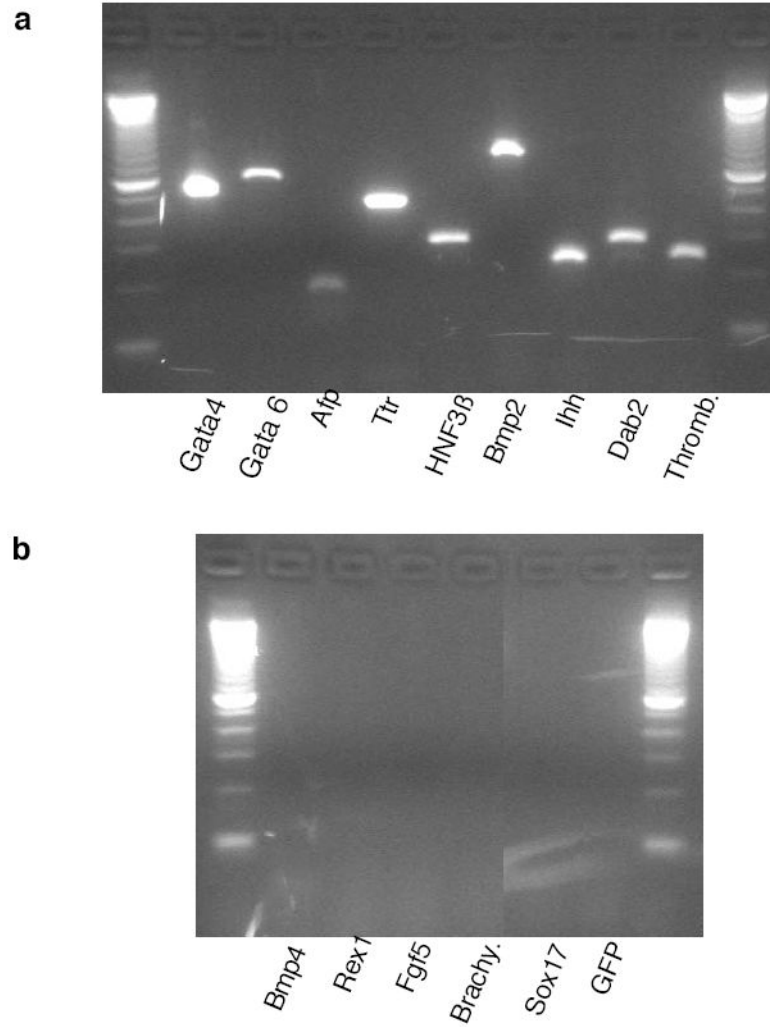
Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.



Supplementary Figure 4.