Role of Histone H3 Lysine 27 Methylation in X-Inactivation

Kathrin Plath, Jia Fang, Susanna Mlynarczyk-Evans, Ru Cao, Kathleen A. Worringer, Hengbin Wang, Cecile C. de la Cruz, Arie P. Otte, Barbara Panning, Yi Zhang

Supporting Online Material

Materials and Methods

Antibodies. Antibodies against di- or tri-methyl-K27 and tri-methyl-K9 were raised in rabbit and/or chicken with the peptides (TARK9STG) and (TKAARK27SAPST) where K9 and K27 are di- or tri-methylated, respectively. All antibodies used were affinity-purified and tested for specificity by ELISA (S1). Antibodies against Ezh2 and Eed have been previously described (S1-S3).

Cell culture and isolation of embryos. Trophoblast stem (TS) cells were kindly provided by Gary Uy (S4) and cultured as described (S5). To induce differentiation of TS cells, we removed FGF4, heparin, and EMFI-CM from the medium. The ES cell lines 2-1tet and T20: ΔSX, have been described previously (S6). ES cell lines were cultured under standard conditions (S7) and differentiation into embryoid bodies was performed as described by Martin et al. (S8). The transformed mouse embryonic fibroblasts carrying a conditional Xist allele have been described before (S9), and Cre-mediated deletion of Xist was performed as described (S10). Tet-inducible HeLa cells (Clontech) were cultured as directed by the manufacturer. Preimplantation embryos were provided by the UCSF transgenic facility.

Immunostaining. TS cells were plated onto glass slides and cultured for 2 days. The cells were then permeabilized for 5 min in cold CSB buffer (3 mM MgCl2, 0.3 M sucrose in PBS) containing 0.2% Triton X-100. After washing with cold CSB buffer twice, cells were fixed in 4% paraformaldehyde for 15 min. Cells were then incubated in blocking solution (1% BSA in PBS) for 30 min before incubating with primary antibody for 1 hour in a humidified chamber. After three consecutive 5-min washes in blocking solution, cells were incubated for a further 30 min with secondary antibody (Jackson Laboratories). Cells were washed again twice by PBS and water, then mounted in Fluorescent mounting medium (Dako). For peptide competition staining, primary antibodies were incubated with 3 or 10 µg/ml of peptides for 30 min at 37°C before adding to the cover slides. The sequences of the competing peptides are: 3mK27 (QLATKAARK3mSAPATG) and 3mK9 (ARTKQTARK3mSTGGKAPGGC) [in addition, 2mK27 (QLATKAARK2mSAPATGGV) and 2mK9 (TARK2mSTGGK) peptides were used in the ELISA analysis]. All somatic cell types and the male mouse ES cell line carrying the Xist cDNA transgene were grown on coverslips. At each time point of the differentiation course, differentiating ES cells were trypsinized and cyto-spun onto slides (Shandon). Cells were treated with ice-cold CSK (100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM Pipes, pH 6.8) for 30 seconds, CSK + 0.5% Triton-X for 30s, CSK again for 30s, and fixed for 10 min in 4% paraformaldehyde (PFA). Preimplantation embryos were treated with 0.5% Triton X-100, fixed in 4% PFA and placed on polylysine-coated slides. For immunofluorescence, cells or embryos were blocked for 30 min (5 % goat serum, 0.2% fish skin gelatin, 0.2% Tween in PBS, or in 0.5 mg/ml BSA in PBS), incubated overnight at 4° degrees or 1 hour at RT in primary antibody, washed in block or 0.2% Tween/PBS, incubated in secondary antibody (Vector Labs) for 1 hour,
washed with 0.2% Tween/PBS, stained with (DAPI) in PBS and mounted in Vectashield mounting medium (Vector Labs).

**Fluorescence in situ hybridization (FISH).** In cases when immunostaining was followed by FISH, immunostaining was carried out as described above, with the exception that tRNA (InVitrogen) and RNase inhibitors (Promega) were added with the primary antibody. After the final immunostaining wash, cells and embryos were fixed with 4% PFA then dehydrated through a 70-85-100% ethanol series. FISH was then performed as described previously (S7). Xist/XIST RNA was detected with a fluorescein-labeled single-stranded RNA (Roche) probe antisense to Xist/XIST.

**XIST/Xist cDNA transgenes, generation of stable cell lines, and induction of Xist RNA expression.** Human XIST cDNA [bases 1 to 16474 (S11)] was cloned into the vector pcDNA5/FRT (InVitrogen) from RT-PCR products and plasmids containing the large exons 1 and 6 by using appropriate oligonucleotides or restriction digests. To generate a tetracycline inducible transgene, we subsequently replaced the CMV promoter by a tetracycline-inducible promoter. In both plasmids, we subsequently introduced the puromycin selectable marker. Stable HeLa cell lines were generated by transfection of the plasmid with Fugene (Roche) and selection with puromycin for more than 10 days. We obtained the mouse ES cell line carrying an X-linked Xist cDNA transgene that lacks the A repeat (which is required for silencing) and is driven by a tetracycline-inducible promoter from R. Jaenisch (Whitehead, Cambridge, MA; cell line ∆SX) (S6). For Xist expression from the inducible XIST/Xist cDNA transgenes, cells were incubated in the presence of 2 µg/µl doxycycline for 24–48 hours.
Supplementary References


Fig. S1. H3-2mK27 is not enriched on the Xi in TS cells. Female TS cells were stained with Ezh2 (green) and H3-2mK27 (red) antibodies, and DAPI (blue). The merged picture demonstrates that H3-2mK27 is not enriched on the Ezh2-marked Xi.

Fig. S2. Characterization of the H3-3mK27 and H3-3mK9 antibodies. (A) H3-3mK9 antibody stains both centromeric heterochromatin and Xi, marked by Ezh2, in female TS cells. The centromeric heterochromatin staining was also observed when the H3-3mK9 antibody was preincubated with an H3-3mK27 peptide, but not when the antibody was preincubated with an H3-3mK9 peptide. The Xi staining was lost when the H3-3mK9 antibody was blocked with an H3-3mK27 peptide, but not when blocked with an H3-3mK9 peptide. (B) ELISA analysis of the H3-3mK27 and H3-3mK9 antibodies. The competing peptide sequences are listed in Materials and Methods and the amounts of the peptides were adjusted to molar concentration equivalent to that of the H3-3mK27 peptide (0.5 µg/ml).

Fig. S3. Enrichment of the Eed-Ezh2 complex and H3-3mK27 on the Xi is transient in differentiating TS cells. (A) Immunolocalization of Ezh2 (green) and H3-3mK27 (red) in nuclei (blue) of female TS cells differentiated for 2, 4, and 10 days (rows). The merged images demonstrate that Ezh2 and H3-3mK27 colocalize on the Xi early but not late after differentiation. (B) Quantification of the data presented in (A). The graphs depict the percentage of cells with an Xi-like accumulation for Ezh2 (circles) and H3-3mK27 (squares), respectively. More than 150 cells were analyzed for each time point.

Fig. S4. A low-power view of the entire blastocyst from which the data were taken that are presented in Fig. 3A. The region of the ICM that is enlarged in Fig. 3A is highlighted by the red
Presented is the merged image of the Eed (green), H3-3mK27 (red), and DAPI (blue) staining.

**Fig. S5.** (A) Wild type HeLa cells were analyzed by immunostaining for EED in combination with *XIST* RNA FISH. DNA is stained with DAPI. The merged picture indicates that these cells do not express *XIST* RNA (green), and EED (red) is evenly distributed throughout the nucleus. (B) Forty-eight hours after *XIST* RNA expression is induced from the *XIST* cDNA transgene in HeLa cells, EED and H3-3mK27 co-localize in the nucleus in an Xi-like domain indicating that both the EED-EZH2 complex and H3-3mK27 are enriched on the Xi. The merge shows the overlay of the stainings for EED (green) and H3-3mK37 (red).