

Fig. S1. Additional information on *Nanog* heterogeneity.

(A) Schematic representation of the two *Nanog* alleles in *Nanog*-GFP reporter cells (TNG; Chambers et al. 2007). Selection with Puromycin leads to a fully NANOG-GFP+ve homogeneous population (Chambers et al. 2007, Navarro et al. 2012). **(B)** H3K9me3 ChIP-qPCR analysed and presented as in Fig. 1C, D ($p=0.02373$ and $8.404e-06$ for the IR and promoter region, respectively). **(C)** Relative mRNA levels (ctl set to 1) of *Nanog*, measured by RT-qPCR and normalised to *Tbp*, upon 3d of PD or CH treatment of FCS+LIF cultured cells. The increase observed upon PD treatment was tested with Student's t test ($p=0.009019$ for E14Tg2a and 0.002947 for the two $\Delta K9$ clones combined). **(D)** Quantification (z score) of NANOG and OCT4 immuno-staining in untreated E14Tg2a cells (ctl; $n=2053$) and after 3d of CH ($n=1554$) or PD ($n=1857$) treatment, as indicated. The changes in NANOG distribution in PD treated cells were assessed with a KS test ($p < 2.2e-16$). **(E)** Representative image of smFISH assays using a *Nanog* mRNA probe in the indicated cell lines. **(F)** Quantification of the number of *Nanog* mRNA molecules per cell for each indicated cell line. The distributions were compared with a Chi-squared test ($p < 2.2e-16$).

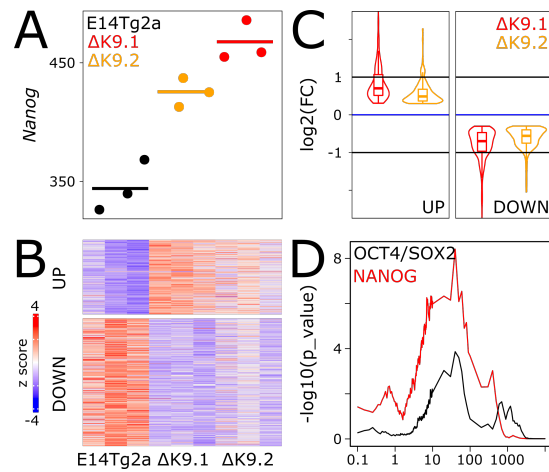


Fig. S2. Gene expression consequences in $\Delta K9$ cells. (A) Confirmation of *Nanog* upregulation in $\Delta K9$ cells by RNA-seq (Transcripts Per Million, TPM). **(B)** Z scored heatmap of genes identified as differentially expressed in $\Delta K9$ cells. **(C)** Violin-Boxplot of gene expression fold-changes measured in $\Delta K9.1$ and $\Delta K9.2$ clones compared to wild-type E14Tg2a cells. **(D)** Statistical association (Y-axis, $-\log_{10}(\text{Fisher exact test p-value})$) between the differentially expressed genes shown in (B) with the presence of NANOG (red) or OCT4/SOX2 (black) binding sites as a function of the distance (X-axis, kb).

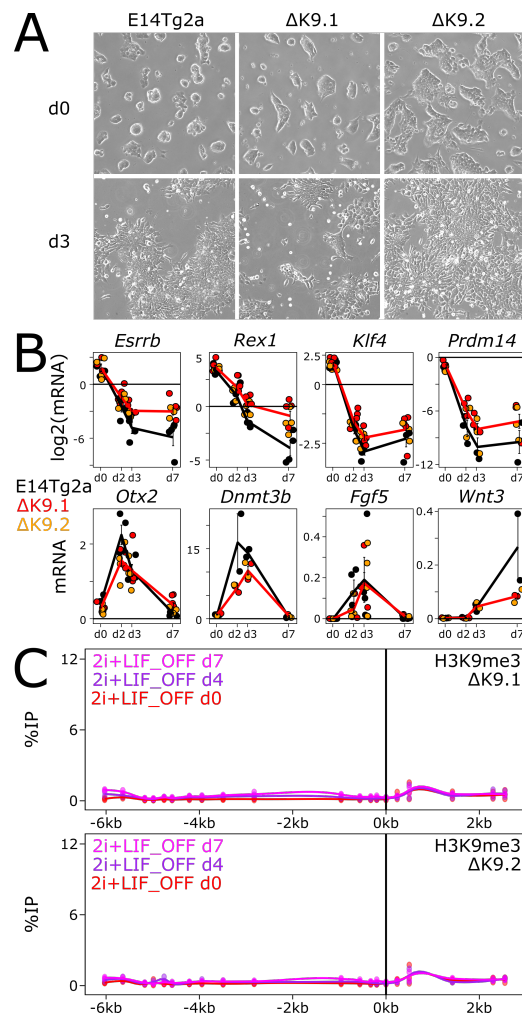


Fig. S3. Differentiation of wild-type and Δ K9 cells upon 2i+LIF withdrawal.

(A) Representative bright-field photomicrographs of wild-type (E14Tg2a) and Δ K9 cells cultured in 2i+LIF (top) and after 3 days of withdrawal (bottom). **(B)** RT-qPCR analysis of markers of naïve pluripotency (top) and differentiation (bottom) after 0, 2, 3 and 7 days of 2i+LIF withdrawal. *Tbp* was used for normalisation. Differences in naïve marker downregulation were assessed with a Student's t test at the day of strongest reduction in wild-types (d7 for *Esrrb* and *Rex1*; d3 for *Klf4* and *Prdm14*; $p=0.02361, 0.007429, 0.01211, 0.08776$, respectively). For differentiation markers, d2 was used ($p=0.0214, 0.1431, 0.1604$ for *Otx2*, *Dnmt3b* and *Fgf5*, respectively) except for *Wnt3* (d7 $p=0.135$). **(C)** Analysis of H3K9me3 at *Nanog* in Δ K9.1 and Δ K9.2 cells analysed and presented as in Fig. 3B, showing a lack of enrichment throughout differentiation.

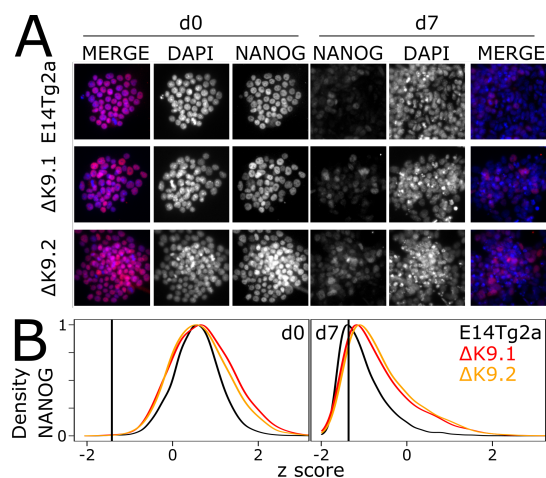


Fig. S4. General retention of low NANOG levels in differentiating Δ K9 cells.

(A) Representative illustration of undifferentiated (left) and differentiated (right; 7d of 2iLIF withdrawal) E14Tg2a, Δ K9.1 and Δ K9.2 cells stained for NANOG and DNA (DAPI).

(B) Quantification of NANOG levels in the conditions described in (A), presented as in Fig. 2C. The vertical line shows the median of differentiated E14Tg2a cells. Note that the vast majority of Δ K9 cells present higher NANOG levels than E14Tg2a cells after 7 days of differentiation (KS test $p < 2.2e-16$).

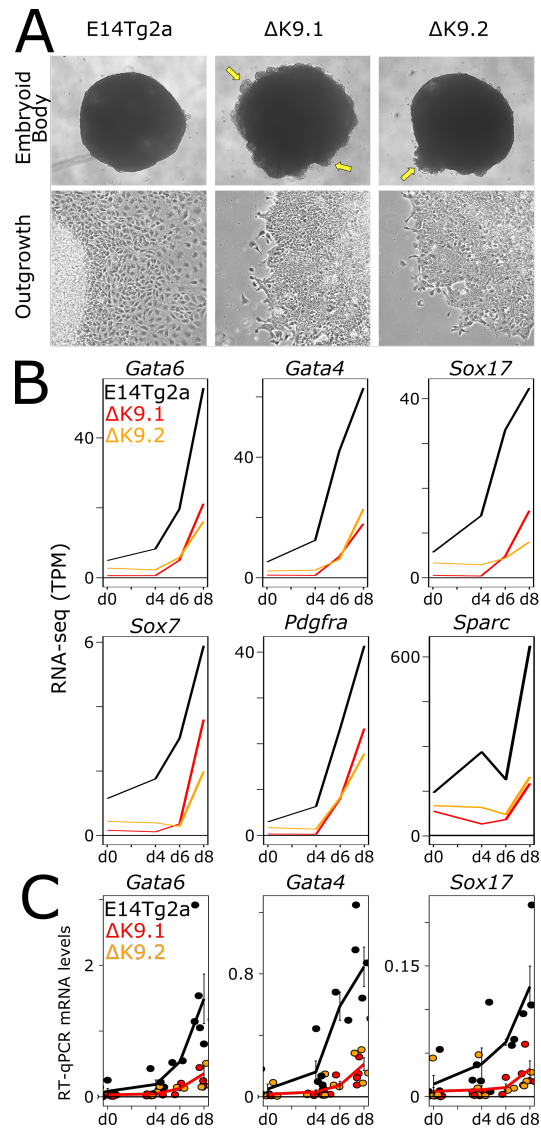


Fig. S5. Differentiation of wild-type and Δ K9 cells into embryoid bodies.

(A) Representative bright-field photomicrographs of wild-type (E14Tg2a) and Δ K9 embryoid bodies (top) and their derived cellular outgrowths (bottom). The arrows point to defective sealing of EBs periphery. **(B, C)** Analysis of endoderm markers after 0, 4, 6, 8 days of embryoid body differentiation, by RNA-seq (B) or by RT-qPCR (C). *Tbp* was used for normalisation.

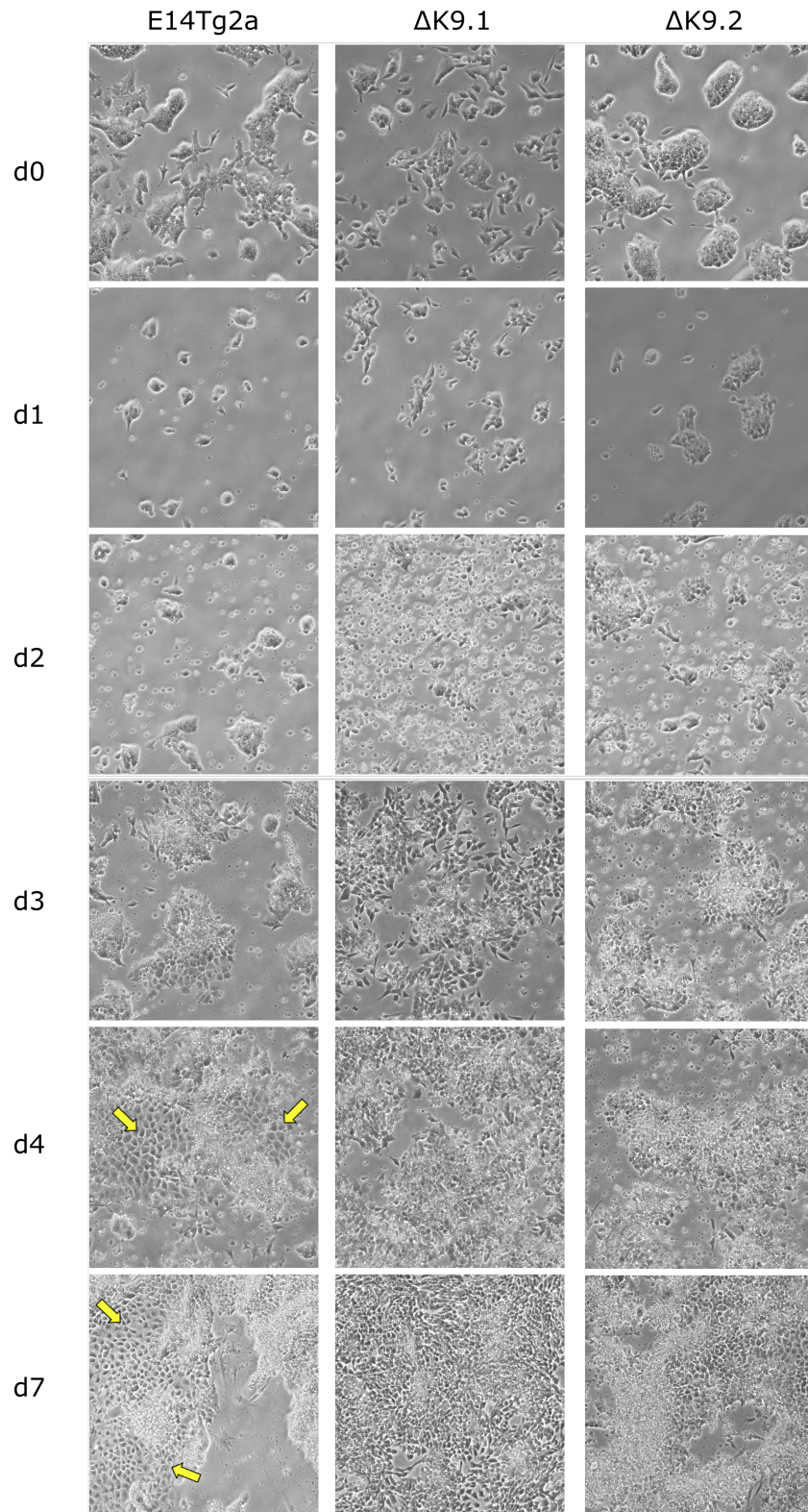


Fig. S6. Directed differentiation of wild-type and $\Delta K9$ cells into primitive endoderm. Representative bright-field photomicrographs of wild-type (E14Tg2a) and $\Delta K9$ cells during their treatment with a primitive endoderm differentiation protocol. The arrows point to clusters of primitive endoderm cells.

Supplementary Materials and Methods.

Cell Culture

General ES cell culture conditions.

ES cells (E14Tg2a; Δ K9 clones; TNG (Nanog-GFP reporter cells), a kind gift of Pr. Ian Chambers; TKO (triple knock-out DNMTs); ZKO (*Zfp57* knock-out), a kind gift of Basilia Acurzio & Andrea Riccio) were cultured (37°C, 7%CO₂) on 0.1% gelatine (SIGMA, Cat# G1890-100G) in DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Gibco, Cat# 31966-021), 10% FCS (Sigma, F7524), 100 μ M 2-mercaptoethanol (Gibco, Cat# 31350-010), 1 \times MEM non-essential amino acids (Gibco, Cat# 1140-035) and 10 ng/ml recombinant LIF (MILTENYI BIOTEC, Cat# 130-099-895). For TNG, when specified, puromycin was used at 2 μ g/ml. When indicated, cells were grown in serum-free 2i-containing medium (1 μ M PD0325901 and 3 μ M CHIR99021; Axon1408 & Axon1386 respectively): 0.5X DMEM/F12 (Gibco, Cat# 31331093), 0.5X Neurobasal (Gibco, Cat# 21103049), 0.5X N2 supplement 100X (Gibco, Cat# 17502048), 0.5X B27 supplement 50X (Gibco, Cat# 17504044), 10 μ g/ml Insulin (Sigma, Cat# I1882-100MG), 2 mM L-Glutamine (Invitrogen, Cat# 91139), 37.5 μ g/ml BSA (Sigma, Cat# A3311-10G), 100 μ M 2-mercaptoethanol (Gibco, Cat# 31350-010), 10 ng/ml recombinant LIF (MILTENYI BIOTEC, Cat# 130-099-895). Cells were seeded at 0.02M to 0.04M cells/cm², media changed every 2 days and cells passaged every 3-4 days. All 2i+LIF analyses were performed after at least 3-4 passages in 2i+LIF. All experiments inhibiting ERK or GSK3b were performed with 1 μ M PD0325901 and 3 μ M CHIR99021, respectively. Cells were karyotyped and regularly tested mycoplasma-free.

Culture conditions of MEFs, XEN and TS cells.

Mouse Embryonic Fibroblasts (MEFs) were derived from F1 129sv/129sv E13.5 male embryos and cultured (37°C, 7%CO₂), for no more than 4 passages, in DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Gibco, Cat# 31966-021), 10% FCS (Sigma, F7524), 100 μ M 2-mercaptoethanol (Gibco, Cat# 31350-010), 1 \times MEM non-essential amino acids (Gibco, Cat# 1140-035). When indicated, MEFs were treated for 3 days with 1 μ M PD0325901. Extra-Embryonic Endoderm (XEN) cell lines (Artus et al., 2010) were routinely cultured (37°C, 7%CO₂) in DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Gibco, Cat# 31966-021), 10% FCS (Sigma, F7524), 100 μ M 2-mercaptoethanol (Gibco, Cat# 31350-010), 1 \times MEM non-essential amino acids (Gibco, Cat# 1140-035) on 0.1% gelatine, without LIF, and passaged every 4 days. Trophectoderm Stem (TS) cell lines (F2 and F3) were initially isolated from F1 129/Sv Hprt-4 Pgk1a \times 129/Sv embryos using a published protocol (Tanaka et al., 1998) and cultured (37°C, 5%CO₂) in RPMI 1640 Medium, GlutaMAX™ Supplement (Gibco, Cat# 61870036) with 20% FCS (Sigma, F7524), 1 mM sodium pyruvate, 100 μ M β -mercaptoethanol (Gibco, Cat# 31350-010), 25 ng/ml hrFGF4 (235-F4-025, R&D) and 1 μ g/ml heparin (H3149-10KU, Sigma) on mitomycin-treated MEFs. Cells were passaged every 2-3 days. For TS analyses, MEFs were removed by adsorption onto gelatinized culture dishes for 1.5 hours.

Derivation of Δ K9 cells.

gRNAs (5'- CAGAGGAGGGCTTAAGAGAT and 5'- CACTCTAACCCAGCTTAAGT) were designed and cloned under the control of a U6 promoter in a vector conferring puromycin resistance, as described (Heurtier et al., 2019). 1 μ g of each gRNA-expressing vector and of a Cas9/mCherry expression vector (Addgene#64324) were lipofected in E14Tg2a cells according to manufacturer's instructions (Lipofectamine 2000; ThermoFisher). Lipofected cells were selected with Puromycin (1 μ g/ml) and FACS-sorted for mCherry fluorescence. Puromycin-resistant and mCherry-positive cells were seeded at clonal density and ~100 clones were picked 10 days later. Clones were screened by 2 independent PCR (LongAmp Taq PCR kit; BioLabs Cat# E5200S) with primers spanning the deletion (DelCTRL_F/R; N12c_F/N17b_R; Table S2), by real-time PCR using primers along the *Nanog* locus (Table S2), and by cloning and sequencing of PCR products (N12c/N17b). Two karyotypically normal independent clones, Δ K9.1 and Δ K9.2, were selected for this study.

Clonal assays.

600 cells were plated in single wells of 6-well plates coated overnight with poly-L-ornithine 0.01% (Sigma, Cat# P4957) at 37°C, washed twice with PBS 1X and coated 2 h with 1X laminin (Sigma, Cat# L2020). After 7 days, cells were fixed and stained using an alkaline phosphatase staining kit (Sigma Aldrich, Cat # 86R-1KT) according to the manufacturer's instructions or by fixation 20 min in PFA 4%, 2 washes with PBS 1X, incubation 15 min at RT in the dark in a staining solution (TrisMaleate 1M, MgCl₂ 1M, 100 mg/ml α -naphthyl-phosphate, 100 mg/ml Fast-Red TR), 2 washes with PBS 1X, 1 wash with milliQ-water. Colonies were counted under a stereo-microscope (NIKON-SMZ1500).

2i_{OFF} differentiation.

Cells were adapted from FCS+LIF to 2i+LIF for 3-4 passages, harvested and seeded at 10,000 cells/cm² on Poly-L-ornithine/Laminin coated cell culture treated surfaces, in 2i+LIF medium but omitting PD0325901, CHIR99021 and LIF. The medium was changed daily.

Embryoid Bodies differentiation.

Embryoid Bodies (EBs) imaged in Fig.S5A (top) were obtained with a hanging-drop protocol where a suspension of 0.1M cells/mL was distributed in 20 μ l drops (2000 cells/drop) onto inverted cover plates of several dishes filled with PBS 1X to avoid evaporation. The day after, the aggregates formed in each drop were pooled and seeded on non-cell culture treated dishes and cultured in 10%FCS-DMEM without LIF for 7 additional days. EB differentiation for outgrowths (Fig.S5A, bottom) and RNA-seq analysis (Fig.S5B) was performed by seeding 0.04M cells/cm² in 10%FCS-DMEM/LIF on 0.1% gelatinized cell culture treated dishes. After 3 days, the aggregates were harvested (2min trypsinization), transferred to non-cell culture treated dishes for 4 days in 10%FCS-DMEM medium without LIF and then replated at very low density on 0,1% gelatinized cell culture treated dishes for 2 and 4 additional days in 10% FCS-DMEM medium without LIF.

Primitive endoderm differentiation.

ES cells cultured in FCS+LIF were harvested and seeded onto 0.1% gelatinized single wells of μ -slide 4 well^{Ph+} ibiTreat (Ibidi GmbH Ref#80446) at 37000 cells/cm² and differentiated as previously described (Anderson KGV et al., 2017). They were first cultured for 24H in endoderm base medium (EBM): RPMI 1640 Medium, GlutaMAX™ Supplement (Gibco, Cat# 61870036) supplemented with 2% B-27 minus insulin (Gibco, Cat# 15285074) and 100 μ M 2-mercaptoethanol (Gibco, Cat# 31350-010). Subsequently, Activin A (100 ng/ml; R&D, Cat# 338-AC-010), CHIR99021 (3 μ M) and LIF (10 ng/ml) were added (inductive PrE medium). The medium was changed every day.

Commitment assays.

Cells cultured in 2i+LIF were plated at clonal density (600 cells per well of 6-well plates) and subject to 2i_{OFF} differentiation, as described above. After each day, the medium was changed into 2i+LIF for 7 days, after which cells were fixed and stained for alkaline phosphatase activity as described.

Preparation of mitotic cells.

To obtain mitotic ES cells (>95% purity as assessed by DAPI staining and microscopy), we used a nocodazole shake-off approach, as described before (Festuccia et al., 2018).

Imaging analyses.

Bright field microscopy.

Cell culture pictures were taken on a Nikon Eclipse Ti-S inverted microscope equipped with: CFI S Plan Fluor ELWD $\times 20$ objective; 89 North PhotoFluor LM-75; Hamamatsu ORCA-Flash 4.0LT camera; NIS Elements 4.3 software.

Immunofluorescence of NANOG/OCT4 in E14Tg2a cells.

Cells were trypsinized, counted and fixed for 10 min in 4% formaldehyde (Sigma Cat#F8775) at room temperature and quenched immediately after with Glycine 125 mM for 5 min at room temperature. After one wash in PBS 1X, they were cytospun at 0.5M cells/200 μ l/spot (4 min – 300 rpm – low speed) on Superfrost+ slides. After washing the cells twice in PBS 1X, they were permeabilized with cold PBS 1X/0.5 % v/v Triton X-100 for 5 min, washed twice with cold PBS 1X, and blocked in PBS1X/1% Donkey Serum (DS) (Sigma, Cat# D9663) for 30 min on ice. Cells were incubated overnight at 4°C with primary antibodies (diluted in PBS1X/1% DS) within a humid chamber. After three washes in cold PBS1X, cells were incubated 1H at room temperature in the dark with secondary antibodies (diluted in PBS1X/1%DS), washed three times in PBS1X and nuclei counterstained with Vectashield antifade mounting medium with DAPI (Vectorlabs, Cat#H-1200). Imaging was performed with an inverted Nikon Eclipse X microscope equipped with: X20/0.45 (WD 8.2-6.9) objective; LUMENCOR excitation diodes; Hamamatsu ORCA-Flash 4.0LT camera; NIS Elements 4.3 software. Cell Profiler (Carpenter AE et al., 2006) was used for quantifications and ggplot2 (Wickham et al. 2016) for plotting in R.

c3/ Comparative immunofluorescence of NANOG (E14Tg2a vs mutant cells Δ K9, TKO or ZKO).

WT and each mutant cell line were trypsinized, counted and resuspended at 1M/ml in FCS free medium (DMEM-Glutamax/100 mM 2-mercaptoethanol/NEAA 1X) into sterile 1.5 mL Eppendorf tubes. Cells were then individually incubated either with 10 μ M Rhodamine Red dye (Invitrogen, Cat#CMTPX C34552) or 1 μ M Deep Red dye (Invitrogen, Cat#C34565) for 30 min at 37°C. The labeled cells were then collected by centrifugation, washed with PBS1X, resuspended in DMEM/10%FCS+LIF medium and mixed at a 1:1 ratio for WT^{Rhod} and mutant^{DeepRed} cells (usually \sim 0.4M each). The opposite labeling (WT^{DeepRed} & mutant^{Rhod}) was also performed with identical results. Mixed cells were seeded into a Poly-L-Ornithine/Laminin coated single well of a μ -slide 4 well^{Ph+} ibiTreat (ibidi GmbH Ref#80446) and incubated for \sim 6H at 37°C and 7% CO₂. Cells were then fixed directly into the well by freshly prepared PFA 4% (Fisher Scientific, Cat# 16431755) for 10 min at room temperature in the dark, washed twice in PBS1X for 10 min and used immediately for immunostaining or stored at +4°C for short time. Cells were washed in PBS-Tw0.1% (PBS1X/0.1 % Tween20 (Sigma, Cat#P9416)) and permeabilised with PBS1X/0.1% Triton X-100 (Sigma, Cat#T8787) for 10 min at room temperature. After three washes with PBS-Tw0.1%, cells were blocked with PBS-Tw0.1%/10% Donkey Serum (Sigma, Cat#D9663) for 30 min on ice in the dark and incubated overnight with the primary antibody (diluted in PBS-Tw0.1%/10% DS). Following three washes with PBS-Tw0.1%, 1H incubation with secondary antibodies at room temperature in the dark and 2 washes with PBS1X, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, Cat# D9542), washed in PBS1X and use immediately for imaging or stored at +4°C for short time. Imaging was performed with an inverted Nikon Eclipse X microscope equipped with: X20/0.45 (WD 8.2-6.9) objective; LUMENCOR excitation diodes; Hamamatsu ORCA-Flash 4.0LT camera; NIS Elements 4.3 software. Quantifications were performed using Cell Profiler (Carpenter AE et al., 2006). For each experiment, E14Tg2a and each mutant cells quantifications were attributed using the FlowJo software. For each experiment, the fluorescence intensity of each mutant cell was normalised to the median of the corresponding E14Tg2a intensities imaged on the same spot. The data was plotted using the ggplot2 package (Wickham, 2016) in R.

Immunofluorescence of NANOG and primitive endoderm markers.

Differentiated cells were processed into their respective well: one wash with PBS1X, fixation with freshly prepared PFA 4% for 10 min at room temperature, three washes of PBS1X, and used immediately for immunostaining or stored at +4°C for short time. Immunostaining was performed as in c3/ for NANOG in combination with primitive endoderm markers. Imaging was performed with an inverted Nikon Eclipse X microscope equipped with a x20 objective; LUMENCOR excitation diodes; Hamamatsu ORCA-Flash 4.0LT camera; NIS Elements 4.3 software. For each well, ~30 pictures were taken by randomly scanning the well. Quantifications were performed using Cell Profiler (Carpenter AE et al., 2006) and plotted using the ggplot2 package (Wickham et al. 2016) in R. Representative images were generated with ImageJ software using identical settings for

Single-molecule RNA Fluorescent In Situ Hybridisation (smFISH).

Cells were grown at low density, put in no RED medium for 24H before collecting them by regular trypsinization and resuspended in DMEM/10% FCS medium. Cells were fixed with 3.7% Formaldehyde (Sigma F8775) for 10 min at RT and the reaction was stopped by addition of 0.125M glycine (SIGMA G7126) for 5 min at RT. Cells were then centrifugated at 1000 rpm at 4°C, washed once in cold PBS/0.1% BSA, resuspended in cold PBS/0.1% BSA/1 mM EDTA at 1 million cells/mL and cytospun at 500 rpm (low speed) for 4 min on SuperFrost slides (Thermo J1800AMNT) – around 500K cells/spot in order to obtain around 40-60 nuclei/field (obj.x60) and no or few clumps. Slides were air dried for 1 min and stored in 70% EtOH at 4 °C for at least one night. The slides are dehydrated for 3 min in 100% EtOH and fully air dried. Each spot was hybridized for 24H at 37°C in a denaturing chamber (2X SSC/10% formamide) with the *Nanog* mRNA probe cocktail (2X SSC, 10% Formamide, BSA 1 µg/mL, 1 µL of *E.Coli* RNAs at 1 µg/mL, 1 µL of probe at 0.25 µM). The slides were washed for 1H at 37 °C in 2X SSC/10% Formamide then 2 times in 2X SSC for 10 min and mounted in Vectashield medium with DAPI (Vector-abcys H-1200). The *Nanog* mRNA probe was designed using Stellaris Probe Designer version 4.2 on Biosearch Technologies website with the maximum masking level (5) and was synthesized by the same company. Image stacks (0.5 µm gap) were acquired using a Nikon Eclipse X microscope equipped with: x63 oil immersion objective (N.A1.4); LUMENCOR excitation diodes; Hamamatsu ORCA-Flash 4.0LT camera; NIS Elements 4.3 software. The analysis was performed using ImageJ to identify smFISH spots using the Find Maxima function with adapted Noise tolerance and maximal projections of the stacks. The identified maxima were converted into grays scale images and, together with minimal projections of the DAPI staining, were imported into Cell Profiler (Carpenter et al., 2006) to count the number of smFISH spots per DAPI-segmented cell. The results were imported into R to compute the frequency of spot detection per cell and plot them with ggplot2 (Wickham, 2016).

Chromatin analyses.

Chromatin preparation.

After trypsinisation, 10⁷ ES cells were crosslinked for 10 min in 3 ml DMEM/10% FCS/1% formaldehyde (Sigma Cat#F8775). Crosslinking was stopped with 125 mM glycine for 5 min at room temperature then 5min in ice. Cells were pelleted and washed with ice-cold PBS1X. Cells were resuspended in 1 ml of ice-cold swelling buffer (25 mM Hepes pH 7.95, 10 mM KCl, 10 mM EDTA) freshly supplemented with 1× protease inhibitor cocktail (PIC-Roche, Cat# 04 693 116 001) and 0.5% IGEPAL (Sigma, Cat#18896). After 20 min on ice, the suspension was passed 50 times in a dounce homogenizer. Cells were then centrifuged and resuspended in 1 ml of ice-cold D3 (0.1% SDS, 15 mM Tris pH 7.6, 1 mM EDTA) buffer, freshly supplemented with 1× PIC. Samples were sonicated using a Covaris M220 - Setpoint @6°C and 10 cycles with the following parameters for each cycle : 60 sec duration, peak

power of 67W, duty factor of 15% and cycles/burst of 500. A delay of 45 sec is added at the end of each cycle. Result of the average power is 10W. After centrifugation (15 min, 14000 rpm, 4 °C), the supernatant was stored at -80 °C until use. 20 µl were used to quantify the chromatin concentration and check DNA size (typically 200-600 bp).

Immunoprecipitation.

15 to 20 µg of chromatin were used for each ChIP after pre-clearing it for 1.5 hours rotating on-wheel at 4 °C in 1 ml of TSE150 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8, 150 mM NaCl) buffer containing 50 µl of protein G Sepharose beads (Active Motif, Cat#37499) 50% slurry, previously blocked with BSA (0.5 mg/ml ; Roche, Cat# 10711454001) and yeast tRNA (1 µg/ml ; Roche Cat# 10109495001). Immunoprecipitations were performed overnight rotating on-wheel at 4 °C in 500 µl of TSE150. 20 µl were set apart for input DNA extraction and precipitation. 50 µl of blocked protein G beads 50% slurry was added for 2 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: 2 × TSE150, 1 × TSE500 (as TSE150 but 500 mM NaCl), 1× washing buffer (10 mM Tris-HCl pH8, 0.25M LiCl, 0.5% IGEPAL, 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-1%SDS. 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.

qPCR analysis.

Input and IP samples were analysed by real-time quantitative PCR performed in duplicates in 384-well plates with a LightCycler 480 (Roche) using 4.6 µl of LightCycler 480 SYBR Green I Master (Roche, 04707516001), 5 µl of sample and 0.2 µl of each primer at 20 µM in a final reaction volume of 10 µl. Standard and melting curves were generated to verify the amplification efficiency (>85%) and the production of single DNA species. PCR primer sequences are listed in Table S2. The 2dCt method was used. All values were corrected to the input and plotted in R using ggplot2 (Wickham et al., 2016).

Antibodies. Antibodies used for all ChIP experiments are listed in Table S2.

Gene expression analyses.

RNA preparation, RT-qPCR and sequencing.

E14Tg2a and ΔK9 cells were differentiated in parallel as embryoid bodies and cells recovered at d0, d4, d6, d8. RNA extraction and DNase treatment of 3 independent assays were made with NucleoSpin RNA Mini kit (Macherey Nagel, Cat# 740955.50) according to the manufacturer's protocol. Reverse Transcription was performed with 1 µg of total RNAs with random hexamers following manufacturer's protocol (Roche 04379012001); qPCR was performed as described above (*Tbp* was used as a reporter; see Table S2 for primer sequences). Stranded, poly-A selected RNA-seq libraries were prepared and sequenced (paired-end 150bp reads; around 50 millions each) by Novogene Co Ltd.

Alignments and quantification.

Stranded paired end RNA-seq reads were aligned to the mm10 genome using STAR (Dobin et al., 2013) and quantified by RSEM (Li and Dewey, 2011), with additional options "--calc-pme --calc-ci --estimate-rspd --forward-prob 0.0 --paired-end". Transcripts per million (TPM) were computed after omitting outliers identified by Principal Component Analysis (30 or more standard deviations away from the mean for PC1 component).

Principal component analysis.

PCA was run in R (prcomp function; with option center=TRUE) using log₂ transformed TPM after selecting genes with at least 1 TPM in at least one sample mean (n=3). The top 1000 PC1 loadings (55% of variance) were used for gene ontology analyses. Data visualisation was made in base R.

Differentially expressed genes.

For all differential expression tests RSEM estimated read counts per sample were rounded for use with DESeq2 (Love et al., 2014), which was run without independent filtering. For differentially expressed genes in undifferentiated ES cells, we selected those with an FDR < 0.05 in ΔK9.1 versus E14Tg2a and ΔK9.2 versus E14Tg2a and same fold-change direction with $\text{abs}(\log_2\text{FC}) > 0.3$. For embryoid body differentiation, we considered genes with absolute $\log_2(\text{FC}) > 1$ and FDR < 0.05 at any day of the differentiation versus undifferentiated cells for either E14Tg2a or ΔK9 cells. The corresponding heatmap was made in R with ComplexHeatmaps package (Gu et al., 2016).

Clustering of differentially expressed genes and developmental annotation.

K-means clustering was computed with R using the function kmeans with options k=6, nstart=50, iter.max=50. Only differentially expressed genes as identified during embryoid body differentiation were used (z scored mean TPM). The number of clusters was chosen as the minimal value identifying at least one cluster with maximal expression at each day of differentiation, including d0. Correlations to developmental gene expression were made by directly plotting the log₂(FC) reported in a previous study using SCNMT-seq around gastrulation on mouse embryos (Argelaguet et al., 2019). The data was downloaded from ftp://ftp.ebi.ac.uk/pub/databases/scnmt_gastrulation. The heatmap was made in R with ComplexHeatmaps package (Gu et al., 2016) and the boxplot with ggplot2 (Wickham, 2016).

Enrichment analyses.

Gene Ontology analyses were performed using Enrichr (<https://maayanlab.cloud/Enrichr/>) and the top association was selected. To determine enrichments of each group of differentially expressed genes in proximity to TF binding sites for Nanog and Oct4/Sox2 (Festuccia et al., 2019), we calculated Fisher tests right tail p-values for the association between differentially expressed genes of each cluster within xbp of a binding site to a background of all genes clustered within xbp of a binding site, for x in [1, 1e+9] bp. Data visualization was made in base R.

Supplementary References.

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