Supplementary Methods

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Animals and embryos production

All experiments were conducted according to the French and European regulations on care and protection of laboratory animals (EC Directive 86/609, French Law 2001-486 issued on June 6, 2001). CD1-Crl female and male mice were purchased from Charles River Laboratories, France.

Embryos were collected from (CD1-Crl) females mated with males of the same genotype. Blastocysts were collected from females at E3.25 in EmbryoMax M2 Medium
(Millipore Bioscience ref MR-015D) and cultured for five hours in EmbryoMax KSOM (Millipore Bioscience ref MR-121D) with 10µM MG132 (Sigma ref M7449-200UL) to prevent blastomere mitotic exit.

Regular ESC culture conditions.

ES cells were cultured on 0.1% gelatine (SIGMA, G1890-100G) in DMEM + GlutaMax-I (Gibco, 31966-021), 10% FCS (Gibco 10270-098), 100 µM 2-mercaptoethanol (Gibco, 31350-010), 1× MEM non-essential amino acids (Gibco, 1140-035) and 10 ng ml^{-1} recombinant LIF (MILTENYI BIOTEC, 130-099-895). Cells were passaged 1:10 every 2–3 days. When indicated, cells were grown in 2i-containing medium - 1 µM PD0325901 (Axon Medchem Bv Axon-1408), 3 µM CHIR99021 (Axon Medchem Bv Axon-1386) - either directly supplemented to FCS/LIF medium (for mitotic shake off and chromatin studies), or added to serum-free medium - 0.5X DMEM/F12 (Gibco 31331093), 0.5X Neurobasal (Gibco 21103049), 0.5% N2 supplement 100X (Gibco 17502048), 1% B27 supplement 50X (Gibco 17504044), 10µg/mL Insulin (Sigma I1882-100MG), 2 mM L-Glutamine (Invitrogen 91139), 0.05% BSA (Sigma A3311-10G), 100 µM 2-mercaptoethanol (Gibco, 31350-010), 10 ng/ml recombinant LIF (MILTENYI BIOTEC, 130-099-895).

Derivation of Esrrb-GFP, Nanog-GFP and Oct4-GFP ESCs.

E14Tg2a cells were stably transfected with CAG-Nanog-GFP-IRES-puromycin^R or CAG-Nanog-GFP-IRES-puromycin^R transgenes. ECKOiE cells (ref fest NCB) were stably transfected with CAG-Esrrb-GFP-IRES-puromycin^R and selected by culture in the absence

of doxycycline. For all cell lines, clones expressing low, comparable and homogeneous levels of the fluorescent proteins were selected.

Derivation of Ccna-GFP Sox2-AID ES cells.

Ccna-GFP cells (Festuccia et al.,NCB,2016) were lipofected with 3ug of a linearised targeting vector designed to insert a 5x Gly linker –Auxin inducible degradation domain-IRES Blasticidin^{R} cassette at stop codon of Sox2 and 1ug of pU6_CBh-Cas9-T2A-mCherry (Addgene no. 64324) driving expression of the gRNA 3'- CAGGGGCAGTGTGCCGTTAA -5'. 48 hours later selection was added and after 2 weeks single colonies were picked, expanded, and correctly targeted cells identified by PCR on genomic DNA and sequencing. ESC lines homozygous for the desired recombination product, were further lipofected with 3ug of a linearised targeting vector designed to insert a CAG-OsTir-T2a-Neomycin^{R} cassette at the TIGRE locus and 1ug of pU6_CBh-Cas9-T2A-mCherry (Addgene no. 64324) driving expression of the gRNA 3'- ACTGCCATAACACCTAACTT-5'. 48 hours later selection was added and after 2 weeks single colonies were picked, expanded, and correctly targeted cells identified by PCR on genomic DNA and sequencing. One hetherozygous line, Ccna-GFP Sox2-AID c1.2, was used for the experiments described.

Imaging.

Embryo immunofluorescence.

Embryos were fixed for 24 hours at 4°C with 2 mM Di(N-Succinimidyl) glutarate (DSG -Sigma Aldrich ref 80424-5MG) in phosphate-buffered saline (PBS1X) and then for 20 min at room temperature in 4% paraformaldehyde (Euromedex ref 15714). Incubation with primary and secondary antibodies was performed in PBS1X, 0.1% Triton-X100, 10% donkey serum. The following antibodies were used: anti-Nanog (1:100 ; rabbit CosmoBio ref REC-RCAB0002PF), anti-Esrrb clone H6705 (1 :100 ; mouse R&D Systems ref PP-H6705-00), Alexa 488-nm anti-rabbit (1:300; Invitrogen ref A21206) and Alexa 647-nm anti-mouse antibody (1:300; Invitrogen ref A31571). Embryos were incubated at room temperature for 2 hours with primary antibodies and for 1 hour with secondary antibodies. Nuclei were counterstained with Hoechst 33342 (1.6 µM; Sigma Aldrich, B2261-25MG), and embryos were placed individually in glass bottom microwell dishes (MatTek Corporation) in PBS1X. Fluorescent images were obtained using a confocal laser-scanning microscope (LSM800; Zeiss) with the

objective Plan-apochromat 20 X /NA 0.8, speed 6, pinhole 1 airy unit, and laser intensities suited for optical section thickness of 2 µm. Images shows one embryo out of the four of the same experiment which harbored mitotic cells. The experiment was performed four time independently.

Culture conditions for all imaging experiments.

Cells were plated on IBIDI hitreat plates coated overnight with poly-L-ornithine 0.01% (Sigma, P4957) at 4 °C, washed and coated 2 h with laminin (Millipore, CC095) 10 µg ml^{-1} in PBS.

Immunofluorescence on fixed cells.

Fixation: Cells growing in IBIDI dishes were washed with PBS twice and fixed in the following conditions. PFA: Cells were incubated for 10 min in 1 ml PBS 1% formaldehyde (Thermo, 28908) at room temperature. DSG and DSG+PFA: Cells were crosslinked in 1 ml of freshly prepared PBS-DSG 2 mM at pH 7.0 (Sigma, 80424-5 mg) for 50 min at room temperature. For DSG+PFA only: After washing in PBS once, cells were incubated for 10 min in 2 ml PBS 1% formaldehyde (Thermo, 28908) at room temperature. Glyoxal and Glyoxal+PFA: Fresh fixative solution was prepared before each experiment mixing 2.835 ml H2O, 0.789 ml ethanol, 0.313 ml glyoxal 40% stock (Sigma-Aldrich, #128465), 0.03 ml acetic acid. The solution was brought to 5 with NaOH. Cells were incubated for 30 min in the fixative solution on ice. For Glyoxal +PFA only: Cells were then washed with PBS and incubated for 10 min in 1 ml PBS 1% formaldehyde (Thermo, 28908) at room temperature.

Staining: All cells were washed twice in PBS and permeabilised with PBS/0.1% v/v Triton-X100 for 15 min at room temperature. Blocking was performed for 30 min at room temperature in PBS/0.1% v/v Triton-X100 supplemented with 3% of donkey serum (Sigma, D9663) (blocking buffer). Primary antibodies (diluted in blocking buffer to the concentrations indicated below) were applied 2 hours at room temperature or overnight at 4°C in a volume of 1ml per dish. After three washes in PBS/0.1% v/v Triton-X100, secondary antibodies (indicated below and diluted to 2µg/ml in blocking buffer) were applied for 1 hour at room temperature. Cells were washed three times in PBS/0.1% v/v Triton-X100 and nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, D9542). Primary antibodies were used at the following concentrations: anti-Nanog rat polyclonal (E-bioscience, Cat # 14-5761-80)1:500; Oct4 (Santa Cruz Biotechnology, Cat # sc-5279), 0.4µg/ml; Sox2 (Santa Cruz Biotechnology, Cat # sc-17320), 0.4µg/ml; ; Esrrb

(Persaeus Proteomics, PP-H6705-00), 2 μg/ml. Secondary antibodies: Alexa Fluor 594 AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch, Cat #711-585-152); Alexa Fluor 488 AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, Cat #715-545-150); Alexa Fluor 488 AffiniPure Donkey Anti-Goat IgG (H+L) (Jackson ImmunoResearch, Cat #705-546-147); Alexa Fluor 488 AffiniPure Donkey Anti-Rat IgG (H+L) (Jackson ImmunoResearch, Cat# 712-545-150). Imaging was performed on a LSM800 Zeiss microscope using a 64x oil-immersion objective.

ES cells treated with hesperadin. E14Tg2a cells growing in IBIDI plates were treated with nocodazole 50 ng ml^{-1} for 4 h and hesperadin (Selleck Chemicals, S1529) 500nM for 15min prior to fixation with DSG/PFA and staining as described above. Primary antibodies: goat anti-Oct4 (Santa Cruz, sc8628; 1:500) and rabbit anti-Aurkb (Abcam, ab2254; 1:500). Secondary antibodies donkey anti-goat and anti-rabbit (711-545-152 Alexa 594 and 705-585-147 Alexa 488 respectively, 1:500, Jackson Immuno Research). Images of 500 nm z-steps were acquired on a Nikon Eclipse Ti inverted microscope equipped with a 63x oil-immersion objective (N.A. 1.4), a Hamamatsu ORCA Flash 4.0LT camera using the NIS Elements 4.3 software.

Spinning-disc live imaging. Esrrb-GFP, Nanog-GFP, Sox2-GFP, Oct4-GFP and Oct4-RFP ES cells growing in IBIDI plates were switched to phenol red-free medium (Gibco, 31053-028) (FCS/LIF culture conditions). Cells were incubated with 500 nM Hoechst-33342 for 20 min before imaging. During the imaging experiment the cells were kept at 37 °C in a humidified atmosphere (7% CO_2). Image of 600 nm z-step stacks were acquired with a 63× oil immersion objective on a Zeiss AxioObserver Z1 microscope equipped with a Yokogawa CSUX1 spinning-disk confocal scanner, a Hamamatsu EMCCD ImageEM X2 camera using the Volocity acquisition software.

Spinning-disc live imaging for FRAP. Cells were transferred to red-phenol-free medium before imaging and kept at 37 °C in a humidified atmosphere (7% CO_2) during the entire duration of the experiments. Images were acquired using a 63× oil immersion objective on a Zeiss AxioObserver Z1 microscope equipped with Yokogawa CSUX1 spinning-disc confocal set-up, a Hamamatsu EMCCD ImageEM X2 camera and Volocity imaging software. For FRAP, 5 frames were acquired before bleaching (20 ms pulse using a 488 nm laser, spot of minimal size) and then recovery was imaged for 1 min or 30 seconds (1 image each 110 ms) for interphase and mitosis respectively (pixel size 0.2 μm). For FLIP, before each

acquisition, two areas of 10 x 10 pixels (2 x 2 μm) located at the opposite end of the cell being analysed were bleached using a 488 mm laser. Bleaching and acquisition cycles were repeated at maximum speed (310 ms), for a total time of 190 seconds. For FRAP, fluorescence recovery was analysed in Matlab as described previously (Mueller et al. 2012), with one modification: the bleached region was located automatically by subtracting the first pre-bleach image from the last post-bleach image and calculating the two-dimensional convolution (conv2 function) of this image with a circle of the typical size of a bleach spot.

Preparation of mitotic populations.

E14tg2a cells were grown to 70–80% confluence in T150 flasks. Vigorous shake-off was performed to detach debris, dying cells or poorly attached cells colonies, medium discarded and flasks washed twice in PBS. After 4-5 h in medium containing 50 ng ml^{-1} nocodazole (Sigma, M1404), flasks were gently washed with PBS. Gentle shake-off was performed in 10 ml of medium, monitoring the process under a microscope to avoid detaching clumps of cells in interphase. The cell suspension was filtered through a 10μm filter (pluriSelect, 43-50010-03) by gravity and cells spun down. Typically, around 10^7 cells were obtained from each preparation. Purity of mitotic preparations was systematically checked under a fluorescence microscope after fixation, cytospinning and DAPI staining. Preparations with more than 5% contamination from interphase were discarded.

Chromatin preparation for TF-binding analysis.

Fixation (DSG + PFA). 10^7 ES cells were crosslinked in 2 ml of freshly prepared PBS-DSG 2 mM at pH 7.0 (Sigma, 80424-5 mg) for 50 min at room temperature with occasional shaking. After pelleting and washing once in PBS, cells were incubated for 10 min in 2 ml PBS 1% formaldehyde (Thermo, 28908). Crosslinking was stopped with 0.125 mM glycine for 5 min at room temperature. Cells were pelleted and washed with ice-cold PBS. *Fixation PFA:* ES cells (10^7) were resuspended in 3 ml of pre-warmed DMEM/FCS/LIF and crosslinked for 10 min at room temperature adding 1% formaldehyde (Thermo, 28908). Crosslinking was stopped with 0.125 mM glycine for 5 min at room temperature. Cells were pelleted and washed with ice-cold PBS.

Chromatin preparation: For interphase preparation exclusively, cells were resuspended in 2 ml of swelling buffer (25 mM Hepes pH 7.95, 10 mM KCl, 10 mM EDTA) freshly supplemented with 1× protease inhibitor cocktail (PIC-Roche, 04 693 116 001) and 0.5% NP-40. After 30 min on ice, the suspension was passed 40 times in a dounce (only for asynchronous populations). For mitosis and interphase preparations: cells were centrifuged and resuspended in 300 µl of TSE150 (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl pH8, 150 mM NaCl) buffer, freshly supplemented with 1× PIC. Samples were sonicated in 1.5 ml tubes (Diagenode) using a Bioruptor Pico (Diagenode) for 7 cycles divided into 30 s ON–30 s OFF sub-cycles at maximum power, in circulating ice-cold water. After centrifugation (30 min, full speed, 4 °C), the supernatant was stored at −80 °C. Five microlitres was used to quantify the chromatin concentration and check DNA size (typically 200–350 bp).

Chromatin immunoprecipitation (ChIP).

For ChIP-qPCR and ChIP-Seq experiments, chromatin from 2.10^6 and 10^7 cells was used respectively. Chromatin was pre-cleared for 3 hours rotating on-wheel at 4 °C in 300 µl of TSE150 containing 50 µl of pG Sepharose beads (Sigma, P3296-5 ML) 50% slurry, previously blocked with BSA (500 µg ml^{-1}; Roche, 5931665103) and yeast tRNA (1 µg ml^{-1}; Invitrogen, AM7119). Immunoprecipitations with anti-Esrrb mouse monoclonal (Perseus Proteomics, H6-705-00) (1 µg per $2*10^6$cells, 5 µg per 10^7 cells), anti-Nanog rabbit polyclonal (Cosmobio, Cat. REC-RCAB001P) (0.6 µg per $2*10^6$cells, 3 µg per 10^7 cells); anti-Oct4 goat polyclonal (Santa Cruz Biotechnology, Cat # sc-5279) (0.6 µg per $2*10^6$cells, 3 µg per 10^7 cells); anti-Sox2 goat polyclonal (Santa Cruz Biotechnology, Cat # sc- 17320) (0.6 µg per $2*10^6$cells, 3 µg per 10^7 cells) were performed overnight rotating on-wheel at 4 °C in 500 µl of TSE150. Twenty microlitres was set apart for input DNA extraction and precipitation. Twenty-five microlitres of blocked pG beads 50% slurry was added for 4 h rotating on-wheel at 4 °C. Beads were pelleted and washed for 5 min rotating on-wheel at room temperature with 1 ml of buffer in the following order: 3 × TSE150, 1 × TSE500 (as TSE150 but 500 mM NaCl), 1× washing buffer (10 mM Tris-HCl pH8, 0.25M LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 mM EDTA), and 2 × TE (10 mM Tris-HCl pH8, 1 mM EDTA). Elution was performed in 100 µl of elution buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8) for 15 min at 65 °C after vigorous vortexing. Eluates were collected after centrifugation and

beads rinsed in 150 µl of TE-SDS1%. After centrifugation, the supernatant was pooled with the corresponding first eluate. For both immunoprecipitated and input chromatin, the crosslinking was reversed overnight at 65 °C, followed by proteinase K treatment, phenol/chloroform extraction and ethanol precipitation.

General library preparation.

End repair: Precipitated DNA was resuspended in 37.5ul of water and mixed with 2ul of 10M dNTPs, 5ul of NEB T4 ligase buffer, 2.5ul of NEB T4 polymerase (M0203L), 0.5ul of NEB Klenow polymerase (M0210L) and 2.5ul of NEB T4 PNK (M0201L). Samples were incubated 30min at 20^{o}C in a thermocycler. DNA was purified with SPRI beads: 90ul of bead suspension (see SPRI bead preparation section below) and 50ul isopropanol were added and samples transferred to a 96 well plate. After incubating for 5 min, the plate was put on a 96S Super Ring Magnet (Alpaqua, A001322), beads were allowed to separate completely, and the supernatant removed without disrupting the bead pellet. Beads were washed twice with 200ul of 70% Ethanol and the supernatant completely removed. DNA was eluted in 21ul of water. A-Tailing: 20ul of sample were mixed with 2.5ul of NEB Buffer #2, 1ul of 5mM dATP, 1.5ul of NEB Klenow 3'-5' exo minus (M0212L), and incubated at 37^{o}C for 30min in a thermocycler. DNA was purified with SPRI beads as before, but using a volume of 45ul of beads and 25ul isopropanol. DNA was elute in 20ul of water. Adaptor ligation: 19ul of sample were mixed with 2.5ul of NEB T4 ligase buffer, 1.25ul of a 0.2uM solution of annealed adapters, and 2.5ul of NEB concentrated T4 ligase (M0202M) and incubated overnight at 16^{o}C. For a description of the adapter preparation and sequence see below. DNA was purified with SPRI beads as before, but using a volume of 35ul of beads and no isopropanol, eluting in 20 ul of water. Library amplification: 19.5 ul of sample were mixed with 1ul of a 1:10 dilution of Quant-iT Picogreen dye (Invitrogen, P11496), 25ul of KAPA HiFi HotStart 2x master Mix (KK2502), 1ul of 10uM PCR 1.0 and 1ul of PCR 2.0 primers (See below).The amplification mix was distributed in two wells of a Roche LightCycler 384 plate (4729749001) and on a Roche LightCycler 480 II instrument (05015243001) using the following program:

Step	Temperature	Duration	
Amplification	98	1 min	1 cycle
	98	10 sec	Number of cycles according to detected amplification
	64	20 sec	
	72	45 sec	
Last cycle	98	10 sec	1 cycle
	64	20 sec	
	72	45 sec	
	Down to 37	For ever	

Fluorescence was monitored and amplification stopped during the exponential phase. Samples were removed from the plate and purified with SPRI beads using 70ul beads, no isopropanol, and eluting in 40ul of water. 1ul was used to measure the DNA concentration with a Qubit 3 and the provided reagents (Invitrogen, Q33218). 1 ng of DNA was used to check fragment size with a D1000 High Sensitivity Screentape and appropriate reagents (Agilent, 5067-5584, 5067-5585) on an Agilent 2200 Tapestation.

Adapters were designed in house. 22.5ul each of 40uM ssDNA Barcoded and Universal adaptor solutions (see below) were mixed with 5ul of NEB buffer 2, and annealed in a thermocycler.

Barcoded adapter: 5'P-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC IIIIII NNNNNNNN ATCTCGTATGCCGTCTTCTGCTTG. 5'P indicates the presence of a 5'phosphate group. IIIIII represent the six base index nucleotides, and NNNNNNNN a stretch of 8 random nucleotides used for single molecule barcoding.

Universal adapter:

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T.

* indicates the presence of a phosphorothioate bond between the last C and T.

For Mnase the libraries were sequenced (PE150) by Novogene Co. Ltd. ChIP-seq was sequenced by the BioMics facility of the Institut Pasteur.

SPRI Bead preparation:

1 ml Sera-Mag™ Magnetic SpeedBeads™, carboxylated, 1 µm, 3 EDAC/PA5 (GE Healthcare Life Sciences, Cat #65152105050250) were washed 3 times with a TE-Tween solution (10 mM Tris HCl pH 8, 1 mM EDTA, 0.05% Tween 20, pH 8.0) and resuspended in TE-Tween-20% PEG 8000 solution (10 mM Tris HCl pH 8, 1 mM EDTA, 0.05% Tween 20, pH 8.0).

MNase-seq and H3 ChIP-seq on MNase digested chromatin.

10^7 ESCs were resuspended in 3 ml of pre-warmed DMEM/FCS/LIF and crosslinked for 10 min at room temperature adding 1% formaldehyde (Thermo, 28908). Crosslinking was stopped with 0.125 M glycine for 5 min at room temperature. Cells were pelleted and washed with ice-cold PBS. 2.5 x 10^6 cells were resuspended in 500ul of MNase buffer (50mM Tris-HCl PH8, 1mM CaCl2, 0.2% Triton) supplemented with protease inhibitor cocktail (PIC-Roche, 04 693 116 001).Cells were pre-incubated for 10 min at 37°C and ½ U, 16 U or 128 U of MNase (Expressed in KUntiz, 1 Kunitz is equivalent to 10 gel units, NEB M0247S) added to the reaction. Cells were incubated for further 10 min at 37°C, inverting the tubes occasionally. The reaction was stopped on ice by adding 500ul of 2x STOP buffer (2% Triton, 0.2% SDS, 300mM NaCl, 10mM EDTA). Tubes were left rotating overnight on a wheel to allow diffusion of the digested fragments. The cell suspension was spun down and the supernatant stored at -80°C.

For Mnase-seq: 250 ul (for ½ U digestion conditions) or 25ul (for 16U and 128U digestion conditions) of chromatin were brought to a final volume of 250ul and 1% SDS concentration. Samples were incubated o/n at 65°C to revert the crosslinking. The next day 250ul of TE and 5ul of 20 mg/ml Proteinase K (Eurobio, GEXPRK) were added and samples incubate 2 hours at 37°C. DNA was phenol/chloroform extracted, and ethanol precipitated. Samples were resuspended in 50ul of TE and 2 ul of RNase (Roche, 11119915001) were added. After 30 minutes incubation at 37°C DNA was purified with SPRI beads as described (see The Library Preparation section), adding 90ul of beads and 50 ul isopropanol, and eluted in 40ul of water. 1ul was used to measure the DNA concentration with a Qubit 3 and the provided reagents (Invitrogen, Q33218). 1 ng of DNA was used to check fragment size with a D1000 High Sensitivity Screentape and appropriate reagents (Agilent, 5067-5584, 5067-5585) on an Agilent 2200 Tapestation. In case genomic DNA of high molecular weight was observed, the samples were absorbed on SPRI beads, using a 0.5x SPRI beads/sample ratio, beads

discarded, and SPRI beads added to the supernatant to reach a 1.8x bead/sample ration. MNase libraries were preparated as described (see the Library Preparation section) using a starting amount of 10-20ng.

For H3 ChIP-seq: 250 ul (for ½ U digestion conditions) or 50ul (for 16U and 128U digestion conditions) of chromatin were brought to a final volume of 500ul adding TSE150 (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl pH8, 150 mM NaCl) buffer, freshly supplemented with protease inhibitor cocktail (PIC-Roche, 04 693 116 001). ChIP was performed as described (See the Chromatin immunoprecipitation section), using 5 ug of anti-Histone H3 rabbit polyclonal (Abcam, Cat # ab1791).

FACS.

Ccna-GFP Sox2-AID ES cells were cultured in the presence of 0.5mM of auxin (Sigma, I5148) for 3 hours. During the last 20 minutes 20µM Hoechst-33342 was added. Cells were trypsinised, filtered through a 40µm cell strainer and kept on ice. ES cells were sorted on the basis of Hoechst and GFP levels in three populations (early G1, late G1 and G2) using a FacsARIA III (Becton-Dickinson), keeping samples and collection tubes at 4 °C. Data were analysed using the FlowJo software suite (Tree Star). Around 10^6 cells were sorted per population before RNA extraction.

RNA isolation and library preparation.

Cells were lysed with 1mL TRIzol (ThermoFisher) and RNAs extracted according to manufacturer's protocol. To eliminate any genomic DNA contamination, this was followed by an additional DNAse I treatment (Qiagen 79254) for 20min at 37°C followed by phenol:chloroform purification. RNAs were resuspended in Ultrapure DNAse/Rnase Free Distilled Water (Thermo 10977035). Ribo-depleted, stranded and paired-end RNA-seq libraries were prepared and sequenced by Novogene Co Ltd.

Western Blot

For Western Blot analysis cell pellets corresponding to 106 cells were resuspended in 100 µl Laemmli Sample Buffer (161-0737 BIO-RAD) containing β-mercaptoethanol. Lysed pellets were boiled for 10 min at 95oC and centrifuged for 10 min at maximum speed at room temperature.

Typically 10 µl per sample was loaded on 4-15% Mini-PROTEAN TGX Stain-Free Gels (4568086 BIO-RAD) and run in 1x SDS-Running Buffer (250 mM Tris/ 1.92 M Glycine/1% SDS) at 10-20 mA using the Mini-PROTEAN Tetra System (BIO-RAD). Proteins were transferred on nitrocellulose membrane (Amersham Protran 10600003) for 1 hour at 300mA using the wet transfer system (BIO-RAD) in 1x Transfer Buffer (10x 0.25M Tris/ 1.92M Glycine) prepared with a final concentration of 20% Ethanol. Membranes were blocked in PBST (PBS 0.1% Tween-20) 5% BSA for 1 hour at room temperature and incubated over night at 4 oC with primary antibodies (diluted in PBST 5% BSA). Excess antibodies were washed with PBST (5 washes, 5 min each) and incubated for 1 hour at room temperature in secondary antibodies HRP-conjugated (diluted in PBST 5% BSA). Membranes were washed 5 times, 10 min each at room temperature and incubated with PIERCE ECL2 Western Blotting Substrate (80196 Thermo Scientific 5 min in dark. After excess reagent was removed, proteins were visualised using the BIO-RAD Chemidoc MP Imaging System and processed using the Image Lab Software (BIO-RAD).

Antibodies used:

Primaries: anti-H3 Abcam ab1791 (1:5000), anti-Sox2 R&D AF2018 (1:500-1:1000)

Secondaries: anti-Rabbit IgG-HRP Thermo Fisher RB230254 (1:10.000), anti-Goat IgG-HRP Thermo Fisher 61-1620 (1:10.000)

ATAC-Seq

For each experiment 5 x 10^4 cells were used. Cells were harvested, counted, washed once with ice-cold PBS and spun down. Supernatants were completely removed and pellets were placed on ice. Each pellet was resuspended in 25 µl TD buffer (2× reaction buffer from Nextera kit; Illumina, cat. no. FC-121-1030). After the addition of 2.5 µl of transposase TDE1 (Nextera Tn5 Transposase from Nextera kit; Illumina, cat. no. FC-121-1030) and 22.5 µl nuclease free water, reactions were mixed and incubated for 30min at 37^{o}C in gentle agitation. Reactions were stopped by adding the appropriate volume of Binding Buffer (Qiagen MinElute PCR Kit) and the DNA was purified using the Qiagen MinElute PCR Kit according to manufacturer's protocol. The purified DNA, eluted in 10 µl, was either stored at -20^{o}C or used directly for library preparation.

Library preparation for ATAC:

Reactions were prepared mixing 10 µl transposed DNA, 2.5 µl 25 µM primer Ad1.noMX, 2.5 µl 25 µM primer Ad2 from (Buenrostro et al. 2013), see also: (https://media.nature.com/original/nature-assets/nmeth/journal/v10/n12/extref/nmeth.2688-S1. pdf), 9 µl Nuclease Free Water, 1 µl of a 1:8 dilution of Quant-iT Picogreen dye (Invitrogen, P11496) and 25ul of KAPA HiFi HotStart 2x master Mix (KK2502). Reaction mix was transferred to qPCR plates and amplification was carried out under the following conditions:

Step	**Temperature**	**Duration**	
Pre-amplification	72	5 min	1 cycle
	98	30 sec	
	98	10 sec	5 cycles
	63	30 sec	
	72	1 min	
Amplification	98	30 sec	1 cycle
	98	10 sec	Number of cycles according to amplification, usually 11-12
	63	30 sec	
	72	1 min	

After amplification libraries were purified with SPRI beads (as described in the general library preparation section), using a sample to bead ratio of 1: 1.4. 1ul was used to measure the DNA concentration with a Qubit 3 and the provided reagents (Invitrogen, Q33218). 1 ng of DNA was used to check fragment size with a D1000 High Sensitivity Screentape and appropriate reagents (Agilent, 5067-5584, 5067-5585) on an Agilent 2200 Tapestation. Libraries were sequenced (PE150) by Novogene Co. Ltd.

Computational Methods.

Data and availability.

Samples are summarised in Table S1. Briefly, at least 2 x Interphase and 2 x Mitosis ChIP-seq samples were sequenced for DSGPFA Esrrb, Sox2, Oct4, Nanog; For sox2 RNA-seq 3 x (EG1, LG1, G2) x (-Auxin, +Auxin) samples were sequenced. For nucleosome positioning and accessibility data , 3 x (0.5U, 16U, 128U) x (Interphase, Mitosis) for MNase-seq, 3 x (0.5U, 16U, 128U) x (Interphase, Mitosis) for MNase H3 Chip and 2 x (Interphase, Mitosis) for ATAC-seq. Public datasets used in this study: Esrrb RNA-seq GSE75066 (Festuccia et al. 2016); Esrrb, Sox2, Oct4 and Naong PFA ChIP-Seq GSE11431 (Chen et al. 2008); Sox2, Oct4, Nanog PFA ChIP-seq GSE11724 (Marson et al. 2008); Sox2, Oct4, Nanog PFA ChIP-seq GSE44288 (Whyte et al. 2013); Sox2 PFA ChIP-seq GSE43275 (Aksoy et al. 2013); Sox2 Interphase and Mitosis PFA ChIP-seq GSE89599 (Deluz et al. 2016); Sox2, Oct4 Interphase Mitosis PFA ChIP-seq GSE92846 (Liu et al. 2017); Oct4 G1, G2/M PFA ChIP-seq GSE78073 (Shin et al. 2016).

ChIP-seq Data Processing.

For all ChIP-seq samples (those generated for this study, and public datasets) reads were aligned with bowtie2 (Langmead and Salzberg 2012) in the mm9 genome, with options "-k 10" for all samples/ Reads were additionally filtered for those with a single discovered alignment (mapping quality: 255) and an edit distance less than 4. For libraries prepared with custom barcodes (Table S1), reads aligning with identical coordinates were collapsed to those with distinct barcodes. For non-barcoded libraries in which duplication rate was > 10%, reads with identical alignment coordinates were collapsed to one, and otherwise duplicate reads were retained. Proper treatment of duplicate reads is essential for identification of those ChIP-seq peaks that are bookmarked. Peaks bookmarked in mitosis are commonly smaller, and thus will contain a lower rate of genuine, non-PCR, duplicates. Inappropriate collapse of genuine duplicates will therefore overestimate bookmarking signal.

RNA-seq Data Processing.

Stranded paired end RNA-seq reads were aligned to the mm9 genome using STAR (Dobin et al. 2013) and quantified by RSEM (Li and Dewey 2011) using the RSEM-STAR pipeline, with additional options "--seed 1618 --calc-pme --calc-ci --estimate-rspd --forward-prob 0.0 --paired-end".

MNase-seq, MNase H3 ChIP-seq and ATAC-seq data processing.

Paired end reads were trimmed by aligning read pairs to discover regions of reverse complementarity surrounded by either our custom adapters for MNase-seq or Nextera sequencing adapters for ATAC-seq. Alignment and trimming was performed with the BioSequences package for Julia 0.6 (Bezanson et al. 2015). Similarly to ChIP-seq processing, reads were aligned to mm9 genome using bowtie2 (Langmead and Salzberg 2012) with options "-k 10 -I 0 -X 1000 --no-discordant --no-mixed", and filtered for reads with a single alignment mean edit distance less than 4 between read pairs. MNase-seq and MNase H3 ChIP-seq were sequenced with barcoded adapters, barcodes with identical position were collapsed.

ChIP-seq Peak calling.

Peaks were called against relevant inputs/controls for all samples using macs2 (Feng et al. 2012) with "callpeak -q 0.2 -g mm". Peaks intersecting with the mm9 blacklist (ENCODE Project Consortium 2012) were excluded along with those on chrM and chrY. To determine a set of candidate binding we merged peaks in all DSGPFA samples in interphase and mitosis, and we further filtered peaks to have an FDR < 0.01 in at least replicates of either interphase or mitosis samples.

ChIP-seq Bookmarking Analysis.

To determine the set of peaks bookmarked for Esrrb, Oct4, Sox2 and Nanog we combined macs2 peak call statistics with RNA-seq style differential expression calls offered by the negative binomial generalised linear model of DESeq2 (Love et al. 2014). Here we refer to a peak as the region identified by DSGPFA samples merged for a given factor. In addition to previous filtering, we restrict our attention to high quality binding regions. First, we required that a peak have a height > 0.8 reads per million (RPM) in one sample, and the maximum called FDR < 10^{-10} over all samples. This resulted in 33,933 peaks for Esrrb, 16,441 peaks for Oct4 and 26,368 peaks for Sox2 and 27,195 peaks for Nanog. As the expectation is that bookmarking does not occur in the majority of binding regions tested, we opted for a strategy of evaluating a differential occupancy signal of interphase and mitosis peaks over input and a differential signal between interphase and mitosis. We counted the total number of unnormalised reads falling into peaks in 2 x interphase and 2 x mitosis samples along all input

samples, and evaluated differential occupancy with DESeq2. We performed Wald tests on the model ~ChipTF + ChipM, where ChipTF is a factor differentiating chip samples from input samples, and ChipM is a factor identify mitosis samples. We tested ChipTF (evidence of a peak over input in interphase), ChipM (difference between interphase and mitosis peaks) and the sum of ChipTF and ChipM (evidence of a mitosis peak over input). In this strategy, we expect the majority of ChipTF comparisons to be found significant, and a minority of Chip M and ChipTF+ChipM to be significant. Thus, the median geometric mean normalisation approach of DESeq is inappropriate. We consequently set the size factors to the library size (total mapped reads) of each sample, and thus the GLM parameters can be interpreted as log rates of the occurrence of reads within peaks. Each test was evaluated for significance with FDR < 0.05 and without the use of independent filtering. To determine the set of bookmarked peaks we required that a peak had differential occupancy with FDR < 0.05 for ChipTF+ChipM and additionally that these peaks had been called with macs2 FDR <0.01 in all mitosis replicates, and that at least one mitosis peak had been called with FDR < 10^{-10}. Combined, this is a conservative strategy that helps ameliorate the effects of contamination from interphase cells. Then, used the significance of ChipM to classify the type of bookmarking: BI (I>M), BS (I=M), BM (M > I). This resulted in Esrrb: Lost (L): 23,787, BI: 2,848, BS: 5,762 and BM:1,534 regions. Sox2: L 25,793, BI: 556, BS:18. Oct4: L: 16,293, BI: 76, BS:25, BM:1 regions. Nanog: L 27,177, BI: 18 regions.

RNA-seq Differential expression analysis.

RSEM estimated read counts per sample were rounded for use with DESeq2 (Love et al. 2014). Genes with at least 10 normalised counts in all replicates of at least one condition were considered for differential expression analysis. For all differential expression tests DESeq2 was run without independent filtering and without any fold change shrinkage, genes with padj < 0.05 are considered differentially expressed. Genes responding to Esrrb-depletion (EKOIE doxycycline inducible Esrrb: GSE75066 (Festuccia et al. 2016)), or Sox2 Auxin-inducible degradation were determined at cell cycle stages early G1 (EG1), late G1 (LG1) and G2. We performed Wald tests under the model ~CC + TF + CC:TF, where CC is a factor indicating cell cycle: EG1, LG1 or G2 and TF is a factor describing the presence or absence of the relevant TF in that sample. We tested the sum of TF and its interaction determine to determine a set of responsive genes in each stage of the cell cycle.

Gene Peak Proximity Enrichments.

We determined whether genes responsive in EG1 to depletion of Esrrb or Sox2 were proximal to candidate Esrrb or Sox2 bookmark peaks. We calculated the distance between the TSS of each gene tested for differential expression to the set of candidate bookmark peaks. We then performed Fisher exact tests for the association between the set EG1 responsive genes to the set of all genes within *x*bp of a peak, for *x* in $[10^0, 10^8]$.

Transcription Factor Motif Detection.

To discover transcription factor binding motifs in the mm9 genome assembly we used FIMO (Grant, Bailey, and Noble 2011) with parameters "--thresh 1e-3 --max-stored-scores 50000000" and supplied a 0-order Markov background file describing the relative nucleotide frequencies in the mm9 assembly. We scanned for Esrrb motif MA0141.3 and Oct4-Sox2 composite motif MA0142.1. We intersected these motifs with Esrrb, Oct4, Sox2, and Oct4-Sox2 peaks. Metaplots are centred on the best scoring motif within each peak.

Correction for MNase-seq Enzymatic Cutting Bias.

We used a *k*-mer approach to correct for the enzymatic sequence preferences of MNase. We took the two 6-mers lying over the end coordinates of each read, such that each 6-mer was composed of two 3-mers, one lying within the read and one lying outside. By evaluating position weight matrices (PWM) of the k-mers at the left and the right of each read, we found a directional model of the cutting bias to be suitable. Specifically, we found the left PWM to be approximately equal to the reverse complement of the right PWM. If γ_i is the rate of occurrence of k-mer k_i in the genome, and ρ_i is the rate of occurrence in MNase cutsites and if k_L and k_R are the left and right k-mers, then we weight each read by $\sqrt{\rho_R \rho_L / \gamma_R \gamma_L}$. Kmers were counted using the BioSequences package in Julia 0.6 (Bezanson et al. 2015).

MNase-seq and ATAC-seq meta plots.

For MNaseq-seq and MNase H3 ChIP-seq, we calculated the rate of occurrence of nucleosomal fragment (140-200bp) mid-points surrounding the central feature of interest, that is, to generate the point clouds in Fig. 5 and Fig. 6 we scored the single base-pair

corresponding to the mid-point of barode-collapsed cutting bias weighted reads. For ATAC-seq we similarly marked the two end points of fragment in the 0-100 bp range shifted inward by +/- 4bp as recommended by (Buenrostro et al. 2013).

To identify nucleosome positioning or accessibility robustly over a range of sites and sequencing depths we employed Gaussian Process Regression on mid-point or cut-site data of MNase-seq or ATAC-seq respectively. We used a squared exponential covariance function and selected hyper-parameters for signal variance, lengthscale and noise variance, optimised on -500bp to +500bp window surrounding the mid-point of each metaplot. For the most, this window contains the activity of interest and the covariances appear relatively stationary within this region as compared to outside where clearly the length and noise scales change as the data loses coherence. We selected hyperparameters by sparse gaussian process regression employing a variational approximation to the likelihood (Titsias 2009) as offered by GPy (GPy, n.d.), with an initial inducing input once per 10bp.

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