PNAS PNAS PNAS PNAS

Supporting Information

Cerase et al. 10.1073/pnas.1312951111

SI Materials and Methods

ChIP and Microarray Analysis. For ChIP, cells in suspension were fixed in 1% formaldehyde for 10 min; quenched with 125 mM glycine for 5 min; and lysed in 1% SDS, 10 mM EDTA (pH 8.0), 50 mM Tris·HCl (pH 8.1), and protease inhibitors for 10 min at 4 °C. Chromatin was sheared to 0.3- to 0.5-kb fragments by sonication (Bioruptor; Diagenode); diluted 1:10 in 1% Triton X-100, 2 mM EDTA (pH 8.0), 150 mM NaCl, and 20 mM Tris·HCl (pH 8.1) in the presence of protease inhibitors (Roche); and precleared with 30 μL of protein A agarose beads (Millipore) for 90 min at 4 °C. Chromatin was subjected to immunoprecipitation overnight at 4 °C using 5 μL of trimethylated histone H3 lysine 27 (H3K27me3) rabbit polyclonal antibody (07-449; Upstate) in 1 mL of diluted chromatin. For all experiments, 2.5 μL of polyclonal anti-mouse IgG (7023; Sigma) was used to measure background levels. Immune complexes were purified using 30 μL of protein A agarose beads for 2 h at 4 °C. Beads were washed three times. DNA was eluted in 1% SDS and 0.1 M $NaHCO_3$ reverse cross-linked at 65 °C overnight, and phenol-chloroform extracted and precipitated with isopropanol. DNA was resuspended in 100 μL of H_2O and analyzed on a Bio-Rad Chromo4 real-time PCR system using SYBR Green PCR Master Mix (Bio-Rad) following the manufacturer's instructions. Enrichment was normalized to 1% of input DNA. For quality control, positive and negative amplification controls were chosen, based on a study by Azuara et al. (1). In order to reduce the number of PCR amplification cycles, DNA from eight ChIP experiments was pooled for each experiment in conditions with and without doxycycline. Whole genome amplification (Sigma) was performed according to the method of O'Geen et al. (2). All experiments were quality-controlled before hybridization on tiling array. Custom arrays were purchased from Nimblegen (average probe length of 60–75mer). A chromosome 17 genomic region, spanning megabase (Mb) 20–85.4 (mm8), was tiled on the array, together with control regions from other chromosomes comprising a total span of ∼2 Mb (Table S1). Cy3/5 labeling, hybridization, and scanning were performed as previously published (2). Peak detection was by TileMap software (http://jilab.biostat.jhsph.edu/software/tilemap/index.htm). A peak was called when five or more tiles were significantly above the local background. X-inactive specific transcript (Xist)-induced new peaks were called when a peak was found in two-thirds of induced samples and never in at least three noninduced samples.

Statistical Analysis. H3K27me3 enrichment/depletion in a given genomic feature is defined as the $\log_2$ ratio between the observed density and the expected density over the total genomic area represented on the microarray. Reference Sequence (RefSeq) gene coordinates were obtained from University of California, Santa Cruz (UCSC) RefSeq Gene Table (mm9) and then "lifted over" into mm8 assembly; the promoter corresponds to the 1,000 bp upstream of the transcription start site, and the 3′ end corresponds to the 1,000 bp downstream of the gene body. Bivalent domain coordinates came from a study by Ku et al. (3). Repeat element coordinates were obtained from the UCSC RepeatMasker track (mm8). To compensate for the underrepresentation of the repeat elements within the microarray, H3K27me3 peaks were scored as overlapping when their border was less than 250 bp distant from the closest repeated element. The statistical significance of the enrichment/depletion was then assessed by the Fisher exact test.

Sample Preparation for 3D Structured Illumination Microscopy. For all experiments, cells were seeded on 18 × 18-mm borosilicate glass coverslips (catalog no. 1.5H, 170-μm ± 5-μm thickness; Marienfeld Superior) 1 d before fixation. For immunostaining, cells were washed two times with PBS and fixed with 2% (vol/vol) formaldehyde/PBS for 10 min following stepwise replacement with 0.05% Tween/PBS (PBST). For permeabilization, cells were incubated in 0.5% Triton X-100/PBS for 10 min and washed subsequently two times in PBST. For immunofluorescence detection in combination with RNA FISH (immuno-RNA-FISH), cells were equilibrated in 2× SSC and incubated in 50% (vol/vol) formamide/2× SSC at 4 °C for 2–4 h. A labeled and denatured full length RNA-FISH probe was added, cells were mounted on slides and sealed with removable rubber cement, and samples were allowed to hybridize at 37 °C overnight. Unbound probes were removed with washing three times (each wash for 5 min) in 50% (vol/vol) formamide/2× SSC and at 42 °C followed by washing three times with 0.05% Tween/2× SSC (SSCT) at 42 °C, and probe detection was carried out in 2% (wt/vol) BSA/0.5% fish skin gelatin (FSG)/2× SSCT for 1 h at room temperature (RT) in a humid chamber.

For subsequent immunostaining, cells were equilibrated in 1× PBST and blocked with 2% (wt/vol) BSA/0.5% FSG/PBST for 1 h. Antibodies were diluted in blocking buffer and incubated for 1 h in a humid chamber at RT. Unbound antibodies were removed by thorough washing with 1× PBST. A detailed description of the immuno-RNA-FISH procedure for superresolution microscopy is provided elsewhere (4). After the immunostaining procedure, cells were postfixed using 4% (vol/vol) formaldehyde/PBS for 10 min and DNA was counterstained with DAPI (2 μg/mL) for 10 min. Samples were mounted in Vectashield antifade mounting medium (Vector Laboratories) and sealed with nail varnish. H3K27me3 antibodies used were purchased from Active Motif and Diagenode (catalog nos. 39535 and CS-069-100, respectively). SUZ12 (clone no. D39F6) rabbit mAb (catalog no. 3737), and Ezh2 (clone no. DC29) rabbit mAb (5246) were purchased from Cell Signaling. Eed mouse mAb has been described previously (5). Ring1b mouse mAb was a gift from Haruhiko Koseki, Yokohama, Japan.

Three-Dimensional Structured Illumination Microscopy and Image Analysis. Three-dimensional structured illumination (SI) microscopy on fixed ES cell samples and TetraSpeck beads (Invitrogen) was performed on a DeltaVision OMX V3 system (Applied Precision Imaging/GE Healthcare) equipped with a 100× magnification/1.40-N.A. PlanApo oil immersion objective (Olympus); Cascade II:512 EMCCD cameras (Photometrics); and 405-, 488-, and 593-nm diode lasers. Somatic cells were imaged with a DeltaVision OMX V3 Blaze system (Applied Precision Imaging/GE Healthcare) equipped with a 60× magnification/1.42 N.A. PlanApo oil immersion objective (Olympus); 405-, 488-, and 592-nm diode lasers; and Edge sCMOS cameras (PCO) (4). In both cases, SI image stacks were acquired with a z-distance of 125 nm and with 15 raw SI images per plane (five phases, three angles). The SI raw datasets were computationally reconstructed with channel-specific measured optical transfer functions (OTFs) and a Wiener filter set to 0.002 using the softWoRx 6.0 software package (Applied Precision) to obtain a superresolution 3D image stack with a lateral (x,y) resolution of ∼110–130 nm and an axial (z) resolution of ∼300 nm (6). Images from the different color channels were registered with alignment parameters obtained from calibration measurements with 0.2-μm diameter TetraSpeck beads using image registration with a linear-fitting model implemented using softWoRx 6.0. The voxel size of

the reconstructed images is 40 nm in *x,y* and 125 nm in *z* with a 32-bit depth. For all subsequent image processing and data analysis, images were shifted to positive values and converted to 16-bit composite tagged image file-stacks and analyzed with ImageJ (http://rsbweb.nih.gov/ij). Colocalization was analyzed in three different ways. First, for a visual qualitative estimation, signal intensities of green and red channels from all voxels of a 3D image stack were plotted as a 2D scatter plot (7). Second, the colocalization degree was quantified based on the red/green signal intensities by calculating Pearson's and Manders' coefficients (8). Pearson's coefficients were calculated on nonthresholded images from the fraction of the stack containing the "Barr body" volume (~15 z-sections covering a thickness of ~2 μm) and its surroundings or similar sized volumes of the controls (SNLs), respectively. Barr bodies were defined as volumes with an enrichment of the respective polycomb repressive complex 2 proteins or Xist RNA, including their close surroundings. The Barr body 3D mask was obtained by applying a Gaussian filter to merged channels, thresholding to remove low-intensity signals, and converting the obtained stack into a binary file that mapped all voxels of interest for coefficient calculation. Manders' coefficients were calculated for the signal intensities of voxels in a similar way. To estimate the threshold for every image stack and for both channels, a separate small 3D volume from an area outside the cell or nucleus was selected. The average intensity of this "background substack" was calculated and served as a base to calculate the threshold for Manders' coefficient calculations (9, 10). Third, nearest neighbor measurements were performed using the TANGO plug-in for ImageJ/Fiji (11). Before import into TANGO, the cropped image stacks were scaled between the mode and the maximum value by an in-house ImageJ script. The red and green signals were segmented according to a set of predefined rules: (*i*) top hat filter with a 2-pixel (px) radius in *x,y* and *z*; (*ii*) Laplace of Gauss filter with a 1-px radius in *x,y* and *z*; and (*iii*) spot detector 3D with Otsu autothresholding. Finally, a minimal distance (nearest neighbor) analysis of centroid *x,y* and *z* positions of all segmented green and red signals/spots within the Barr body mask was performed. Statistical differences in colocalization coefficients, as well as nearest neighbor distances, of different experiments were analyzed by pairwise *t* test comparison with Bonferroni correction of the level of significance. A minimum of 10 cells were analyzed with an average of 15 z-sections per cell. A similar analysis was done for TetraSpeck beads.

1. Azuara V, et al. (2006) Chromatin signatures of pluripotent cell lines. *Nat Cell Biol* 8(5):532–538.
2. O'Geen H, Nicolet CM, Blahnik K, Green R, Farnham PJ (2006) Comparison of sample preparation methods for ChIP-chip assays. *Biotechniques* 41(5):577–580.
3. Ku M, et al. (2008) Genomewide analysis of PRC1 and PRC2 occupancy identifies two classes of bivalent domains. *PLoS Genet* 4(10):e1000242.
4. Markaki Y, Smeets D, Cremer M, Schermelleh L (2013) Fluorescence in situ hybridization applications for super-resolution 3D structured illumination microscopy. *Methods Mol Biol* 950:43–64.
5. de Napoles M, et al. (2004) Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. *Dev Cell* 7(5):663–676.
6. Schermelleh L, et al. (2008) Subdiffraction multicolor imaging of the nuclear periphery with 3D structured illumination microscopy. *Science* 320(5881):1332–1336.
7. Ronneberger O, et al. (2008) Spatial quantitative analysis of fluorescently labeled nuclear structures: Problems, methods, pitfalls. *Chromosome Res* 16(3):523–562.
8. Manders EM, Stap J, Brakenhoff GJ, van Driel R, Aten JA (1992) Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. *J Cell Sci* 103(Pt 3):857–862.
9. Bolte S, Cordelières FP (2006) A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc* 224(Pt 3):213–232.
10. Costes SV, et al. (2004) Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophys J* 86(6):3993–4003.
11. Ollion J, Cochennec J, Loll F, Escudé C, Boudier T (2013) TANGO: A generic tool for high-throughput 3D image analysis for studying nuclear organization. *Bioinformatics* 29(14):1840–1841.

Fig. S1. Microarray H3K27me3 peak detection in positive and negative controls. (*A*) HoxC cluster (positive control region) is depicted, showing genes (blue), CpG islands (CGI; green), and H3K27me3 peaks (red) compared with UCSC-deposited H3K27me3 peaks from the Broad Institute (1) (assembly mm8). Tiles are shown in olive green. (*B*) Negative control region, Sox2 promoter. (*C*) Venn diagrams showing H3K27me3 domains at promoters in the region of interest for three noninduced samples vs. H3K27me3 domains at promoters, as defined by Ku et al. Orange, magenta, and green indicate three different noninduced biological replicas, and gray represents the data from Ku et al. (overlaps are 96%, 89%, and 87%, respectively). (*D*) Randomly selected preexisting H3K27me3 peak on chromosome 17. Genomic coordinates are from mm8 assembly.

1. Mikkelsen TS, et al. (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature* 448(7153):553–560.

Fig. S2. Validation of H3K27me3 new peaks by real-time PCR. (*A*, *Left*) Example of a new H3K27me3 peak. Peaks are present in at least two-thirds of doxycycline (dox)-induced samples but absent in noninduced samples. (*A*, *Right*) Example of a nonpeak interval. (*B*, *Upper*) Quantitative PCR (qPCR) of five randomly selected H3K27me3 new peaks and five nonpeak intervals. (*B*, *Lower*) Suz12 ChIP. Three biological replicas analyzing noninduced and doxycycline-treated samples are shown. Arrows indicate examples shown in *A*. Error bars represent SEM in three independent experiments. A single asterisk (*) and two asterisks (**) indicate statistical ($P \leq 0.05$) and highly statistical ($P \leq 0.001$) significance, respectively (*t* test). (*C*) Same as in *B* at 72 h after Xist induction.

Fig. S3. Gene expression analysis in undifferentiated 3E ES cells following Xist induction for 72 h. (*A*) Histogram of deregulated genes mapping to different chromosomes (red, down-regulated; blue, up-regulated). (*B*) Pie graph of down-regulated genes on chromosome 17 divided into weakly and strongly down-regulated genes (also Fig. 2). (*C*) Quantitative RT-PCR validation of randomly selected genes from three predefined categories: weak, strong, and non–down-regulated genes. Error bars indicate SD of three biological replicas. A single asterisk (*) and two asterisks (**) indicate statistical ($P \leq 0.05$) and highly statistical ($P \leq 0.001$) significance, respectively (*t* test).

Fig. S4. Segmentation of partially overlapping signals (*A*) and well-separated signals (*B*) using the TANGO plug-in for ImageJ/Fiji (11). The second row shows individual spots segmented by the applied algorithm from the 3D SI microscopy (3D-SIM) images (shown in the first row) with different shades of green and red, respectively. Evaluation masks were used to define the Barr body volume for segmentation and analysis. The centroid *x,y,z* positions of each segmented spot within the mask were used for nearest neighbor measurements. An overlay of the input image (white) and the segmented structures in the red and green channels (third row) illustrates the robustness of the segmentation procedure, with morphological characteristics of the spots being largely preserved. The example datasets show Eed (green) and Ezh2 (red) in *A* and Xist (green) and Eed (red) in *B*.

Fig. S5. Three-dimensional SIM of 0.2-μm diameter TetraSpeck beads (*A*) and immunofluorescence (IF) staining in SNL cells with one primary antibody against H3K27me3 and simultaneous detection with Alexa 488- (green) and Alexa 594- (red) conjugated secondary antibodies (*B*). Scales and magnifications are shown. Graphs illustrate nearest neighbor distance and colocalization analysis of TetraSpeck beads and for H3K27me3 from ∼15 z-stacks through a single cell or throughout the beads. A box plot [±1.5 interquartile range (IQR)] and mean (SD) of nearest neighbor green-red (g-r) distance (*Upper*) and a green-red scatter plot with Pearson's (Ps), Manders' 1 (M1), and Manders' 2 (M2) correlation coefficients (*Lower*) are displayed for the single depicted cell. Averaged values for multiple samples (10 cells per 10 TetraSpeck similar-sized regions) are shown in Fig. 4 *A* and *B*.

Fig. S6. Three-dimensional SIM of immuno-RNA-FISH with Xist RNA (red) and Suz12 (green) (*A*), Xist RNA (green) and Eed (red) (*B*), and IF detection of Ezh2 (red) and Eed (green) (*C*). Panels show a maximum intensity z-projection of DAPI (*Left*), a zoomed-in section surrounding the Xist RNA domain (*Center*), and a magnified view of four serial z-sections of 125-nm z-distance (*Right*) in the center of the Barr body region. Graphs illustrate colocalization analysis from ∼15 z-stacks. A box plot (±1.5 IQR) and mean (SD) of nearest neighbor green-red distance (*Upper*) and a green-red scatter plot with Ps, M1, and M2 correlation coefficients (*Lower*) are displayed for the single depicted cell. Values shown apply to the single depicted cell. Averaged values from multiple cells are shown in Fig. 4 *A* and *B*.

Fig. S7. Three-dimensional SIM of immuno-RNA-FISH of a C2C12 cell with Xist RNA (red) and Eed (green) (*A*), 3E ES cell with Xist RNA (red) and Ring1b (green) (*B*), and Ezh2-Ring1b IF staining (*C*). Panels show a maximum intensity z-projection of the DAPI-stained cell nuclei (*Left*), a magnified view on the Xist RNA-enriched Barr body region (*Center*), and four serial z-sections. (*Right*) Four serial z-sections with a 125-nm z-distance centered on the Barr body are shown. Graphs illustrate colocalization analysis from ~15 z-stacks. A box plot (±1.5 IQR) and mean (SD) of nearest neighbor green-red distances (*Upper*) and a green-red scatter plot with Ps, M1, and M2 correlation coefficients (*Lower*) are displayed for the single depicted cell. Averaged values from multiple cells are shown in Fig. 4 *A* and *B*. Arrows indicate sites of colocalization of green and red signals.

Table S1. List of control microarray tiling regions

Non-Ch17 H3K27me3 controls	± Control	UCSC coordinates (mm8)	Length
Hox			
HoxA	+	Chr6: 52,000,000–52,230,900	230,901 bp
HoxB	+	Chr11: 95,970,848–96,200,766	229,919 bp
HoxC	+	Chr15: 102,700,887–102,870,000	169,114 bp
HoxD	+	Chr2: 74,400,000–74,590,000	190,001 bp
Constitutive on X			
Constitutive H3K9/27me3	+	ChrX: 99,580,000–99,760,000	180,001 bp
ChIP controls			
Nanog	–	Chr6: 122,672,213–122,675,251	3,039 bp
Sox2	–	Chr3: 34,835,954–34,842,937	6,984 bp
Math1/Atoh1	+	Chr6: 64,656,735–64,660,811	4,077 bp
Sox1	+	Chr8: 12,392,333–12,397,508	5,176 bp
Msx1	+	Chr5: 38,106,742–38,112,705	5,964 bp
Random			
ChrX (1)	Random	ChrX: 72,400,000–72,590,000	190,001 bp
ChrX (2)	Random	ChrX: 5,400,000–5,590,000	190,001 bp
Chr12	Random	Chr12: 33,900,000–34,100,000	200,001 bp
Chr18	Random	Chr18: 23,900,000–24,100,000	200,001 bp
Total			1.8 Mb

Genomic coordinates are from mm8 assembly.