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Activation of transcription factor circuitry in 2i-induced ground state pluripotency is independent of repressive global epigenetic landscapes

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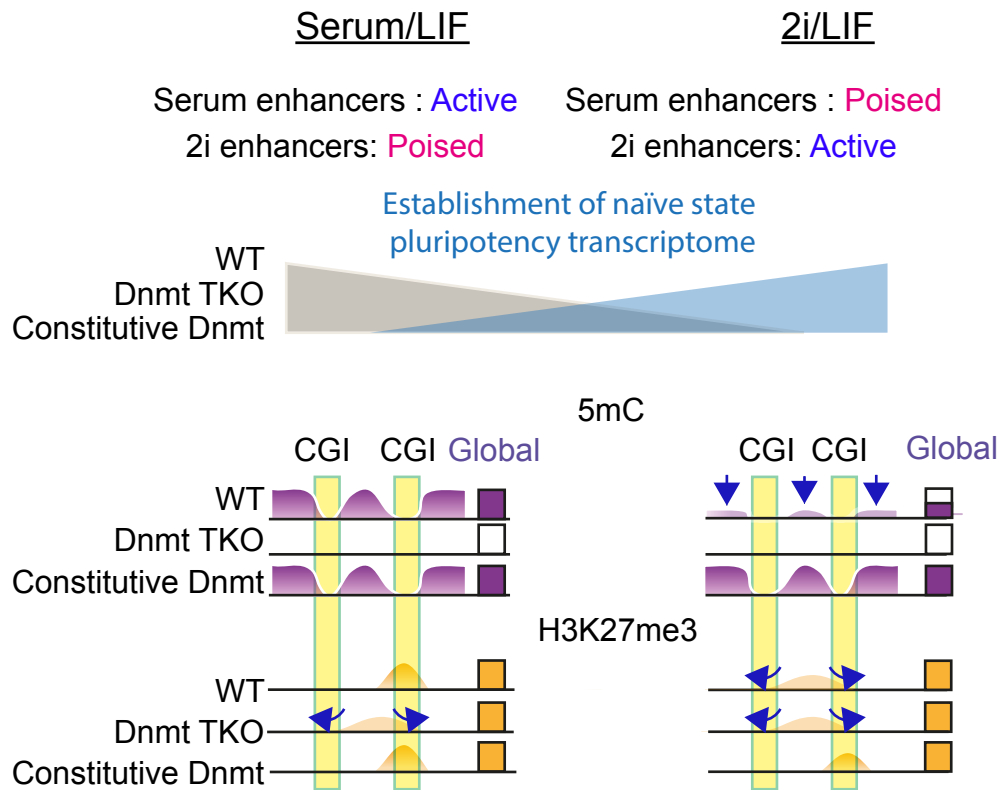


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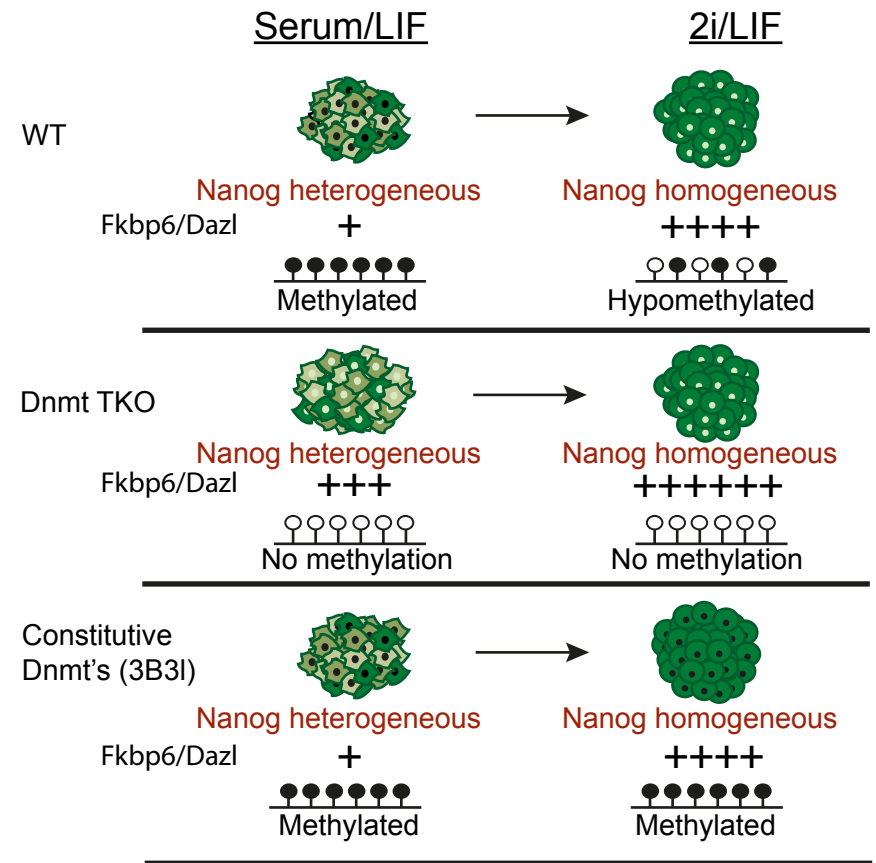
Activation of transcription factor circuitry in 2i-induced ground-state pluripotency is independent of repressive global epigenetic landscapes

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Graphic summarising our major findings that the 2i induced transition of mESCs from serum/Lif (S/L) conditions is not dependent on global DNA methylation states. This transition is driven by signal induced transcription factor changes.



Morphological and transcriptional changes are comparable for wild type (WT), hypomethylated (TKO) and constitutively methylated (3B3I) mESCs to the extent that Fkbp6 and Dazl genes are activated in 2i conditions despite having fully methylated promoters in 3B3I mESCs.

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3 **1 Activation of transcription factor circuitry in 2i-induced ground state pluripotency is**
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5 **2 independent of repressive global epigenetic landscapes**
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10 28 **Running title** Decoupling of DNA methylation role in resetting pluripotency circuitry

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31 Abstract

32 Mouse embryonic stem cells (mESCs) cultured with MEK/ERK and GSK3 β (2i) inhibitors
33 transition to ground state pluripotency. Gene expression changes, redistribution of histone
34 H3K27me3 profiles and global DNA hypomethylation are hallmarks of 2i exposure, but it is
35 unclear whether epigenetic alterations are required to achieve and maintain ground state or occur
36 as an outcome of 2i signal induced changes. Here we show that ESCs with three epitypes, WT,
37 constitutively methylated, or hypomethylated, all undergo comparable morphological, protein
38 expression and transcriptome changes independently of global alterations of DNA methylation
39 levels or changes in H3K27me3 profiles. *Dazl* and *Fkbp6* expression are induced by 2i in all three
40 epitypes, despite exhibiting hypermethylated promoters in constitutively methylated ESCs. We
41 identify a number of activated gene promoters that undergo 2i dependent loss of H3K27me3 in
42 all three epitypes, however genetic and pharmaceutical inhibition experiments show that
43 H3K27me3 is not required for their silencing in non-2i conditions. By separating and defining
44 their contributions, our data suggest that repressive epigenetic systems play minor roles in mESC
45 self-renewal and naïve ground state establishment by core sets of dominant pluripotency
46 associated transcription factor networks, which operate independently from these epigenetic
47 processes.

49 Introduction

50 Mouse embryonic stem cells (mESCs) can be derived from the inner cell mass (ICM) of
51 blastocysts following their *in vitro* culture (1,2). Culture medium developed for mESCs contains
52 leukemia inhibitory factor (LIF) in the presence of serum or bone morphogenetic protein 4, which
53 induces phosphorylation and activation of downstream transcription factors Stat3 and Smad1
54 (3,4). Cultured mESCs have considerable self-renewing capacity via expression of pluripotency

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3 55 genes that maintain their identity; they can contribute to germline chimeras and can differentiate
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5 56 into all three primary germ layers *in vitro* (5). Pluripotency in mESCs depends upon the
6
7
8 57 coordinated action of a gene regulatory network assembled from transcription factors (e.g. Oct4,
9
10 58 Sox2, Nanog; the OSN network), which is subject to modulation by multiple signalling pathways
11
12 59 in response to environmental cues that support self-renewal or initiate differentiation (6,7). Under
13
14 60 serum/LIF (serum) culturing conditions, a fine balance between renewal and pro-differentiation
15
16 61 signals results in populations of mESCs that are heterogeneous and metastable, exhibiting a
17
18 62 dynamic equilibrium in expression states for several pluripotency factors (8). Importantly, during
19
20 63 the process of derivation of mESCs from pre-implantation stage embryos the cells adopt mature
21
22 64 heterochromatin and DNA/histone modifications that deviate from their *in vivo* ICM counterparts
23
24 65 (9). Overall, the cellular and chromatin identity of mESCs in serum cultures partly resembles that
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28 66 of ICM cells, epiblast cells and germ cells (10,11).

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32 67 Culturing mESCS in 2i/LIF conditions containing inhibitors of MEK/ERK and GSK3 β
33
34 68 leads to a more homogeneous population, which more closely resemble ICM cells in terms of
35
36 69 gene expression and epigenetic signatures, termed the naïve ground state (11). Transfer of mESCs
37
38 70 from serum to 2i conditions consolidates developmental naïvety by endowing these cells with
39
40 71 additional pluripotency features (12). These include enhanced *Nanog* and *Klf2* expression and
41
42 72 epigenetic changes, such as global DNA hypomethylation and redistribution of H3K27me3 (a
43
44 73 repressive histone modification mark) from bivalent CpG island promoters (13). It has been
45
46 74 proposed that 2i conversion of mESCs represents a remodelling of the epigenome in concert with
47
48 75 a reconfiguration of the gene regulatory network, which enables unbiased developmental
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50 76 plasticity (14).

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57 78 Although DNA methylation is essential for the maturation of an embryo, it is striking that
58
59 79 globally hypomethylated mESCs lacking de novo and maintenance cytosine methyltransferases

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3 80 cultured in serum grow robustly and self-renew (15-17). TKO (*Dnmt3a*^{-/-}, *Dnmt3b*^{-/-}; *Dnmt1*^{-/-})
4
5 81 cells express typical markers of pluripotency (*Oct4*, *Rex1*, *Fgf4* and *Nanog*) to the same extent as
6
7 82 their wild-type (WT) J1 counterparts, strongly suggesting that DNA methylation is dispensable
8
9 83 for mESC maintenance. Lack of DNA methylation restricts the capacity of these cells to
10
11 84 differentiate into early embryonic stages but is permissive for extraembryonic lineages *in vivo*
12
13 85 (18). Transcriptional repression associated with DNA methylation is essential for maintaining
14
15 86 somatic cell fates and identity (18,19). In serum cultures, different pluripotent states defined by
16
17 87 high-low expression states of *Stella*, *Nanog* and *Rex1*, respectively, are correlated with the
18
19 88 relative global methylation status of mESCs. This suggests a potential role for DNA methylation
20
21 89 in the generation and maintenance of variability in stem cell populations (8,13,20). However, the
22
23 90 direct contribution of DNA hypomethylation in driving mESCs to a naïve gene expression pattern
24
25 91 is still unclear. In addition, the subsequent global redistribution of repressive H3K27me3 marks
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27 92 observed in hypomethylated 2i mESCs may be causally required to stabilise and maintain the
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29 93 ground state, or it may be an indirect consequence of decreased DNA methylation levels (13).
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95 To explore the potential contributions of DNA methylation in relation to mESC identity and
96 stability in serum and 2i cultures, we compared the response of WT mESCs with either
97 constitutively hypomethylated or constitutively methylated mESCs. We observed a similar
98 response in cell morphology, attainment of uniform expression of pluripotency genes (*Nanog*
99 and *Esrrb*) and deep silencing of differentiation genes. We identified a set of core
100 transcriptional changes that occur in the transition to 2i in all three epitypes, suggesting that
101 these changes affect signalling regulated genes and occur independently of DNA methylation
102 state in mESCs. In addition, we identified a subset of CpG island genes that undergo signal-
103 induced transcriptional changes that coincide with a depletion of H3K27me3 in all three cell
104 types under 2i conditions, however, H3K27me3 is not required for their silencing in serum

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3 105 cultured mESCs. Our data suggest that altered patterns of DNA methylation and H3K27me3 do
4
5 106 not define naive state identity, which is primarily dictated by transcriptional and signalling
6
7 107 networks. Instead these epigenetic transitions may be part of dynamic chromatin state changes
8
9 108 prior to differentiation, when epigenetic regulatory mechanisms have a role in defining gene
10
11 109 expression states (21). This general conclusion is in broad agreement with recent work that
12
13 110 studied the impact of PRC2 function in the transition of mESCs to a 2i ground state, in which
14
15 111 loss of Eed (a PRC2 component) to prevent H3K27me3 deposition had a minimal effect on the
16
17 112 2i transcriptome, implying that it is largely dispensable for establishment of the ground state
18
19 113 (22,23). This chimes with our recent work showing that although DNA methylation has a role
20
21 114 in shaping major aspects of PRC directed 3D genome organization in mESCs, this also does not
22
23 115 contribute to maintenance of the 2i ground state (24).
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117 **Materials and methods**

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119 **Cell Culture**

120 All cell lines used were generated from male WT J1 mESCs that were originally derived from
121 the 129S4/SvJae strain, except *Dnmt1^{tet/tet}* cells, derived from R1 mESCs. mESCs were cultured
122 in serum conditions as previously described (25,26), except ESGRO LIF (Millipore) was used at
123 500U/mL for all lines apart from *Dnmt1^{tet/tet}* mESCs which were cultured at 1000U/mL. WT J1
124 mESCs were grown on Mitomycin C inactivated SNLP feeder cells in serum cultures, all other
125 cell lines were grown feeder free on 0.2% gelatin-coated flasks. 2i culturing conditions were as
126 previously described (25,26); inhibitors were used at 1 μ M PD0325901 (MEK inhibitor,
127 Stemgent) and 3 μ M CHIR99021 (GSK3 inhibitor, Stemgent) and ESGRO LIF was used at
128 1000U/mL. Doxycycline-inducible Prdm14 overexpressing TKO cells were established by

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3 129 transfecting PB-TET-FlagPrdm14-IRES-Neo, PB-CARtTA Adv, pCAG-Pbase and pGG131
4
5 130 (pCAG-DsRed-IRES-Hygro)(27) into TKO cells using Fugene 6 HD (Promega) followed by
6
7
8 131 sorting red cells using a BD FACSAria flow cytometer; single cell clones were expanded and
9
10 132 characterised. KOVI-31 and 3B31 mESCs were generated as described in McLaughlin et.al. (25).
11
12 133 *Dnmt1^{tet/tet}* doxycycline regulateable mESCs were a gift from R. Chaillet lab, for derivation
13
14 134 procedure see Borowczyk et al. (28). *Dnmt1^{tet/tet}* doxycycline regulateable mESCs were treated
15
16
17 135 with 10 μ M EPZ6438 (EZH2 inhibitor) for 9 days.

136 **siRNA knockdown**

137 RNAiMAX (ThermoFisher) was used to transfect 2 x 10⁵ mESCs/6 well and 1 x 10⁵ mESCs
138 per 12 well (on coverslips coated with 0.2% gelatin) with 50nM siRNA Tet1 plus 50nM siRNA
139 Tet2 or 100nM scrambled siRNA (see supplementary information, Table S1 for sequences).
140 mESCs were cultured post transfection in either serum for 72h or 48h in serum followed by 24h
141 in 2i; medium was changed every day.

142 **Embryoid body differentiation**

143 mESCs were detached with trypsin and spun down prior to resuspension in mESC basal medium
144 with 20% FCS and plating as 200 cells/20 μ l as hanging drop cultures. After 2 days cells
145 aggregated and EBs were cultured in suspension in mESC basal medium for 3 days prior to 3-6
146 days of adherent culture on 0.2% gelatin on glass coverslips. Patches of cardiomyocytes were
147 recorded, and EBs were fixed with 4% PFA for 15min and stored at 4°C covered in PBS prior to
148 staining.

149 **Differentiation to Epiblast stem cells**

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3 150 6 X 10⁴ mESCs / 6 well were seeded in standard serum conditions on human fibronectin
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6 151 (Millipore, FC010) for 24h and subsequently cultured in EpiSC culture medium supplemented
7
8 152 with N2 (Gibco, 17502048) and B27 (Gibco, 17504044) consisting of a 1:1 mixture of DMEM
9
10 153 F-12 (Thermo Fisher, 21331020) and Neurobasal medium (Thermo Fisher, 21103049), 0.1mM
11
12 154 non-essential amino acids (SIGMA), 2mM L-glutamine, 0.1mM beta mercaptoethanol (Thermo
13
14
15 155 Fisher), Activin A (Peprotech, 120-14E) at 20ng/mL and FGF2 (R&D systems, 3139-FB) at
16
17 156 10ng/mL. Cells were passaged every 3-5 days with accutase (Gibco, A1110501) and reseeded as
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19 157 clusters of cells at 4 X 10⁴ cells/ 6 well, which resulted in a stable Epiblast stem cell line after
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21
22 158 several passages.

25 159 **RT-qPCR**

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28 160 Total RNA was extracted using an RNeasy kit (Qiagen), following manufacturer's protocol and
29
30 161 using Qias shredder columns (Qiagen) to homogenise the samples and RNase free DNase kit
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32 162 (Qiagen) to perform DNA digestion. 1µg RNA was converted to cDNA using random primers
33
34 163 (Promega) and Superscript III (Thermo Fisher) following manufacturer's protocol. qPCR
35
36 164 reactions with gene specific primers (see Table S1) were carried out using SYBR green (Roche
37
38 165 or Thermo Fisher) on a Lightcycler 480 System II (Roche). TBP was used for normalisation;
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40 166 relative quantification was done by the 2^{-dCt} method. 3 biological replicates were used for
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42 167 statistics and standard deviation was calculated to generate error bars following MIQE guidelines
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44 168 (Table S3).

51 170 **Western blotting**

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55 171 Protein was extracted using RIPA buffer (Thermo Fisher) supplemented with protease inhibitor
56
57 172 cocktail (Roche) followed by boiling in 1x LDS sample buffer and 1x reducing reagent (both
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59 173 Thermo Fisher) for 5 min at 95°C. 30, 15 or 7.5µg of protein was loaded per sample and run in

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3 174 4-12% Bis-Tris gels (Thermo Fisher) in 1x MOPS or MES buffer (Thermo Fisher). Dry blotting
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5 175 was performed using an iBlot™ device (Thermo Fisher) and PVDF iBlot™ gel transfer stacks.
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8 176 Membranes were blocked in 0.1% Tween-20 in PBS with 1:10 dilution of western blocking
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10 177 reagent (Thermo Fisher) for a minimum of 45min at room temperature followed by primary
11
12 178 antibody incubation at 4°C overnight and secondary antibody incubation for 1h at room
13
14 179 temperature (for antibodies and dilutions see; supplementary information, Table S2). HRP
15
16 180 stained Western blots were developed using SuperSignal West Pico Chemiluminescent substrate
17
18 181 (Thermo Scientific) and imaged using an ImageQuant LAS 4000 (GE Healthcare). Near infrared
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20 182 stained blots were imaged on an Odyssey Fc system (LI-COR, Nebraska, US).
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25 26 184 **Immunocytochemistry**

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29 185 mESCs on glass coverslips coated with gelatin were stained using standard
30
31 186 immunocytochemistry protocols. Briefly, mESCs were fixed with 4% PFA, 10min and incubated
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33 187 overnight with primary antibodies (see; supplementary information, Table S2) at 4°C, followed
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35 188 by incubation with appropriate Alexafluor-conjugated secondary antibodies (Thermo Fisher) at
36
37 189 room temperature for 1h. Nuclei were labelled using DAPI (4',6-diamidino-2-phenylindole).
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39 190 Imaging was done using a Zeiss AxioScope 2 microscope with Zeiss optics or a Nikon A1
40
41 191 Confocal system with Zeiss optics. Embryos were stained following procedure previously
42
43 192 described (9). Briefly, blastocyst stage embryos were fixed in 4% PFA overnight at 4°C followed
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45 193 by permeabilisation in 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 30 min and 2 washes with
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47 194 PBS-0.01% Tween (Sigma-Aldrich; PBST) prior to blocking with 5% donkey serum (Sigma-
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49 195 Aldrich) in PBST for 2h at room temperature. Primary antibodies (see; supplementary
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51 196 information, Table S2) were incubated overnight at 4°C in 1% donkey serum in PBS, followed
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53 197 by 3 washes in PBST. Secondary antibodies conjugated to either FITC or TRITC (Jackson
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55 198 Laboratories) were used at 1:200 for 1h at room temperature, followed by washes. Samples were
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3 199 counterstained with DAPI in Vectashield mounting medium (Vectashield). Images were captured
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5 200 with a Zeiss laser confocal microscope (LSM510 Meta) and LSM software.
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10 202 **Alkaline phosphatase staining**
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13 203 Alkaline phosphatase staining was performed using Alkaline phosphatase staining kit II
14 204 (Stemgent) according to manufacturer's instructions and imaged using a Nikon Eclipse Ti-S
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16 205 microscope.
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23 207 **Methylation analysis**
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26 208 Genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) following
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28 209 manufacturer's protocol or standard phenol chloroform precipitation and eluted in water. DNA
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30 210 was treated with RNase A/T1 Cocktail (Ambion) overnight at 37°C followed by ethanol
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32 211 precipitation. Quantitation of 5mC in genomic DNA was done by isocratic high performance
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34 212 reverse phase liquid chromatography (HPLC) as previously described (29) with the following
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36 213 alterations. A Dionex UM 3000 HPLC system was used complete with a column chiller, C18
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38 214 column (250mm x 4.6 mm 5µM APEX ODS, Grace Discovery Sciences), and column guard
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40 215 (Phomenex). The mobile phase was 50mM ammonium phosphate (monobasic) pH4.1. The
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42 216 column was chilled to 8°C to improve peak separation. Deoxyribonucleotides (dNMPs) were
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44 217 detected at their extinction maxima using a Dionex 3000 multiple wavelength detector: dCMP,
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46 218 276nm; 5mdCMP, 282nm. Quantifications were calculated from the area under each peak using
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48 219 the respective extinction coefficients (dCMP, 8.86×10^3 ; 5mdCMP 9.0×10^3).
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56 221 **Bisulfite sequencing**
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3 222 Bisulfite sequencing was carried out as described (30). For details of primers utilised see;
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5 223 supplementary information, Table S1. Bisulfite conversion of DNA was performed using the EZ-
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7 224 DNA methylation gold kit (ZymoResearch), products were cloned into pGEM-T Easy (Promega)
8
9
10 225 and minimum 24 clones were picked per condition for plasmid preparation followed by
11
12 226 sequencing using BigDye, version 3.1 chemistry (Thermo Fisher). Bisulfite sequencing DNA
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14 227 methylation analysis (BISMA) was used to calculate percent methylation in bisulfite sequencing
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16 228 reads.
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22 230 **Agilent Expression Array and data analysis**

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25 231 Total RNA was isolated using RNeasy kit (Qiagen) and Cy3 labelled aRNA was prepared using
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27 232 an Amino Allyl MessageAmp II aRNA kit (Ambion). Samples were hybridised to SurePrint G3
28
29 233 mouse GE 8x60k microarrays (Agilent) and scanned using NimbleGen MS200 (Roche). Results
30
31 234 were analysed with custom-written scripts implemented in R (<http://www.R-project.org>).
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33 235 Differential expression was calculated using the Bioconductor package linear models for
34
35 236 microarray data (limma v3.20.8). P-values were corrected for multiple testing using the
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37 237 Benjamini-Hochberg test and probes with $P < 0.05$ were deemed significant.
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44 239 **meDIP**

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48 240 meDIP was carried out as described (31). In short, 20 μ g of genomic DNA was diluted in TE to
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50 241 400 μ l and sonicated using a Covaris plus sonicator to 150-700bp range with a mean of 300bp.
51
52 242 6 μ g of fragmented DNA was diluted to 450 μ l in TE and denatured by incubation in a
53
54 243 thermoshaker for 10min at 99 $^{\circ}$ C. 10% of the sample was taken at this stage as an input fraction
55
56 244 and stored at 4 $^{\circ}$ C. Samples were incubated with 15 μ l of 5mC antibody (Eurogentec) overnight at
57
58 245 4 $^{\circ}$ C. M280 Dynabeads were used for immunoprecipitation. DNA was purified using a PCR clean

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3 246 up kit (Qiagen) and samples were eluted into 20µl; 10µl was subjected to whole genome
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5 247 amplification using a SEQXE WGA kit (SIGMA) per manufacturer's instructions, except no
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7 248 SYBR green was added at the amplification step and samples were amplified for a total of 18
8
9
10 249 cycles at which point the volume required to get 2.1µg of the lowest sample was the volume used
11
12 250 from each sample for further primer removal and clean up using a PCR clean up kit (Qiagen).
13
14 251 We performed proton sequencing on libraries made from 100ng of DNA per sample, using the
15
16 252 Ion XpressPlus Fragment Library Kit (Thermo Fisher). DNA was end repaired, purified, ligated
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18 253 to Ion-compatible barcoded adapters (Ion Xpress™ Barcode Adapters 1–96, Thermo Fisher),
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20 254 followed by nick-repair to complete the linkage between adapters and DNA inserts. The adapter-
21
22 255 ligated library was then amplified (10 cycles) and size-selected using two rounds of AMPure XP
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24 256 bead (Beckman Coulter) capture to obtain fragments of approximately 100–250bp. Samples were
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26 257 then pooled at a 1:1 ratio and sequenced on an Ion Proton P1 microwell chip (Thermo Fisher).
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35 259 **RNA sequencing**

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39 260 Total RNA was extracted from mESCs using an RNeasy kit (Qiagen). RNA was analysed using
40
41 261 a bioanalyser and confirmed to be above RIN 9. Samples were treated with DNase (Ambion),
42
43 262 and sample integrity verified on the Agilent Bioanalyser with the RNA Nano-chip. Illumina Tru-
44
45 263 seq paired end strand specific sequencing (Illumina, USA) was carried out on a NextSeq-550
46
47 264 sequencer (Edinburgh Wellcome trust Clinical Research Facility, Western General Hospital,
48
49 265 Edinburgh, UK). 500ng of Total RNA underwent ribosomal RNA depletion prior to purification
50
51 266 (EPZ6438 treated *Dnmt1*^{tet/tet} mESCs) or PolyA-selection (J1, TKO and 3B31 mESCs),
52
53 267 fragmentation, random hexamer cDNA generation and purification with AMPure XP beads
54
55 268 (Beckman-Coulter, USA). Multiple indexing adapters were ligated to ds cDNA with subsequent
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57 269 hybridisation onto flow cells, and DNA fragment enrichment by 15 cycle PCR for sequencing.
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3 270 Completed libraries were quantified by qPCR using the KAPA Illumina Library Quantification
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5 271 Kit (Illumina, USA) before multiplexing in two equimolar pools and running on two flow cells
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8 272 on the Illumina NextSeq 550. Resulting FastQ files were mapped to the reference genome
9
10 273 (mm10) using the Tophat alignment tool (V2) on Illumina Basespace software and reads per
11
12 274 kilobase per million (RPKM) scores calculated for each gene. Differential gene expression was
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15 275 carried out using DEseq with cut offs of Log2 fold change >2 and adjusted P-values <0.05 within
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17 276 replicates applied.

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22 23 24 278 **H3K27me3 ChIP-seq**

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27 279 ChIP was performed exactly as described (25). Sequencing libraries and Ion proton sequencing
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29 280 was carried out as described for MeDIP-seq above. For details of antibodies see; supplementary
30
31
32 281 information, Table S2.

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37 38 39 283 **High content Western blotting – DigiWest**

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42 284 DigiWest was performed essentially as described in Treindl et.al. 2016 (32). Briefly; cell pellets
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44 285 were lysed in RIPA Buffer containing protease Inhibitor Mix M (Serva), PhosSTOP (Roche
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46 286 Applied Science) and PMSF (Thermo Scientific), incubated for 30 min on ice and protein was
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48
49 287 quantified using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were stored
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51 288 at -80°C until further use. The NuPAGE SDS-PAGE gel system (Thermo Fisher) was used for
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54 289 protein separation and blotting; 8µl protein per sample was separated using 4-12% Bis-Tris gels
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56 290 and transferred onto PVDF membranes (Millipore). For high content Western analysis, the
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58 291 DigiWest procedure and data analysis was performed as described in Treindl et al. (32). In brief;
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3 292 data generated by a Luminex instrument were analysed using a dedicated analysis tool. To
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5
6 293 compare different samples, obtained values were normalised to β -actin; log₂ transformed and
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8 294 analysed in MEV 4.9.0 software. Two factor ANOVA was performed in which factor 1 was
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10 295 defined as 2i treatment and factor two as cell type (J1 vs. TKO vs. 3B31). P-value based on 1000
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12 296 permutations was set to 0.005 and hierarchical clustering based on Euclidean Distance was
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14
15 297 performed. Significant results were plotted in a heatmap. DigiWest data are composed of 96
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17 298 individual measurements derived from 96 molecular weight fractions per sample and all values
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19 299 obtained for a given antibody were used to create images that mimic a Western blot image.
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21 300 Background subtracted Luminex data were scaled to values from 0 and 1 where the highest
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23 301 measured signal intensity was set to 1. Scaled data were loaded into the MEV 4.9.0 software
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25 302 package, and a graph was generated by applying a grayscale colour scheme; 0 (white) to 1 (black).
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28 303 Heatmaps were saved as images and transferred to Photoshop wherein a Gaussian diffusion was
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30 304 applied with a radius of half the element height.
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306 **Bioinformatic processing and analysis of meDIP-Seq and H3K27me3 ChIP-seq datasets**

307 i. *Mapping and data normalisation.* Analysis was done as described previously (31). In short,
308 reads were mapped to reference genome using Torrent TMAP software. Data was binned into
309 200bp windows across the genome and normalised first by total read count and then sequencing
310 noise removed by subtracting the matched input sequence (sheared non-immunoprecipitated
311 DNA).

312 ii. *Peak finding and mapping.* Windows of enrichment were defined as any 200bp window where
313 the signal was enriched over background input noise . Peaks of 5mC and H3K27me3 were called
314 based on threshold levels from J1-serum cells. We defined peaks as regions where at least 2
315 windows (each 200bp) in a three-window region (600bp) were above the 95th percentile of 5mC

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3 316 scores from the J1-serum dataset. These peaks were mapped to one of six unique genomic
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5 317 compartments (promoter core: TSS +/-100bp, promoter proximal: TSS +1kb, promoter distal:
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7 318 TSS +1kb to + 2kb, exonic, intronic or inter-genic: not associated with any of the above) as
8
9
10 319 defined from Refgene annotations.

11
12 320 *iii. Sliding window analysis of 5mC states over regions of interest.* Average patterns of DNA
13
14 321 modifications and histone tail marks were plotted across a series of genomic features (promoters
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16 322 +/-2kb, gene bodies +/-25% total gene length, Oct4/Sox2/Nanog binding sites +/- 100% element
17
18 323 length) using a sliding window based approach. This calculates average levels of each
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20 324 modification across a certain step size relative to coordinates of choice and average patterns
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22 325 across these features are then plotted. Oct4/Sox2/Nanog binding sites were taken as defined in
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24 326 Galonska et al. (22).

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31 32 33 328 **Retrotransposon analysis**

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36 329 J1, TKO and 3B31 mESC RNA-seq was processed as described (25). For retrotransposon
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38 330 analysis, adaptors were removed from RNA-seq reads using TrimGalore! 0.4.1 (paired end,
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40 331 illumina, stringency 3), and aligned to the mm9 mouse genome using TopHat 2.1.0 (very
41
42 332 sensitive, inner distance 23 ± 56 , no coverage search, max multihits 1). Read co-ordinates were
43
44 333 intersected with UCSC genome browser RepeatMasker track co-ordinates using BEDTools
45
46 334 2.25.0, filtered to ensure that each pair or singleton was assigned to only one repeat location, and
47
48 335 the number of sequences belonging to each type of repeat summed. Repeats belonging to LTR,
49
50 336 LINE and SINE Repeatmasker classes that were significantly upregulated ($FDR < 0.05$, $\logFC >$
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52 337 0) were identified using the edgeR package in R. Read counts between samples were normalised
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54 338 relative to the number of reads mapping to the genome.

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340 **Data availability**

341 Data generated or used in this study can be found NCBI GEO Series accession numbers: J1
342 serum/2i MeDIP-seq & 3B3l serum/2i MeDIP-seq: GSM1865089, GSM1865091, GSM2700270,
343 GSM2700271, GSM1865090 & GSM1865092; WT serum/2i H3K27me3 ChIP-seq GSE23943;
344 3B3l serum/2i H3K27me3 ChIP-seq: GSM2700276, GSM2700277; TKO serum/2i H3K27me3
345 ChIP-seq: GSM2700278, GSM2700279, GSM2700284 & GSM2700285; J1, TKO and 3B3l
346 serum/2i gene expression data (microarray): GSE72302; J1 and 3B3l serum/2i gene expression
347 (RNA-seq): GSE121171; TKO gene expression (RNA-seq): GSE130686; EPZ gene expression:
348 GSE101928. DigiWest Luminex reads can be found in; supplementary information, Dataset 2.

350 **Results**

352 **mESCs reset to a naïve ground state in 2i independently of their initial epigenetic state**

353 To investigate the role of repressive epigenetic marks in ground state pluripotency we cultured
354 WT, hypomethylated, and constitutively methylated mESCs and analysed their response to 2i
355 conditions. As a hypomethylated cell type we utilised TKO mESCs lacking functional *Dnmt3a*,
356 *Dnmt3b* and *Dnmt1* (15). To generate constitutively methylated 2i/mESCs, we utilised
357 *Dnmt3a/3b* knockout mESCs rescued by exogenous constitutive expression of *Dnmt3b* and
358 *Dnmt3l*, as detailed in the cell line derivation schematic, hereafter termed 3B3l (Fig. 1a) (25,33).
359 WT J1, TKO and 3B3l mESCs were cultured in 2i medium for 14 days. All three cell types
360 showed characteristic mESC morphologies in serum conditions and acquired archetypal three-
361 dimensional dome-shaped colony morphology after 3 days of 2i exposure (Fig. 1b) (26).
362 Expression of a key marker of 2i transition, *Prdm14*, was upregulated in all three cell lines (Fig.
363 1c) (34). In WT J1 mESCs Transcripts corresponding to de novo methyltransferases (*Dnmt3a*,

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3 364 *Dnmt3b* and *Dnmt3l*) were downregulated while the maintenance methyltransferase (*Dnmt1*)
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5 365 remained unchanged, as observed by microarray analysis and confirmed by RT-qPCR (Fig. 1c
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7 366 and S1a). As expected, only the *Dnmt3l* transcript was downregulated in TKO cells, as the
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9 367 *Dnmt3a*, *Dnmt3b* and *Dnmt1* genes are inactivated. In addition, we confirmed retention of
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11 368 *Dnmt3b* and *Dnmt3l* transcripts in 3B3l mESCs (Fig. 1c and S1b). We also detected
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13 369 homogeneous expression of Nanog and Esrrb in WT J1, TKO and 3B3l mESCs cultured in 2i by
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15 370 immunocytochemistry, in contrast to heterogeneous staining in their serum cultured counterparts
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17 371 (Fig. 1d).

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23 372 To analyse changes in DNA methylation in WT J1, TKO and 3B3l mESCs when
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25 373 converted to a 2i mediated ground state, we performed high performance liquid chromatography
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27 374 (HPLC) quantification of total 5mC levels, which revealed various degrees of hypomethylation
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29 375 in 2i (Fig. 1e). As reported previously, 5mC levels in 2i are reduced by 50% in WT mESCs (J1-
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31 376 2i) (Fig. 1e). Notably, 3B3l cells cultured in 2i (3B3l-2i) exhibited global DNA methylation levels
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33 377 equivalent to WT J1-serum cells (Fig. 1e). Hence, maintenance of *Dnmt3b* and *Dnmt3l*
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35 378 expression can prevent cells from exhibiting global hypomethylation despite increased *Prdm14*
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37 379 levels during 2i adaptation (Fig. 1c and S1b). Loss of DNA methylation upon 2i exposure is
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39 380 linked with *Prdm14* directed repression of *Dnmt3a/Dnmt3b/Dnmt3l* or alternatively, a deficiency
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41 381 of UHRF1 and H3K9me2, required for maintenance methylation (35-37). Although we observed
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43 382 the reported 2i induced reduction of UHRF1 and H3K9me2 levels in WT mESCs that is
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45 383 hypothesised to reduce *Dnmt1* activity, these alterations were not replicated in TKO-2i or 3B3l-
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47 384 2i mESCs (Supplementary information, Fig. S1c, d) (35). The observation that overexpression of
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49 385 *Dnmt3b* and *Dnmt3l* (or deletion of *Prdm14*, see below) can maintain methylation levels in 2i
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51 386 mESCs is incompatible with the primary role proposed for UHRF1 in regulating global DNA
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53 387 methylation levels in 2i mESCs (35,36). Bisulfite sequencing analysis of loci (major satellite,
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55 388 *Dazl* and *Fkbp6* promoters) known to undergo hypomethylation in 2i demonstrated that 3B3l-2i

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3 389 methylation was maintained at most of these sites (major satellite, *Dazl*) or underwent a slight
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5 390 decrease (*Fkbp6* 10% decrease, $p < 0.05$, 2-tailed Mann-Whitney U-Test), but still remained
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7 391 significantly more highly methylated than in J1-2i cells (*Fkbp6* 75.3% in 3B31-2i compared with
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9 392 19.7% in J1-2i, $p < 0.00001$, 2-tailed Mann-Whitney U-Test) (Fig. 1f).

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13 393 To further characterise WT J1, TKO and 3B31 mESCs cultured in 2i we performed several
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15 394 experiments to confirm their pluripotency and 2i mediated signalling responses. All three
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17 395 epitypes exhibit heterogeneous alkaline phosphatase activity in serum and this increases to 100%
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19 396 homogeneous alkaline phosphatase activity in mESCs cultured in 2i (Supplementary information,
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21 397 Fig. S1e). To analyse changes in signalling pathways following 2i culture we utilised the western
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23 398 blotting analysis system DigiWest to simultaneously assay 72 proteins (32). This confirmed the
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25 399 absence of Dnmt1 protein in TKO mESCs and maintenance of Dnmt3l in 3B31-2i at levels
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27 400 significantly above J1-2i and TKO-2i (Supplementary information, Fig. S1f). As expected,
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29 401 culturing mESCs in 2i resulted in upregulation of β -catenin, one of the downstream targets of the
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31 402 GSK3 β pathway and downregulation of phospho-c-Myc, a downstream target of the MEK
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33 403 pathway (Supplementary information, Fig. S1f) (26). Hence, relevant signalling pathways are
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35 404 intact in all three cell lines utilised in the study. Several other proteins exhibit similar expression
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37 405 changes in WT J1, TKO and 3B31 mESCs based on culture conditions e.g. vimentin, which may
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39 406 account for changes in cell shape during 2i adaptation (Supplementary information, Fig. S1f).
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41 407 Moreover, ANOVA analysis of significant analytes ($P < 0.005$) resulted in clustering of DigiWest
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43 408 samples according to culture conditions (Supplementary information, Fig. S1g, Dataset 2);
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45 409 implying that signal transduction pathways that respond to 2i in WT J1, TKO and 3B31 mESCs
46
47 410 induce similar protein expression and modification perturbations. In addition, we found that J1
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49 411 and 3B31 mESCs were capable of forming differentiated embryoid body (EB) outgrowths when
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51 412 plated onto gelatin. TKO mESCs formed EBs but failed to form significant outgrowths and very
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53 413 few cells stained positive for α -smooth muscle actin (mesodermal marker) and Tuj1 (ectoderm)

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3 414 (Supplementary information Fig. 2a and data not shown). This is in agreement with previous
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5 415 observations that lack of DNA methylation in TKO mESCs leads to impaired differentiation to
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7 416 ecto- and mesoderm (18). WT J1 and 3B31 mESCs formed ectoderm and mesoderm lineages;
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10 417 both generated beating cardiomyocytes staining positive for α -smooth muscle actin, as well as
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12 418 cells positive for Tuj1 (Supplementary information, Fig. S2b). It is note-worthy that the
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14 419 α -smooth muscle actin marker not only reports on a mesodermal lineage but also attests the
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16 420 earliest differentiation from pluripotent mouse ESCs into mesendoderm, (ME) the intermediate
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18 421 stage equivalent to the embryonic primitive streak, from which both mesoderm and endoderm
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20 422 are derived (38). However, we did not observe significant endodermal marker expression for
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22 423 either cell type with this differentiation protocol; perhaps indicating that a more directed
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24 424 differentiation approach may be required to generate this lineage with these cell lines (data not
25
26 425 shown). In addition, WT J1 and 3B31 mESCs both generated a stable epiblast stem cell line
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28 426 when treated with appropriate cytokines, however TKO mESCs were unable to differentiate to
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30 427 this lineage (Supplementary information, Fig. S2c). Taken together this data suggests that 3B31
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32 428 mESCs can transition to a naive ground state and differentiate into specialised cell types unlike
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34 429 TKO cells, which are differentiation impaired.
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41 430 In order to investigate alterations in repressive chromatin marks in response to DNA
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43 431 methylation changes and 2i signalling we performed immunocytochemistry (ICC) for
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45 432 H3K27me3 and H2AK119ub in WT J1, TKO and 3B31 mESCs cultured in serum and 2i, as well
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47 433 as in early and late blastocysts. As reported previously, both H3K27me3 and H2AK119ub are
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49 434 redirected to pericentric heterochromatin (PCH) in a high proportion of TKO cells under serum
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51 435 conditions (Fig. 2a) (39). A similar proportion exhibited PCH localisation for both modifications
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53 436 in TKO-2i cells (Fig. 2a). In contrast, no significant PCH association of H3K27me3 and
54
55 437 H2AK119ub was observed in J1-2i or 3B31-2i cells (Fig. 2b; Supplementary information, Fig.
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57 438 S3). This contrasts with a recent report suggesting that 2i-ESCs are distinguished from other
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3 439 pluripotent cells by a prominent enrichment in H3K27me3 and low levels of DNA methylation
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5 440 at PCH (40). However we note that Tosolini and colleagues culture their 2i-mESCs on Laminin
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7 441 which also leads to the appearance of H3K27me3 in conjunction with H3K9me3 at PCH in serum
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9
10 442 grown mESCs, whereas we follow the original SOP of Ying et al., 2008 and grow ESCs on gelatin
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12 443 coated plastic (26). It is possible that the ability of Lamins to confer proliferative stimulation on
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14 444 mESCs may cause additional changes that affect H3K27me3 deposition when mESCs are
15
16 445 transitioned to 2i (41). To verify the situation *in vivo*, we isolated blastocyst stage embryos and
17
18 446 stained them for H3K27me3 (9). Early and late male blastocysts show a marked increase in
19
20 447 H3K27me3 levels in the ICM, in contrast to very low trophectoderm (TE) levels (Fig. 2c). Early
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22 448 ICM patterns display euchromatic distribution away from DNA-dense foci similar to WT J1
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24 449 mESCs. This pattern is maintained in the epiblast; however, in some cells of the late ICM margin
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26 450 proximal to the blastocoele, H3K27me3 foci colocalise with regions of high DNA density in a
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28 451 pattern similar to TKO cells (Fig. 2c). This indicates redistribution of H3K27me3 might be a
29
30 452 response to developmental cues that involves DNA hypomethylation of the ICM (42,43). During
31
32 453 the meiotic prophase of spermatogenesis, H3K27me3 is also observed to accumulate on PCH
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34 454 from the mid pachytene stage onward, during which DNA methylation reprogramming occurs as
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36 455 evidenced by hypomethylated minor satellite repeats (44,45).
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457 **Inhibition of MEK1/2 and GSK3 β is necessary to drive TKO cells to the naïve state**

458 As a reduction in DNA methylation has been postulated to be a hallmark of the naïve state, we
459 analysed TKO mESCs to address whether they would convert more easily to the ground state
460 than WT mESCs. We checked the sensitivity of J1 and TKO cells to the two inhibitors at various
461 dilutions in defined media (SFES) with a constant LIF concentration. Both cell lines responded
462 comparably and neither achieved ground state morphology when cultured in diluted 2i medium
463 (Supplementary information, Fig. S4a). We observed comparable trends in key transcript changes

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3 464 in both J1 and TKO mESCs in diluted 2i, but alterations were not to the same extent as in
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5 465 complete 2i (Supplementary information, Fig. S4b). We also evaluated the effect of individual
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7 466 inhibitors, PD0325901 and CHIR99021. TKO cells acquired a 3D-dome shaped colony
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9 morphology similar to 2i adaptation, upon exposure to CHIR99021 + LIF (CH), while exhibiting
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11 very distinct needle-shaped flat cell morphology upon PD0325901 + LIF (PD) treatment
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13 (Supplementary information, Fig. S4c-d). *Nanog* expression was lower in TKO cells incubated
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15 with CH alone compared to PD or 2i cultured cells (Supplementary information, Fig. S4e).
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17 Prolonged culture in no inhibitor medium (SFES) or single inhibitor (CH or PD) resulted in
18 471
19 decreased cell viability and spontaneous differentiation compared to 2i cells, in agreement with
20 472
21 reports for WT cells (Supplementary information, Fig. S4d) (26). Associated gene expression
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23 changes (notably *Dnmt3l*, *Eomes* and *Lin28a*) did not occur to the same extent with single
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25 inhibitors as compared to inhibiting both pathways (Supplementary information, Fig. S4f).
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32 476 As *Prdm14* is suggested to be a key player in driving cells to the 2i ground state, we
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34 477 generated a TET-inducible *Prdm14* TKO mESC line to test if its overexpression in
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36 478 hypomethylated mESCs is sufficient to drive these mESCs into a ground state (Supplementary
37
38 information, Fig. S5a-b) (36). Upon induction of *Prdm14* no morphological changes were
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40 observed in TET-ON-*Prdm14* TKO clones (Supplementary information, Fig. S5c). Additionally,
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42 although direct targets of *Prdm14* such as *Dnmt3l* and *Lefty2* were down regulated, we did not
43 481
44 observe transcript changes associated with 2i adaptation such as upregulation of *Dmgdh* (highest
45 482
46 expression change in 2i versus serum in wild-type mESCs) or *Eomes* (stem cell maintenance
47 483
48 gene) and downregulation of *Pax6* and *Fgf15* (differentiation genes), in any of the TET-ON-
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50 *Prdm14* TKO clones irrespective of the level of *Prdm14* overexpression (Supplementary
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52 information, Fig. S5d) (46). This suggests *Prdm14* upregulation and 5mC depletion alone is not
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54 sufficient to reprogram serum cultured mESCs to a ground state in terms of morphology
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3 488 (Supplementary information, Fig. S5c) or gene expression (Supplementary information, Fig.
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5 489 S5d).

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10 11 12 491 **Gene expression patterns are determined by culture conditions**

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16 492 In order to delineate gene expression changes caused by 2i signalling from gene expression
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18 493 changes caused by a difference in DNA methylation, we analysed gene expression in WT J1,
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20 494 TKO and 3B3l mESCs cultured in serum and 2i. Pearson correlation analysis of expression
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22 495 profiles revealed robust clustering based on culture conditions rather than genotype (Fig. 3a). The
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24 496 number of differentially expressed genes between TKO and WT J1 mESCs in 2i compared to
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26 497 serum culture conditions is reduced and consolidates as 3.3 times less upregulated and 2.9 times
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28 498 less downregulated genes (Supplementary information, Fig. S6a, b). Moreover, in spite of being
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30 499 hypomethylated in 2i, the transcriptome of WT J1-2i mESCs differs greatly from that of TKO-
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32 500 serum (Supplementary information, Fig. S6c). In agreement with previous reports, we observed
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34 501 a large number of genes (>3500) differentially expressed in 2i wild-type cells compared to serum
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36 502 conditions (1823 up- and 2208 down- regulated, fold change ≥ 2 , p.adj. ≤ 0.05 , eBayes (limma),
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38 503 Benjamini-Hochberg corrected) (Fig. 3b; Supplementary information, Dataset 1) (13,36,47).
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40 504 There is an overlap of 843 genes between all three 2i cell lines that exhibited similar changes in
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42 505 expression (318 up- and 525 down-regulated) (Fig. 3b; Supplementary information, Dataset 1).
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44 506 Moreover, transcription factors associated with naïve pluripotency are upregulated in WT J1,
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46 507 TKO and 3B3l in 2i compared to respective serum cells; with the exception of *Klf2* which
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48 508 exhibited increased expression in TKO and 3B3l mESCs in serum (Fig. 3c). Importantly, these
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50 509 factors are equivalently expressed in all three epitypes in 2i. Functional pathway analysis of the
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52 510 common gene list upregulated in 2i revealed enrichment of genes related to germ cell and
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54 511 embryonic development, while genes downregulated in 2i are linked with differentiation (Fig.

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3 512 3d). Based on pathway analysis, we focused on genes associated with the GO terms ‘Cell Fate
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5 513 Commitment’ (CFC: GO:0045165) and ‘Stem Cell Maintenance’ (SCM: GO:0019827), which
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8 514 were differentially expressed ($FC \geq 1.5$, $p_{adj} < 0.05$, eBayes (limma), Benjamini-Hochberg
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10 515 corrected) between WT J1-serum and J1-2i mESCs. These gene sets showed a similar trend in
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12 516 TKO and 3B31 mESCs following adaptation to 2i (Fig. 3e, f; Supplementary information, Fig.
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14 517 S6d and S7) and we also observed this in WT and *Prdm14*^{-/-} mESCs, (Supplementary
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16 518 information, Fig. S6e, f) (36). In addition, hierarchical clustering analysis linked these cell types
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18 519 together based on culture condition rather than genotype (Supplementary information, Fig. S6g).
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20 520 Our results emphasise that mESCs can achieve a “transcriptional ground state” in 2i through
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22 521 enhanced expression of a number of pluripotency associated genes, including *Nanog*, *Esrrb* and
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24 522 *Klf4*, irrespective of global methylation status, and that hypomethylation is dispensable for this
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26 523 transition (Fig. 3f).
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35 525 **Epigenetic changes in the 2i mediated ground state**

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39 526 To study functional consequences of DNA methylation rearrangement in the reprogramming of
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41 527 mESCs to ground state, we generated genome-wide patterns of 5mC in WT J1 and 3B31 mESCs
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43 528 cultured in serum and 2i by genome wide MeDIP-seq assays (31). We validated the levels of
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45 529 5mC enrichment across the genome in J1 and 3B31 epitypes and report upon an approximately
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47 530 50% reduction in the number of both 5mC enriched loci (termed windows, see material and
48
49 531 methods) and HPLC quantified 5mC scores in J1-2i mESCs (Fig. 1e and Fig. 4a). The number of
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51 532 enriched windows in 3B31-serum was similar to those in WT J1-serum; as expected this cell line
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53 533 did not exhibit the typical loss of 5mC upon 2i exposure (Fig. 4a). Interestingly, although global
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55 534 levels of 5mC differ between the epitypes upon transition to ground state, by western blot there
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59 535 was no obvious change in total H3K27me3 levels in WT J1 and 3B31 mESCs in serum or 2i in

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3 536 agreement with two reports, but H3K27me3 levels were higher in TKO-2i cells (Fig. 4b) (13,40).
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5 537 In contrast, a recent proteomic study found higher H3K27me3 levels in WT mESCs cultured in
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7 538 2i conditions; despite this they also report, by ChIP-seq, a redistribution of H3K27me3 profiles
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9 539 in 2i culture conditions (23). Although we report an overall reduction in 5mC levels within these
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11 540 cells (as evident from a drop of 5mC peaks to 4.7% in the J1-21 cells with respect to the serum
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13 541 state. Fig. 4c), the underlying genomic patterns remain largely unchanged (Fig. 4d;
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15 542 Supplementary information, Fig. S8a, b)
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24 544 To refine our assessment, we interrogated 5mC and H3K27me3 patterns across a number
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26 545 of functional genomic compartments (Fig. 4). Focusing first on promoter and gene body regions,
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28 546 we discovered that although levels and patterns of 5mC are lost upon transition to 2i in WT J1
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30 547 cells, these are maintained across the same sets of promoters and gene bodies in J1-serum, 3B31-
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32 548 serum and 3B31-2i mESCs (Fig. 4a and 4c-e). Analysis of locus specific patterns also revealed
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34 549 that 5mC marked promoters identified in WT J1 serum mESCs remain similarly marked in both
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36 550 3B31-serum and 2i cells (Fig. 4d, e). To analyse H3K27me3 patterns we utilised a published E14
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38 551 mESC dataset as our WT reference data (GSE23943) as their deposition at bivalent promoters is
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40 552 reported to be dependent on global DNA methylation levels (13,43,48). H3K27me3 patterns are
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42 553 typically reduced upon transition to 2i in WT E14 mESCs but broadly maintained over promoter
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44 554 and genic regions in the constitutively methylated cell line in 2i (Fig. 4d, f). To delineate 5mC
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46 555 mediated changes from 2i signalling changes in H3K27me3 distribution; we compared patterns
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48 556 of H3K27me3 between TKO-serum and TKO-2i mESCs. Interestingly we note a reduction in the
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50 557 H3K27me3 signal, which manifests in loss from a number of promoter elements (Fig. 4f, g).
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52 558 Stratification of the total promoter set based on regions which lose H3K27me3 between TKO-
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54 559 serum and TKO-2i revealed a subset of 2i specific, DNA methylation independent regulation loci
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3 560 across all epitypes (Fig. 4f). Visualisation of these loci (specific examples; *Neurog3*, *Hoxc12-13*
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5 561 and *Tbx3*) uncovered a strong loss of H3K27me3 levels in 2i conditions irrespective of global
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7 562 DNA methylation state – highlighting a small set of common 2i signalling induced epigenetic
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9 563 changes in all three mESC epitypes (Fig. 4h).

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13 564 Analysis over enhancer and pluripotency factor binding elements revealed that such loci
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15 565 are typically depleted in 5mC and H3K27me3 in both serum and 2i conditions for all three
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17 566 epitypes (Fig. 4d; Supplementary information, Fig. S8b) (22). Analysis over annotated replication
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19 567 origin sites revealed a general loss of 5mC in WT J1-2i conditions but higher levels of 5mC at
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21 568 medium and late replicating sites in both WT J1-serum, 3B31-serum and 3B31-2i mESCs (Fig.
22
23 569 4d) (49). Similarly, there is a general loss of H3K27me3 in WT E14-2i cells with higher levels
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25 570 of H3K27me3 present in early replication origins in WT E14-serum, 3B31-serum and 3B31-2i
26
27 571 lines (Fig. 4d). Additionally, DNA methylation is lost at repeat sequences (including major
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29 572 satellite and LINE-1 retrotransposons) in WT J1-2i but maintained in 3B31-2i mESCs (Fig. 4d).

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35 573 Analysis of RNA-seq data for WT J1, TKO and 3B31 mESCs cultured in serum and 2i
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37 574 revealed that some differences exist between culture conditions and epitypes tested in terms of
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39 575 retrotransposon expression (Supplementary information, Fig. S8c-f). Consistent with previous
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41 576 observations, retrotransposon derepression in response to 2i or changes in DNA methylation was
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43 577 relatively modest in mESCs (Supplementary information, Fig. S8c), and smaller in magnitude
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45 578 than observed for differentially expressed genes (50-52). A subset of 44 (28%) of the 156
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47 579 retrotransposons upregulated when WT J1 mESCs transition from serum to 2i is also upregulated
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49 580 when TKO and 3B31 mESCs transition from serum to 2i (Supplementary information, Fig. S8d).
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51 581 These retrotransposons, which include RLTR13B2::ETNERV3-int and RLTR44C::RLTR44-int
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53 582 long terminal repeats and associated internal sequences, are upregulated in 2i regardless of
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55 583 methylation state (Supplementary information, Fig. S8f). Some elements in this subset, such as
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3 584 RLTR10D::IAP-d-int, appear to exhibit a combinatorial response to 2i and DNA methylation
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5 585 such that they are upregulated in response to hypomethylation and further upregulated in response
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7 586 to 2i (Supplementary information, Fig. S8f). These elements exhibit similar behaviour to *Dazl*
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9 587 and *Fkbp6* genes (Fig. 5b), although it is also possible that the mixed response of these elements
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11 588 reflects distinct responses of individual genomic copies of that repeat. Interestingly, most (149 of
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13 589 203) of the retrotransposons that are upregulated in response to DNA hypomethylation in serum-
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15 590 cultured mESCs, including RLTR44B and MMERVK10D3_LTR, are not upregulated when WT
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17 591 J1 mESCs transition from serum to 2i (Supplementary information, Fig. S8e-f). Thus, different
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19 592 retrotransposons respond to the 2i environment, changes in DNA methylation, or a combination
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21 593 of both in mESCs. Finally, imprinted loci exhibit a greater level of both 5mC and H3K27me3
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23 594 retention during 2i reprogramming in both WT J1 and 3B31 mESCs, arguing that maintenance of
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25 595 5mC levels may be functionally required at such sites (Fig. 4d).

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33 34 35 597 **Analysis of epigenetic changes over transcriptionally altered genes.**

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38 598 As we observed a consistent change in transcription in all three epitypes when converted to 2i
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40 599 mediated ground state, we set out to elucidate whether specific changes to 5mC and H3K27me3
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42 600 may be fundamental drivers of these changes. For the total arrayed gene set for all three cell types
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44 601 as well as for gene sets exhibiting strong changes in gene expression when cultured in 2i versus
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46 602 serum, we did not detect a relationship between alterations in promoter 5mC levels and the
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48 603 expression status of linked genes (Fig. 5a & Supplementary information, Fig. S9a). To validate
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50 604 this result, we selected two candidate methylation regulated genes; *Dazl* and *Fkbp6*, and
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52 605 examined the effect of 2i signalling on their expression in WT J1, TKO and 3B31 mESCs by RT-
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54 606 qPCR (Fig. 5b) (13,53). These two transcripts are highly upregulated in TKO compared to WT
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56 607 J1 mESCs, supporting a role for promoter demethylation in their upregulation. However, they are

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3 608 both further upregulated in 2i in TKO and 3B31 mESCs and we observed comparable changes in
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5 609 *Dazl* protein levels by DigiWest analysis (Fig. 5b and Supplementary information, Fig. S9b). The
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7 610 promoters of these genes exhibited demethylation in WT J1-2i but maintained DNA methylation
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10 611 in 3B31-2i as evidenced by meDIP and locus-specific bisulfite sequencing (Fig. 5b and 1f).
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12 612 Hence, at transcript level, regardless of whether their promoters were hypomethylated or
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14 613 hypermethylated, both *Dazl* and *Fkbp6* responded in the same way to 2i signalling, resembling
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17 614 what we observed for a subset of 2i mediated changes in DNA repeat expression (Fig. 5b and
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19 615 Supplementary information, Fig. S8c-f).
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23 616 We did observe a relationship between loss of H3K27me3 from genic regions of genes
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25 617 transcriptionally induced in 2i in all three epitypes; a relationship which was not observed over
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27 618 transcriptionally repressed genes (Fig. 5c). To test whether these genes are regulated by 2i
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29 619 mediated signalling, we assayed their expression levels in mESCs with altered H3K27me3 levels;
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31 620 mESCs exposed to an inhibitor of the polycomb protein, EZH2 methyltransferase (EPZ6438), or
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33 621 through analysis of published expression data from polycomb regulatory complex mutant mESCs
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35 622 (*Eed*^{-/-}) (54,55). We found that 2i induced genes, which lost H3K27me3 in WT, 3B31 and TKO
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37 623 mESCs, were not induced in these H3K27me3 perturbed serum cultured cells (Fig. 5d;
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39 624 Supplementary information Fig. S10), indicating that changes in expression of these genes are
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41 625 also direct consequences of 2i signalling and not due to changes in H3K27me3 deposition.
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50 627 **Relationship between gene regulatory network and epigenetic landscape**

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54 628 A recent study has shown that the differential binding of a core network of the pluripotency
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56 629 factors Oct4, Sox2 and Nanog (OSN) is important in reaching ground state following 2i exposure
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58 630 (22). However, it is not known how the epigenetic landscapes at these key regulatory sites are
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3 631 related to factor binding events and establishment of specific gene regulatory networks. We first
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5 632 set out to elucidate how the transcriptional landscape at genes near differentially bound OSN sites
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8 633 is affected during 2i transition by identifying the nearest neighbouring gene (first gene within
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10 634 10kb of an elevated or reduced OSN site in 2i, referred to as distal elements) and analysed their
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12 635 expression changes in our cell panel. We discovered a similar number of distal genes that are
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14 636 induced and repressed near 2i specific OSN sites upon 2i conversion but observed a large
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17 637 reduction in transcription at distal genes where OSN factors are reduced in 2i, arguing that
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19 638 transcription from a subset of genes is linked to binding of distal factors (Fig. 6a and
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21 639 Supplementary information, Fig. S11a). When we rank 2i driven changes in gene expression at
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23 640 these distal genes in WT J1 mESCs (i.e. $\log_2 \text{FC J1-serum} > \text{J1-2i}$, Fig. S11a), we observe similar
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25 641 transcriptomic changes following 2i conversion in WT J1, 3B31 and TKO mESCs (Fig. 6a,
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28 642 Supplementary information, Fig. S11a, b).

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32 643 Enhancer elements and OSN binding sites are typically DNA hypomethylated, including
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34 644 in 3B31 mESCs. We therefore tested the hypothesis that active DNA demethylation could play a
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36 645 key role in the maintenance of this epigenetic state and that if perturbed would affect
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39 646 constitutively methylated mESCs to a greater degree than WT cells, potentially abrogating their
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41 647 ability to reach ground state. To do so we used an siRNA approach to reduce the levels of the
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43 648 DNA demethylation machinery members Tet1 and Tet2 in WT J1 and 3B31 mESCs cultured in
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45 649 serum or for 24h in 2i (see methods). Compared to scrambled siRNA controls, transfection of
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48 650 published siRNAs against Tet1 and Tet2 (Supplementary information, Fig. S11c) was sufficient
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50 651 to reduce the 5hmC signal in these cells to undetectable levels by immunocytochemistry (Fig.
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52 652 6b) (56). This did not interfere with cell viability as evaluated by morphology, Esrrb staining or
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55 653 pluripotency marker expression assessed by qRT-PCR, indicating the 5hmC pathway was not
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57 654 required for the serum to 2i transition in WT J1 and 3B31 mESCs (Fig. 6b, c).

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Discussion

In this paper we address some widely held assumptions regarding the epigenetic identity of ground state ES cells (57). Early observations reported that the transition of ES cells to ground state, dependent on ground state promoting signalling pathways, is accompanied by a reduction in DNA methylation levels (20,34). This major epigenetic state change, which also involves altered H3K27me3 deposition, was viewed as integral to the cells functional transition through gene expression changes (13). Surprisingly, however, this premise had not been fully tested. Our study reports three unexpected important new insights: the reduction in DNA methylation is not a prerequisite for ES cells to achieve the ground state transition; this global DNA hypomethylation does not lead to transcription alterations of the genes undergoing demethylation; and H3K27me3 redistribution induced by the reduction in DNA methylation does not lead to transcription changes of the genes affected, which are dependent on changes in transcriptional factor networks (22). Our conclusions obtained by a novel route of comparing serum to 2i transitions of wild type, hypomethylated and constitutively DNA methylated mESCs, are in alignment with work using PRC2 deficient mESCs that defined 2i induced transcription factor and proteomic changes respectively (22,23). These papers showed that PRC2 function is not required for mESC pluripotency, self renewal and the 2i transition. Significantly Van Mierlo et al. also observe in 2i cultured PRC2 deficient mESCs a relative increase in DNA methylation levels that appears to be due to enhanced protein expression of Dnmt3a, Dnmt3b and Dnmt3l (23). This observation again challenges the view that it is perturbation of the Dnmt1/Uhrf1 axis that is responsible for hypomethylation in 2i cultured mESCs (35). Consistent with a direct impact of PRC2 deficiency on DNA methylation levels in 2i cultured mESCs, hypermethylation is also observed at the CGI shores of a distinct class of bivalent genes in serum/LIF cultured PRC1/2 mutant mESCs (43). This implies that DNA methylation and Polycomb, two distinct epigenetic repression systems, have direct as well as indirect impacts on each other. Collectively our data

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3 681 and data from Van Mierlo et al. and Galonska et al. support the view that during the 2i transition
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5 682 changes in H3K27me3 and/or DNA methylation are secondary effects, which do not influence
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8 683 specific gene expression in 2i mESCs (22,23). These conclusions raise the question to what extent
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10 684 the epigenetic state of a cell defines its identity. In the case of embryonic stem cells the answer
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12 685 points to an epigenetic ground state that is decoupled from the pluripotency ground state, with
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14 686 both the gene regulatory network and the epigenetic network poised to interconnect at or after
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16 687 differentiation. Of relevance here is the previous suggestion that enhanced germline gene
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18 688 expression in ground state mESCs is primarily a consequence of global DNA hypomethylation
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20 689 (50). We tested this premise and found that patterns of gene expression including germline, are
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22 690 similarly dependent on 2i culture conditions, and occur independently of DNA methylation
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24 691 status. Our observations imply that global reduction of 5mC is not required for gene regulatory
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26 692 networks to orchestrate changes in transcription resulting in altered stem cell states. This is in
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28 693 line with the observation that in some species extensive reprogramming of DNA methylation in
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30 694 early embryogenesis is not observed (58,59).

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38 39 40 696 **H3K27me3 regulated gene expression**

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43 697 All three mESC epitypes showed similar morphological changes, enhanced expression of
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45 698 pluripotency factors and signalling induced changes at a discrete set of H3K27me3 marked genes
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47 699 (e.g. *Tbx3*, *Hoxc12* and *Hoxc13*). Notably, expression of these H3K27me3 marked genes was not
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49 700 activated in serum in the absence of polycomb repression. This agrees with our recent work on
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51 701 the identification of DNA methylation mediated changes in chromatin compaction that occurs
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53 702 when WT mESCs are shifted from serum to 2i growth conditions (25). Chromatin decompaction
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55 703 at polycomb marked regions is prevented in 3B31-2i mESCs when global DNA methylation is
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57 704 maintained, but inhibition of decompaction also does not appear to have phenotypic
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3 705 consequences. Similarly, polycomb dependent chromatin compaction in hypomethylated TKO
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5 706 cells is predicted to be already altered in serum conditions, but, as we have shown, does not
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7 707 significantly impact on the ability of these cells to transition to a ground state under 2i conditions.
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10 708 These observations support the idea that DNA methylation and polycomb processes are not
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12 709 instrumental for the transition of mESCs to a ground state (22,60). It is noteworthy that during
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14 710 early cleavage-stage mouse embryos, H3K27me3 is absent from promoters under
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16 711 hypomethylated conditions (43,61). It is only later that bivalent domains (H3K4me3/H3K27me3)
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18 712 and topological associated domains are newly established in the inner cell mass/trophectoderm,
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20 713 which constitutes the chromatin environment that supports the gene regulatory networks
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22 714 necessary for subsequent development (61,62). Our results widen the impact of the observation
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24 715 that H3K27me3 is dispensable for repression of bivalent genes and de novo silencing in 2i (22).
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31 32 33 717 **DNA methylation regulated gene expression**

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36 718 Our data provide a set of core transcriptomic changes that occur independently of methylation in
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38 719 the conversion of mESCs to the ground state. Despite hypomethylated TKO-serum mESCs
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40 720 showing some intermediate gene expression states, they firmly cluster with WT-serum mESCs
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42 721 and have no less requirement for 2i signalling for conversion to a uniform ground state. Our
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44 722 orthologous experiments demonstrate that constitutive DNA methylation is also not an
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46 723 impediment to 2i adaptation. The impact of DNA methylation loss at promoter regions is
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48 724 relatively restricted here, but even at bona fide methylation dependent genes (*Dazl* and *Fkbp6*),
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50 725 their induction in 2i conditions occurs independently of whether their promoters are constitutively
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52 726 hypomethylated or methylated. The promoter methylation that can be directly overridden in
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54 727 mESCs contrasts with observations of its inhibitory effect in somatic cells to date, and needs
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56 728 further investigation of these different cellular contexts (53). Changes in retrotransposon
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3 729 expression in response to hypomethylation or 2i signalling are not dramatic in contrast to
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5 730 retrotransposon derepression in hypomethylated somatic cells, or mESCs defective in *Setdb1*-
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7 731 dependent histone methylation (30,51,52). Tellingly, neither *Prdm14* inactivation in WT cells,
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9 732 nor its overexpression in TKO mESCs affects 2i mediated transition to the naïve state (36).
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11 733 *Prdm14*^{-/-} mESCs in 2i exhibit global levels of DNA methylation comparable to WT mESCs
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13 734 cultured in serum, which offers strong support that *Prdm14* is a key driver of DNA demethylation
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15 735 in 2i cultures, and not inhibition of Dnmt1 activity by a deficiency of UHRF1 and H3K9me2
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17 736 factors (20,27,35). Analysis of changes in gene subsets related to stem cell maintenance and cell
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19 737 fate commitment in microarray data from *Prdm14*^{-/-} and WT mESCs in 2i with hierarchical
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21 738 clustering of these datasets are in complete agreement with the present study suggesting that the
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23 739 role of *Prdm14* in antagonising FGFR signalling and repression of *de novo* Dnmt enzymes maybe
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25 740 incidental to ground state conversion (20). The characteristic changes that 3B31 mESCs undergo
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27 741 in 2i in terms of morphology, pluripotency marker homogeneity and gene expression changes in
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29 742 stem cell maintenance and cell fate commitment gene subsets supports this view. This indicates
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31 743 that serum/LIF associated patterns of DNA methylation and H3K27me3 are compatible with the
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33 744 2i state. In addition, OSN enhancers associated with canonical Wnt and ERK signalling pathways
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35 745 appear to function equivalently in all three epitypes and in the case of 3B31 mESCs did not depend
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37 746 on Tet mediated demethylation for activity in 2i conditions. We conclude that reorganisation of
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39 747 core pluripotency factors in 2i is independent of the global epigenetic (DNA methylation and
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41 748 H3K27me3) state of the cells.
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50 749 Our results raise new hypotheses regarding the role of DNA methylation and PRC2
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52 750 (Polycomb Repressive Complex 2) in the 2i transition. In the blastocyst embryo, the early ICM
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54 751 state exists during a short-lived window in the lead-up to embryonic differentiation or the
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56 752 derivation of mESC lines, which are thought to acquire late epiblast characteristics in *in vitro*
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58 753 cultures (9). This is accompanied by higher levels of DNA methylation, more restricted *Prdm14*
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3 754 expression, fluctuating pluripotency gene expression and emergence of differentiation marks and
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5 755 heterochromatin maturation marks as well as a shorter cell cycle time (10,63). From this
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8 756 perspective, the conversion of serum mESCs to 2i conditions can be regarded as a reprogramming
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10 757 of these cells to an earlier pluripotent state, which does not maintain these late markers, but their
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12 758 presence is not a hindrance to regaining a 2i mediated ground state. This may be especially
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14 759 relevant to redefining the hallmarks of naïve pluripotency in other mammals that may have
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16 760 distinct developmental epigenetic reprogramming patterns (14). In mouse early preimplantation
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18 761 embryos, a state of low DNA methylation is actively and passively maintained through
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20 762 mechanisms of DNA demethylation, Dnmt1 exclusion, cell division, and Prdm14 expression,
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22 763 while chromatin is dynamic and more dispersed (61,62,64). The organisation of condensed
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24 764 chromatin domains and chromocenters appears after implantation and also in epiblast stem cells,
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26 765 for which DNA methylation is required (64).

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32 766 We propose that global hypomethylation is a consequence of transcriptional and
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34 767 signalling changes in 2i medium, but is not essential for adaptation of mESCs to the
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36 768 “transcriptional ground state” as defined by core transcription changes that occur irrespective of
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38 769 methylation in the conversion of mESCs to the ground state. This implies that signalling induced
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40 770 gene regulatory networks, and not epigenetic mechanisms, dominate to regulate transcriptional
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42 771 changes required for mESCs to achieve ground state. This is compatible with studies showing
43
44 772 that inactivation of many epigenetic regulators (*Ezh2*, *Eed*, *Dnmt1*, *Uhrf1*, *Ring1b*, *G9a*, *Glp*,
45
46 773 (*Suv39h1* and *Suv39h2*), *Jarid1b* and *Tet1-3*) is compatible with development up to blastocyst
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48 774 stages and derivation of self-renewing mESCs but incompatible with later stages of development
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50 775 when pluripotency networks are attenuated and alternative differentiation associated GRNs are
51
52 776 prominent (65). It is clear that altered patterns of DNA methylation and polycomb in the naive
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54 777 state do not define its identity through gene regulation but may reflect genomic reprogramming
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56 778 to earlier patterns present in pre-blastocyst embryos, with 2i conditions reversing chromatin
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3 779 transitions that may develop into the epigenetic control of gene expression in differentiated cells.
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5 780 A key question is, when do these repressive epigenetic pathways become operational and possibly
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8 781 outweigh signalling cues, and what roles do these early transitions play in subsequent
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10 782 differentiation to stable somatic lineage identities. It is plausible that this is linked with core
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12 783 transcription factor changes enabling essential regulatory roles for epigenetic processes in
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14 784 coordinating and maintaining differential patterns of gene expression.
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56 799 **Author Contributions**

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2
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4
5 801 based assays and development of antibody based semi-conductor sequencing protocols), S.K.,
6
7 802 F.T. and M.F.T. (DigiWest), B.R. (HPLC), S.P. and T.W. (ICC of embryos); Investigation, R.S.,
8
9 803 H.K.M., J.P.T., S.K., F.T., M.F.T., B.R., S.P. and T.W.; Formal analysis, H.K.M. and SP (ICC),
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13 805 ChIPseq data); I.R.A. (RNA-seq repeat analysis); F.T. (DigiWest analysis). Data curation, R.S.
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310 **Competing interests**

311 The authors declare no competing interests.

312 Supplementary information is available on this journal's website

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Figure legends

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Fig. 1. J1, TKO and 3B3l mESCs achieve ground state in 2i.

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a, Schematic showing cell line derivation. An expression construct for the co-factor *Dnmt3l* was

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added to *Dnmt3a/3b* knockout mESCs rescued by exogenous constitutive expression of *Dnmt3b*

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(3B) to create a *Dnmt3a/3b* knockout expressing both *Dnmt3l* and *Dnmt3b* (3B3l). *Dnmt3l* was

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also added to a to *Dnmt3a/3b* knockout mESCs (KOVI) to create KOVI-3l. **b**, Morphology

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images of mESCs in indicated culture conditions. Scale bar 100µm. **c**, Expression levels of

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indicated *Dnmt*'s and *Prdm14*. For each gene, fold change with respect to (wrt) average J1-serum

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3 1049 probe value is represented (as log₂) with SEMs. * indicates $p < 0.05$ (unpaired t-test). **d**,
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5 1050 Immunocytochemistry images of indicated conditions showing Nanog (green) and Esrrb (red)
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7 1051 with DNA counterstaining (DAPI, blue) and merge. Scale bar 100 μ m. **e**, HPLC quantification of
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9 1052 5mC levels of indicated mESCs, bars represent mean \pm SD of 2-3 biological replicates. **f**, Bisulfite
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11 1053 sequencing data for indicated gene promoters and cell lines, black: methylated CpG, grey: un-
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13 1054 methylated CpG, white: missing data. Numbers represent percentage of methylated CpGs. *
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15 1055 (black: methylated CpG, grey: un-methylated CpG, white: missing data). * $p < 0.05$, ** $p =$
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17 1056 0.00578, 2-tailed Mann-Whitney U-Test.
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24 1058 **Fig. 2. Epigenetic state of heterochromatin in mESCs and H3K27me3 dynamics during**
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26 1059 **mouse blastocyst development.**
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30 1060 **a-b**, ICC of H3K27me3 (green) and H2AK119Ubiquitin (green) in indicated cells with DAPI
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32 1061 (blue) and merge (turquoise represents overlapping signal). White outlined nuclei are 'zoomed
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34 1062 in' images of representative nuclei. Numbers represent the percentage of cells where green foci
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36 1063 overlap with DAPI stained heterochromatin. Scale bar 50 μ m. **c**, ICC for H3K27me3 (green) with
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38 1064 DAPI (pseudocoloured red). Whole and part embryo confocal projections and highlighted cells
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40 1065 from the inner ICM and the ICM margin (yellow represents overlapping signal), as indicated;
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42 1066 scale bars 10 μ m. Arrowheads indicate three cells showing heterochromatic H3K27me3 staining.
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48 1068 **Fig. 3. Gene expression changes in mESCs upon culturing in 2i.**
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52 1069 **a**, Heatmap showing Pearson correlation and clustering of microarray data for indicated cell lines
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54 1070 and culture conditions. **b**, Venn diagram showing the number of genes changing in expression
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56 1071 ($FC \geq 2$, $p_{adj.} \leq 0.05$ eBayes (limma), Benjamini-Hochberg corrected) in J1-2i (red), TKO-2i
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58 1072 (green) and 3B3l-2i (yellow) compared to their serum counterparts. **c**, Log₂ expression values of
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3 1073 indicated genes in mESCs in serum and 2i. Bars represent mean \pm SD. * indicates $p < 0.05$
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5 1074 (unpaired t-test). **d**, GO-Term analysis showing genes up- or down-regulated in J1, TKO and
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7 1075 3B3l mESCs. **e**, Heatmap representing expression levels of 'Cell Fate Commitment' genes
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9 1076 differentially expressed ($FC \geq 1.5$, $p.adj. \leq 0.05$ eBayes (limma), Benjamini-Hochberg corrected)
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11 1077 in J1-2i compared to J1-serum in indicated cells. * marks genes which are differentially expressed
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13 1078 ($FC \geq 2$, $p.adj. \leq 0.05$ eBayes (limma), Benjamini-Hochberg corrected) between TKO-serum and
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15 1079 J1-serum. **f**, Same as **e** for 'Stem Cell Maintenance' genes.
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22 1081 **Fig. 4. 5mC and H3K27me3 modifications in J1, 3B3l and TKO mESCs in serum and 2i.**

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25 1082 **a**, Windows enriched in 5mC relative to input sequence for indicated cell lines and conditions. **b**,
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27 1083 Fluorescent western blot of whole cell protein extracts showing levels of H3K27me3 for indicated
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29 1084 cell lines cultured in serum or 2i. β -tubulin was used as a loading control. **c**, Total number of
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31 1085 5mC peaks (see materials and methods) for indicated cell lines and conditions. **d**, Heatmap
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33 1086 indicating 5mC (red) and H3K27me3 (purple) levels; 0-10, see scale, for indicated cell lines and
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35 1087 conditions across indicated genomic features, covering regions as described. Q4-Q1 indicates
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37 1088 low to high gene expression. **e**, Sliding window analysis of average genic 5mC patterns across
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39 1089 indicated cell lines. Plots represent length normalised gene bodies with flanking regions ($\pm 25\%$
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41 1090 of total gene length). Stratification of genic patterns by promoter 5mC enrichment (upper box) or
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43 1091 depletion (lower box). Numbers of genes per set are shown above. **f**, Boxplots representing
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45 1092 average H3K27me3 signal on promoters (± 1 kb from TSS) for all promoters or promoters
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47 1093 preferentially losing H3K27me3 in TKO-2i relative to TKO-serum. * indicates $p < 0.05$
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49 1094 (Wilcoxon test). **g**, Heatmap representing normalised H3K27me3 reads across promoters (± 5 kb
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51 1095 TSS). Bottom graphs represent averaged profiles. Heatmaps are ranked by E14 serum levels. **h**,
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53 1096 Examples of H3K27me3 pattern across *Neurog3*, *Hoxc13*, *12*, *11* and *Tbx3* for indicated cell lines
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55 1097 and culture conditions.
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6 1099 **Fig. 5. Epigenetic changes over transcriptionally altered genes in mESCs in serum and 2i.**

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9 1100 **a**, Boxplots of average promoter 5mC in indicated cell lines over the total gene set, genes
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11 1101 expressed under 2i conditions and genes repressed under 2i conditions. * indicates $p < 0.05$
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13 1102 (Wilcoxon test). **b**, Examples of 5mC data at the promoters of the methylation regulated genes
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15 1103 *Dazl* and *Fkbp6* alongside RT-qPCR data for serum and 2i conditions. Promoter regions are
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17 1104 marked by grey shadows. Barcharts show expression analysis of indicated genes by RT-qPCR,
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19 1105 values represent mean \pm SD of expression with respect to (wrt) TBP. * indicates $p < 0.05$
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21 1106 (unpaired t-test). **c**, Scatter plot of fold change in expression 2i-serum (y-axis) versus fold change
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23 1107 in average genic H3K27me3 levels (2i-serum) in WT (left), 3B31 (centre) and TKO (right) cells.
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25 1108 Yellow = 2i induced genes, blue = 2i repressed genes. **d**, Boxplot detailing expression changes
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27 1109 serum to 2i (log₂ fold changes) across WT, TKO, 3B31, *Eed*^{-/-} and EPZ6438 treated *Dnmt1*^{tet/tet}
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29 1110 mESCs. Left: genes with overlapping H3K27me3 loss/expression gain in J1/TKO/3B31 mESCs.
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31 1111 Right: genes with overlapping H3K27me3 gain/expression loss in J1/TKO/3B31 mESCs.
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39 1113 **Fig. 6. Relationship between, gene regulatory networks, epigenetic marks and culture**
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41 1114 **conditions in mESCs.**

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45 1115 **a**, Z-score normalised heatmaps ranked by relative expression level of the nearest gene to an
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47 1116 OSN binding site (within +/-10kb) with increased binding in 2i (top) or increased binding in
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49 1117 serum (bottom) for indicated cell lines. Hierarchical clustering groups by culture condition. (b-c)
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51 1118 siRNA knockdown of Tet1 and Tet2 for indicated cell lines. **b**, Immunocytochemistry for
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53 1119 indicated cell lines. Scale bar is 100 μ m. Left: DAPI (blue), 5hmC (green) and merge. Right:
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55 1120 DAPI (blue), Esrrb (green), Tet1 (yellow), Cy3-siRNA (red) and merge. **c**, Expression analysis
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57 1121 of indicated genes by RT-qPCR in J1-36 (blue) and 3B31 (orange) mESCs in 72h serum or 48h
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1122 serum + 24h 2i transfected with either scrambled siRNA or siRNAs for Tet1 + Tet2. Values
1123 represent mean +/- S.D. of three technical replicates with respect to (wrt) to the housekeeping
1124 gene TBP (TATA Binding Protein), normalised to serum siScrambled values.

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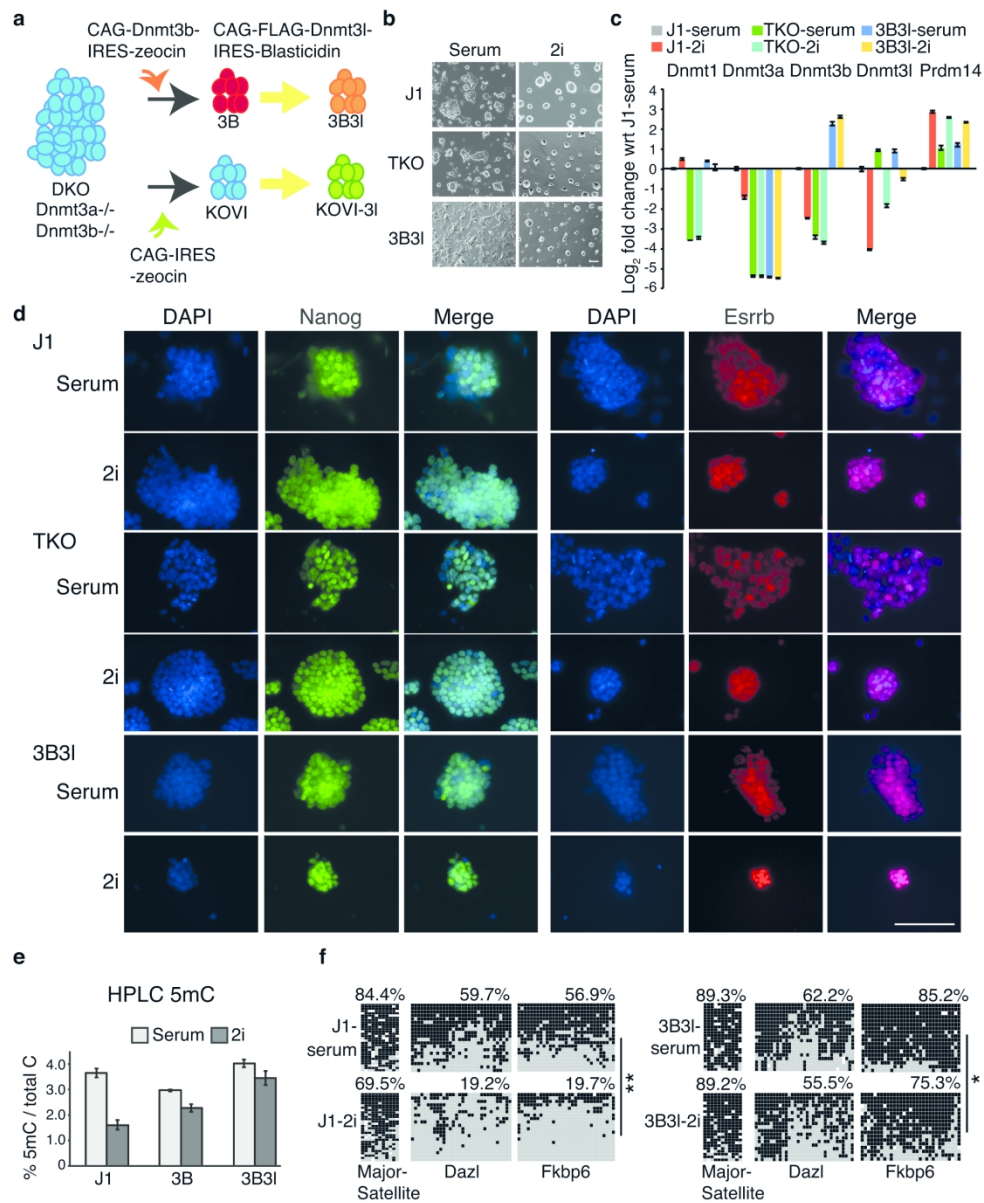


Figure 1: J1, TKO and 3B3I mESCs achieve ground state in 2i.

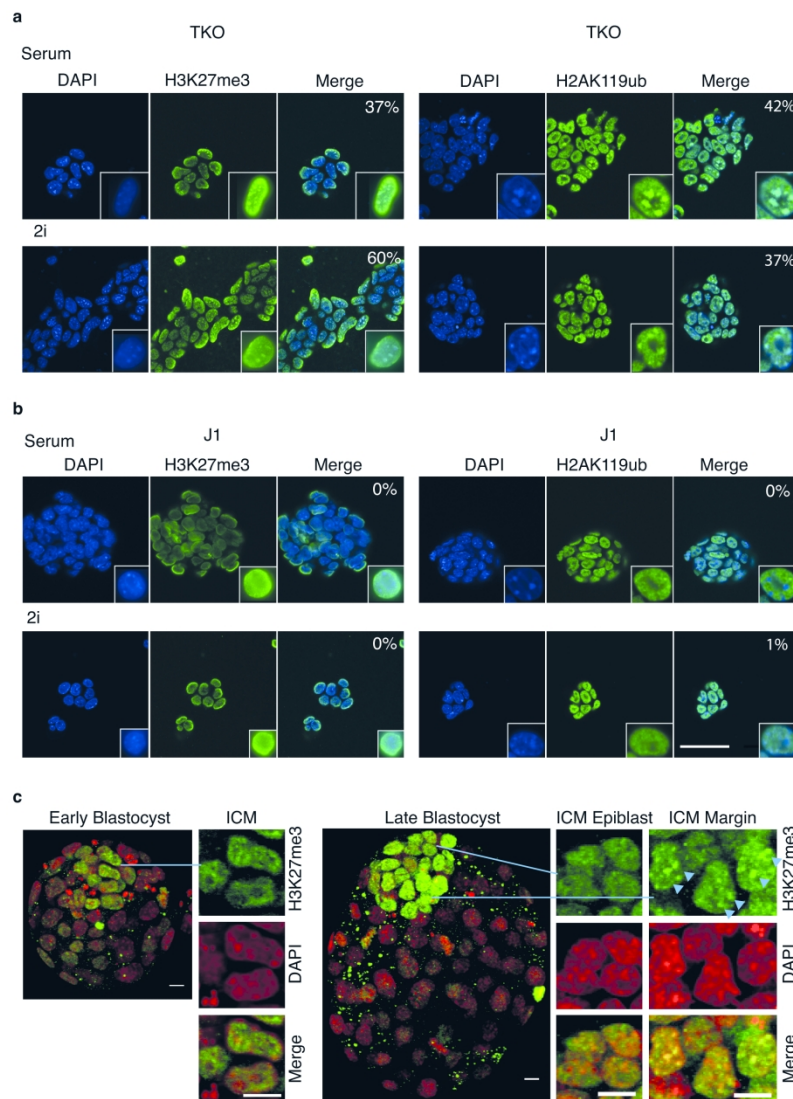


Figure 2: Epigenetic state of heterochromatin in mESCs and H3K27me3 dynamics during mouse blastocyst development.

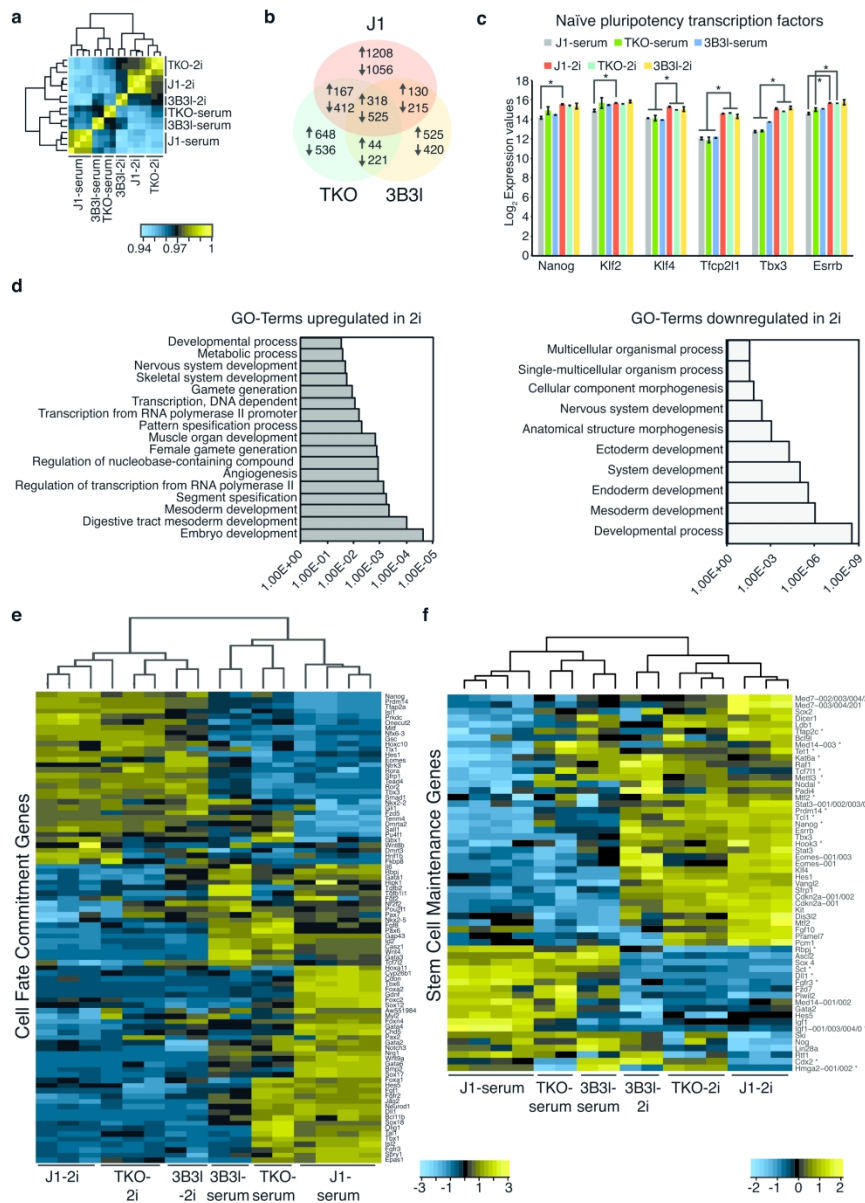


Figure 3: Gene expression changes in mESCs upon culturing in 2i.

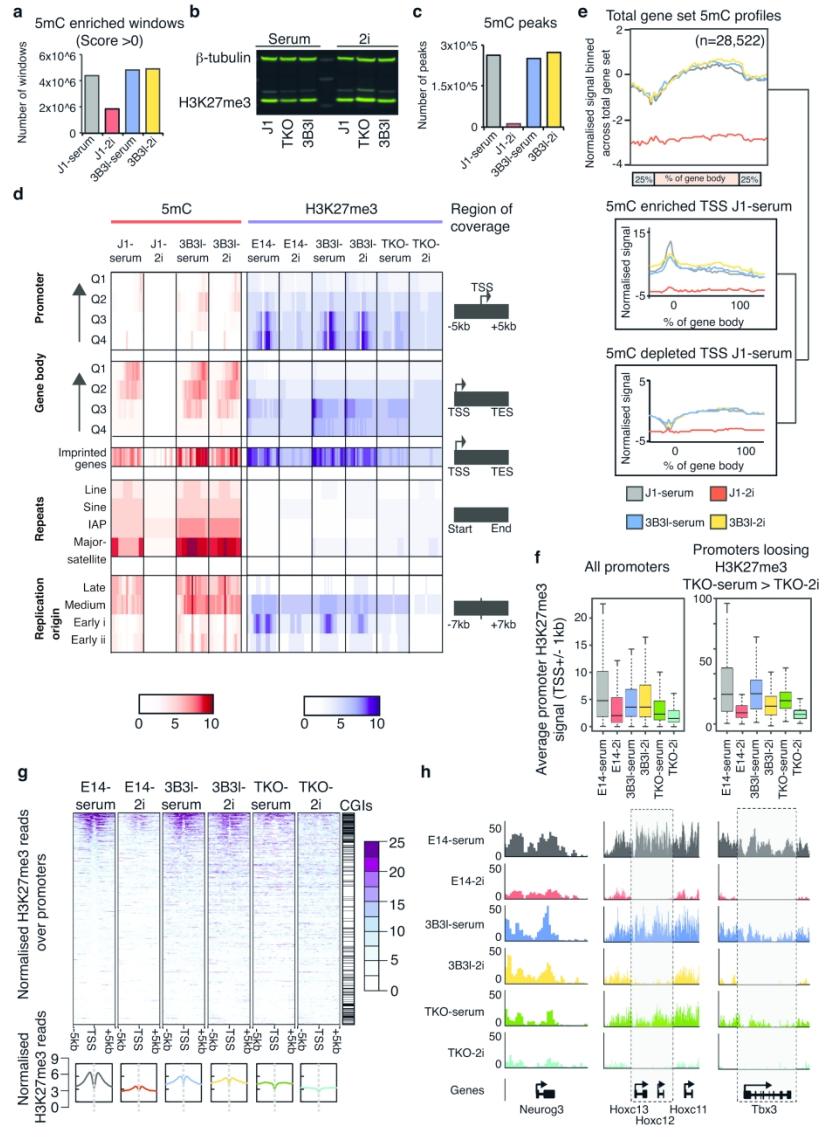


Figure 4: 5mC and H3K27me3 modifications in J1, 3B3I and TKO mESCs in serum and 2i.

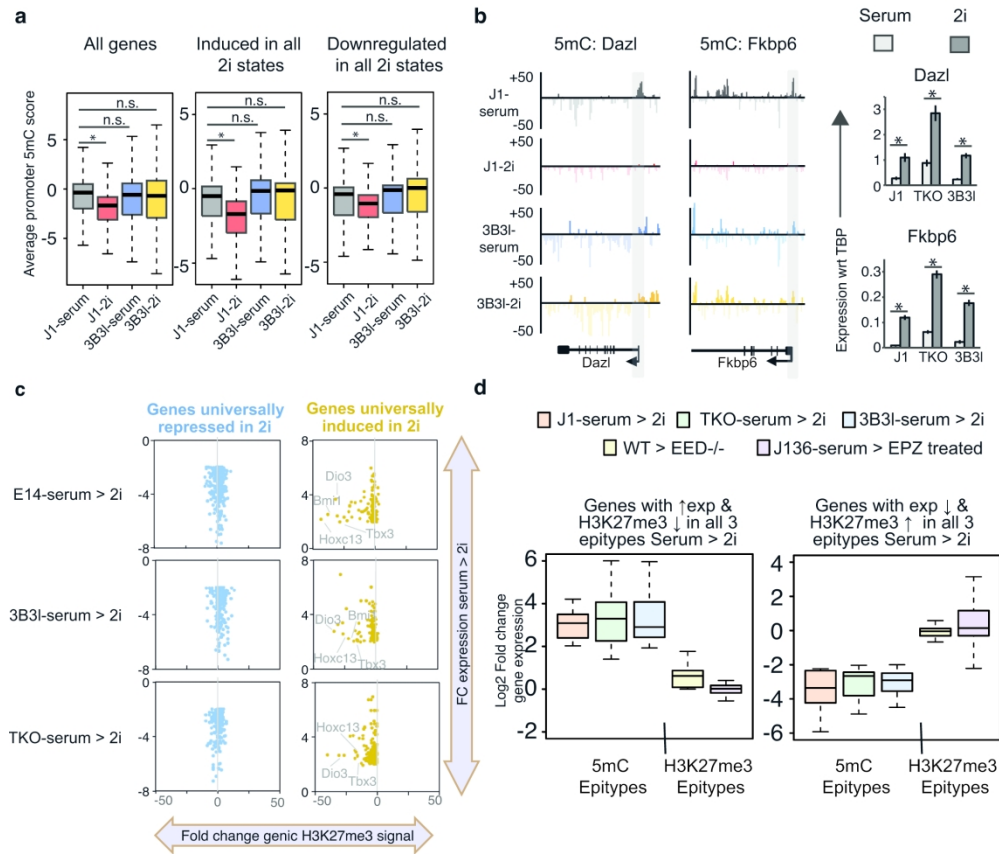


Figure 5: Epigenetic changes over transcriptionally altered genes in mESCs in serum and 2i.

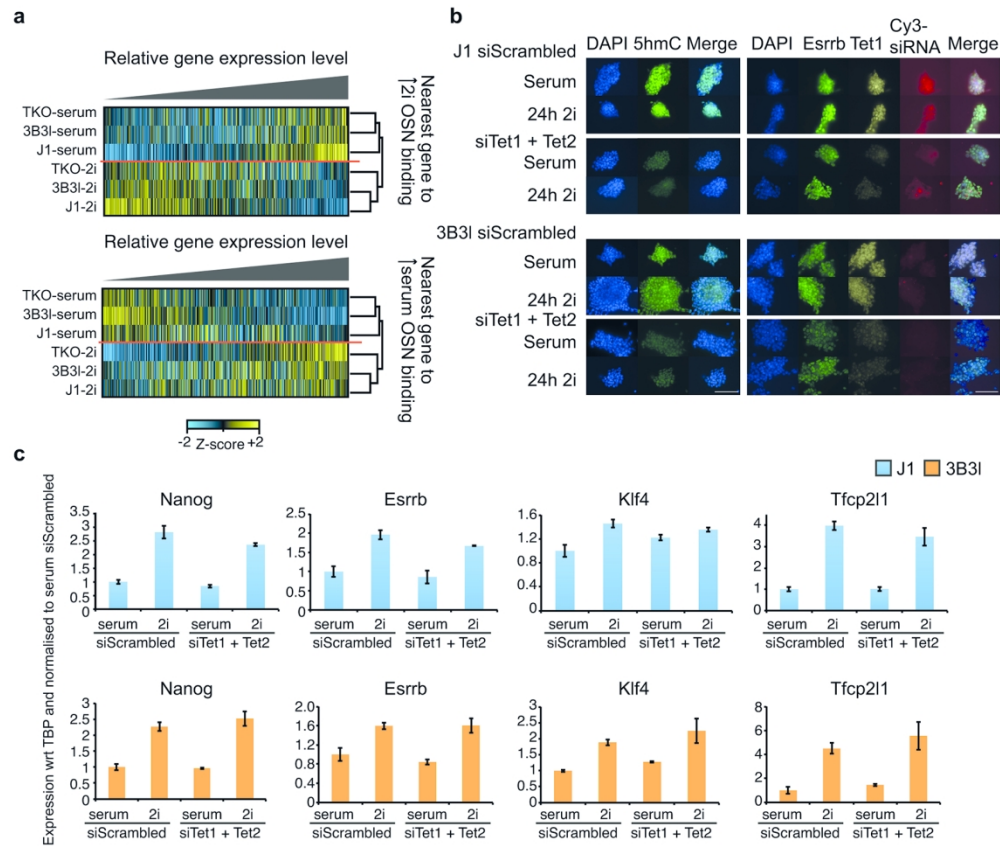


Figure 6: Relationship between, gene regulatory networks, epigenetic marks and culture conditions in mESCs

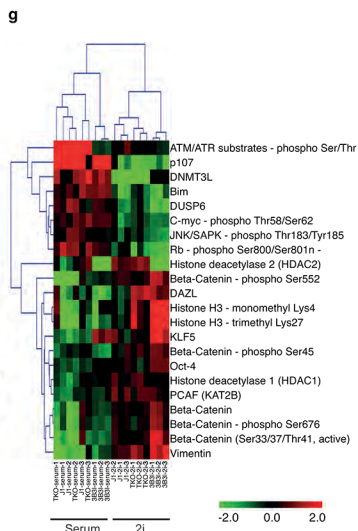
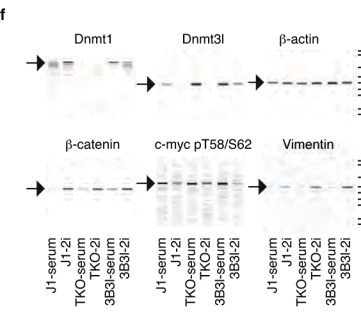
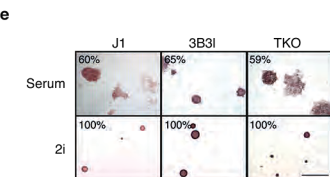
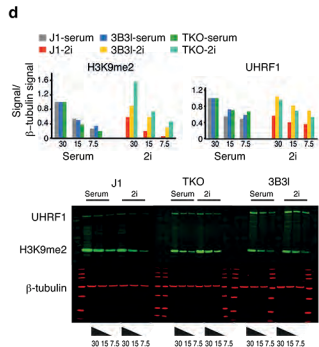
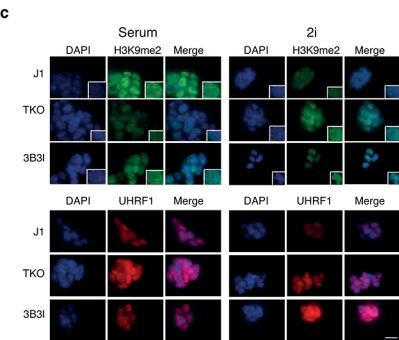
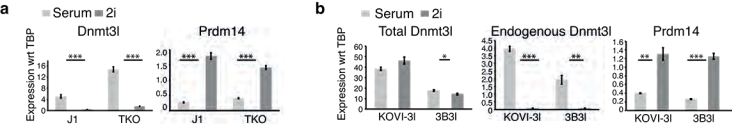


Figure S1: Cell line derivation and characterisation of J1, TKO and 3B3I mESCs in response to 2i culturing

a, Expression analysis of indicated genes by RT-qPCR in TKO and J1 mESCs cultured in serum (light grey) and 2i (dark grey) for 2 weeks. Values represent mean \pm S.E. of gene expression wrt (with respect to) the housekeeping gene TBP (TATA Binding Protein) * $p < 0.001$, (unpaired t-test).

b, Expression analysis of indicated genes by RT-qPCR in KOVI-3I and 3B3I mESCs cultured in serum (light grey) and 2i (dark grey) for 2 weeks. Values represent mean \pm S.E. of gene expression wrt the housekeeping gene TBP. * $p < 0.05$, ** $p < 0.005$, (unpaired t-test).

c, Immunocytochemistry of indicated cell lines showing (top) DAPI (blue), H3K9me2 (green) and merge or (bottom) DAPI (blue), UHRF1 (red) and merge. Scale bar is 20 μ M.

d, Fluorescent western blots showing H3K9me2 and UHRF1 levels relative to β -tubulin and subtracted for local mean background. Bar-charts show a representative quantification of a dilution series of protein 30, 15 or 7.5 μ g with corresponding western blots shown below.

e, Representative images of alkaline phosphatase (AP) staining for indicated cell lines. Numbers indicate the percentage of colonies homogeneously stained for AP in the given condition.

f, Representative DigiWest blots showing levels of indicated proteins for indicated mESC lines and culture conditions.

g, Hierarchical clustering (Euclidean distance) and heatmap representing analytes deemed significant (P value < 0.005) after two-factor ANOVA was performed on DigiWest results.

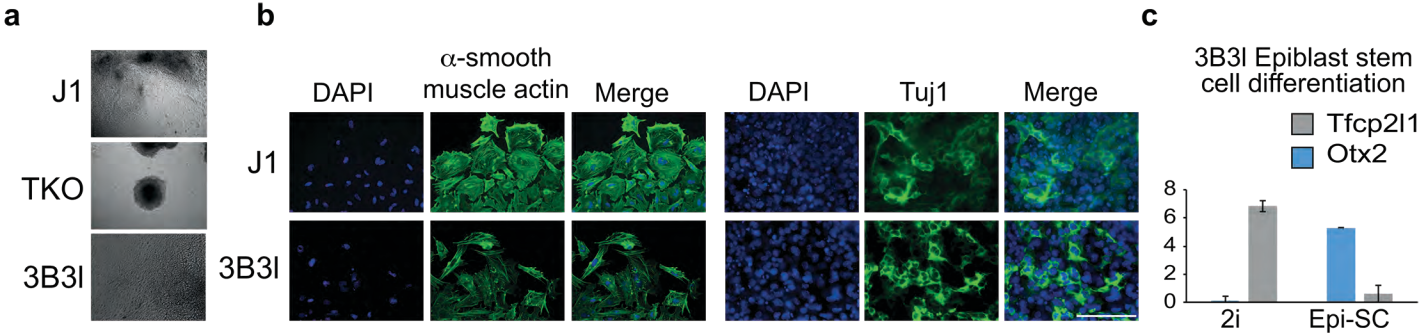


Figure S2: Embryoid bodies generated from J1, TKO and 3B3I mESCs

a, Images of embryoid body outgrowths of indicated cell lines. **b**, Images showing DAPI (blue), α -smooth muscle actin and Tuj1 (green) and merged images of indicated cell lines, scale bar 100 μ m. **c**, Expression analysis of indicated genes by RT-qPCR for 3B3I-2i or 3B3I Epiblast-stem cells, values represent mean \pm S.E. of expression wrt TBP from 3 technical replicates.

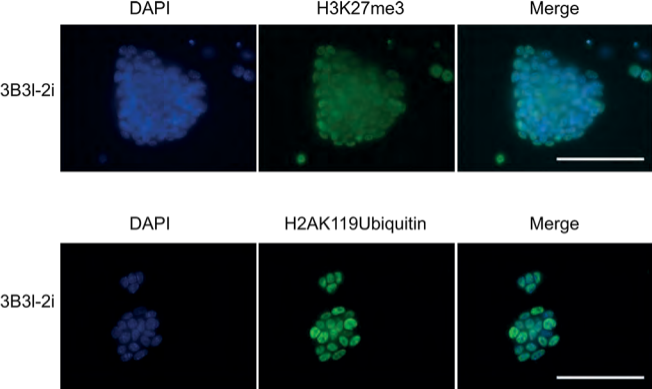


Figure S3: Epigenetic state of heterochromatin in 3B3I mESCs in 2i.

ICC of H3K27me3 (green) and H2AK119Ubiquitin (green) in indicated cells with DAPI (blue) and merge (turquoise represents overlapping signal), scale bar 100 μ m.

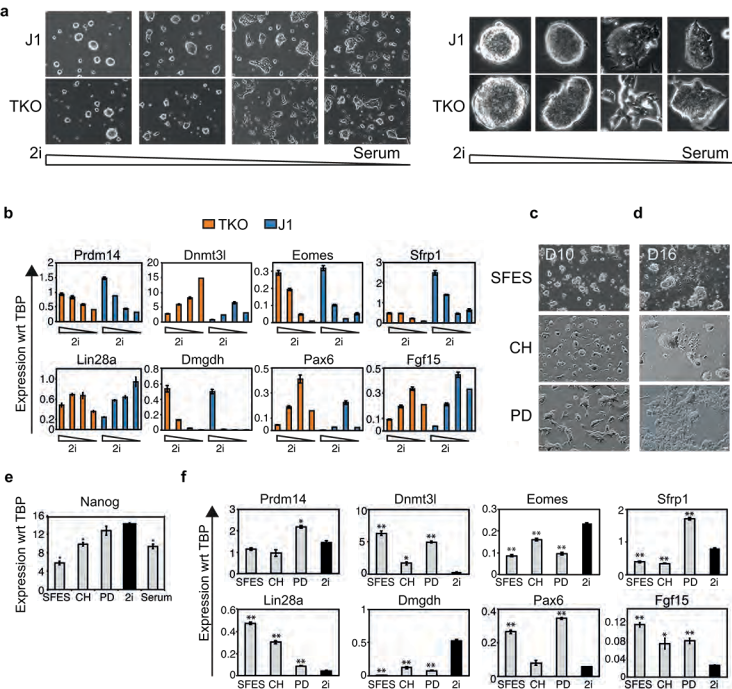


Figure S4: 2i titration and effect of individual inhibitors on J1 and TKO mESCs

a, Morphology images of J1 and TKO cells cultured in either 2i, 1/2 2i, 1/4 2i or Serum. The panel on the right represents 'zoomed-in' images of a representative colony for each condition. Scale bar 100 μ m. **b**, Expression analysis of indicated genes by RT-qPCR in TKO (orange) and J1 (blue) mESCs in 2i, 2i (1:2), 2i (1:4) and serum for 2 weeks. Values represent mean \pm S.E. of gene expression wrt the housekeeping gene TBP (TATA Binding Protein). **c-d**, TKO cells were cultured in SFES (2i basal media + LIF) with no inhibitors or with 2i or 1i (CH or PD) as indicated. Morphology images of indicated cells after 10 and 16 days in culture, scale bar 100 μ m. **e**, Expression analysis of Nanog by RT-qPCR after 10 days in culture, values represent mean \pm S.E. * $p \leq 0.002$ compared to TKO-2i (unpaired t-test). **f**, Expression analysis of indicated genes by RT-qPCR, values represent mean \pm S.E. * $p \leq 0.02$, ** $p \leq 0.0005$ (unpaired t-test) compared to TKO-2i. Experiment was done as biological and technical triplicates; representative dataset shown.

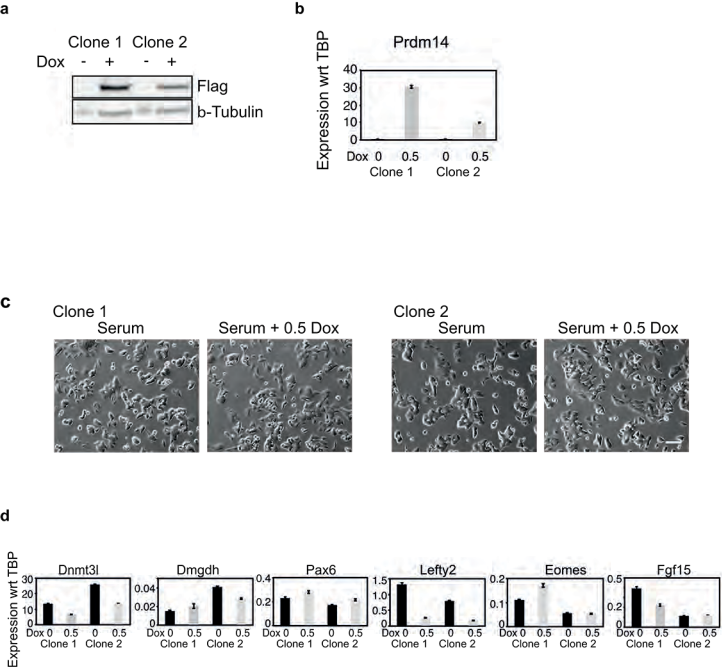


Figure S5: Over-expression of Prdm14 in TKO mESCs

a, Western blot analysis of whole cell protein extract from 2 independent clones of TET-ON-Flag-Prdm14-TKO cells following doxycycline (0.05 mg/ml) induction for 14 days. Blots were probed with M2-anti-Flag antibody to check for induced Prdm14, β -tubulin was used as loading control. **b**, RT-qPCR of Prdm14 to confirm overexpression, values represent mean \pm S.E. of expression wrt TBP from 3 technical replicates * $p < 0.0001$ (unpaired t-test). **c**, Morphology images of TET-ON-Flag-Prdm14-TKO cells +/- doxycycline treatment, scale bar 100 μ m. **d**, RT-qPCR for indicated genes Prdm14-TKO mESCs. Values represent mean \pm S.E. of expression wrt TBP of 3 technical replicates. * $p < 0.02$, ** $p < 0.002$ (unpaired t-test).

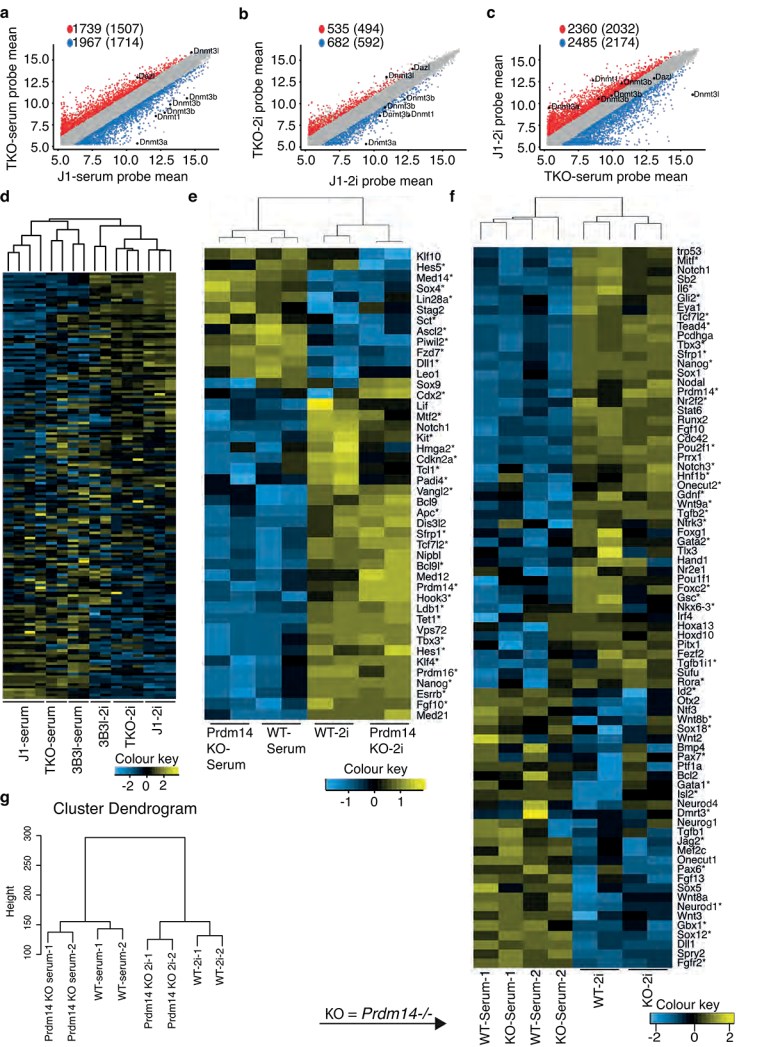


Figure S6: Expression array analysis of J1, TKO, WT and Prdm14^{-/-} (KO) cells in serum and 2i. Prdm14 WT and KO Data is a subset of GEO Series GSE42580.

a-c, Scatter plot of probes (genes) shown as log₂ expression values, red and blue dots indicate differentially expressed probes (FC ≥ 2, p ≤ 0.05) and grey dots = unchanged expression. Selected genes have been highlighted to illustrate the difference between the two cell types. Numbers on top represent differentially expressed probes; numbers in parenthesis represent the equivalent number of genes a, TKO-serum versus J1-serum b, TKO-2i versus J1-2i c, J1-2i versus TKO-serum. **d**, Heatmap for all the probes related to the GO term 'Stem Cell Maintenance' in indicated cell types. **e**, Heatmap representing expression levels of stem cell maintenance genes differentially expressed (FC ≥ 1.5) in either WT or Prdm14^{-/-} mESCs in 2i compared to their serum counterparts. **f**, Heatmap representing expression levels of cell fate commitment genes differentially expressed (FC ≥ 2) in WT cells in 2i compared to serum conditions. * Indicates genes overlapping with datasets of present study. **g**, Hierarchical clustering of the expression array data including all the probes (Euclidean distance and Ward.D2 method).

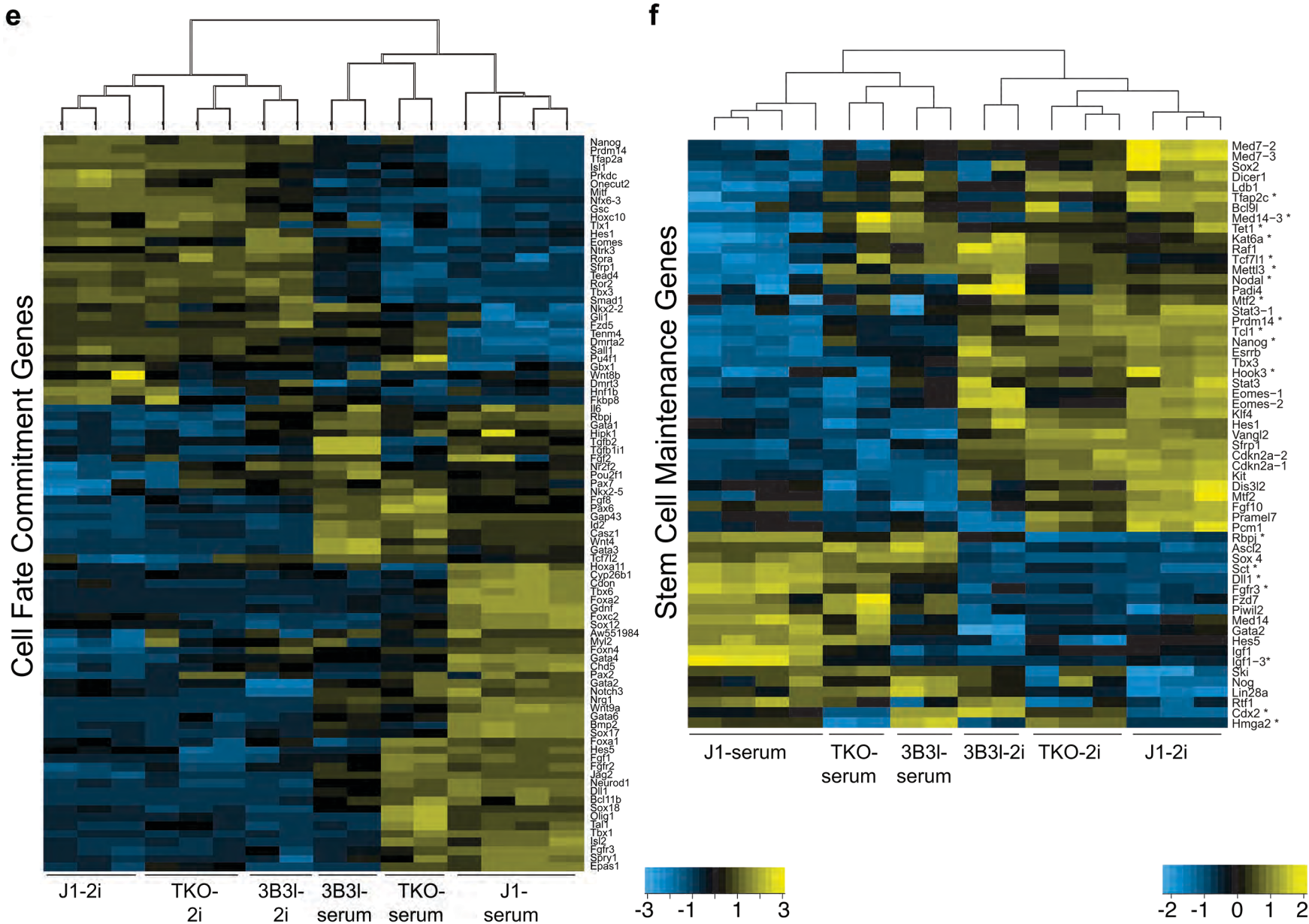


Figure S7 derived from Fig. 3. Gene expression changes in mESCs upon culturing in 2i e-d.

A portion of Figure 3 was enlarged so that the gene names on the right can be read.

e, Heatmap representing expression levels of 'Cell Fate Commitment' genes differentially expressed ($FC \geq 1.5$, $p_{adj.} \leq 0.05$ eBayes (limma), Benjamini-Hochberg corrected) in J1-2i compared to J1-serum in indicated cells. * marks genes which are differentially expressed ($FC \geq 2$, $p_{adj.} \leq 0.05$ eBayes (limma), Benjamini-Hochberg corrected) between TKO-serum and J1-serum. **f**, Same as e for 'Stem Cell Maintenance' genes.

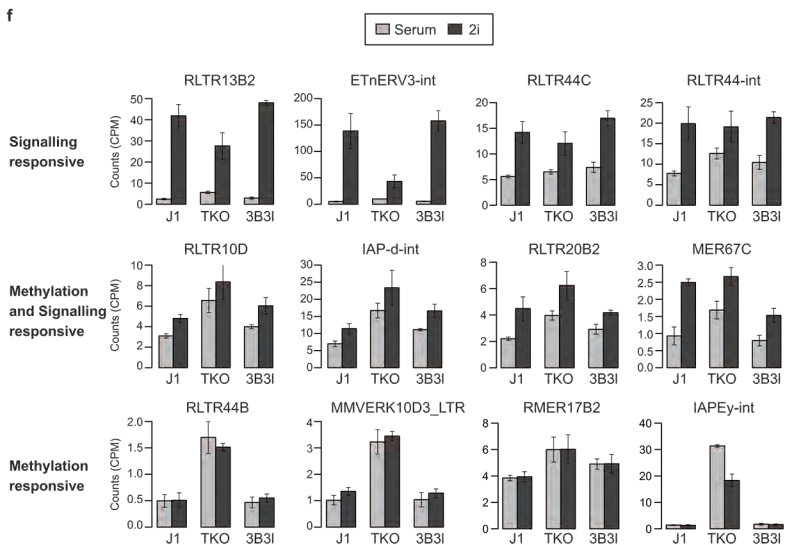
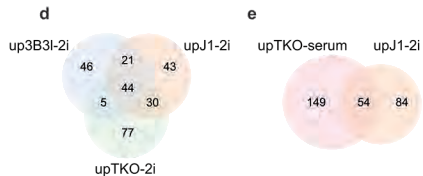
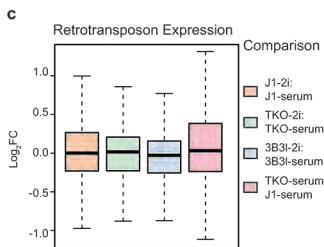
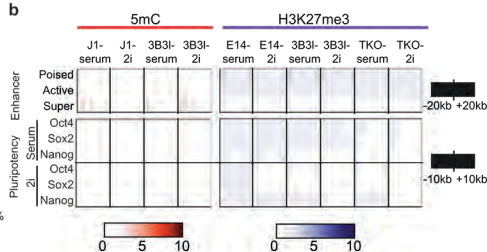
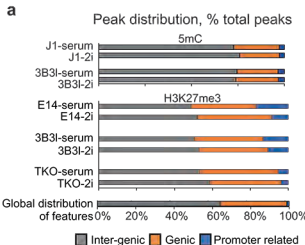


Figure S8: Genome wide analysis of 5mC and H3K27me3 peak distribution in mESCs in serum and 2i.

a, Plot of the distribution of 5mC and H3K27me3 peaks for indicated cell lines and conditions, promoters (TSS +/- 1kb: blue), genic (orange), inter-genic (grey). **b**, Heatmap indicating 5mC (red) and H3K27me3 (purple), levels; 0-10, see scale, for indicated cell lines and conditions across pluripotency transcription factor binding elements, Oct4, Sox2 and Nanog, +/- 10kb. **c**, Boxplot showing changes in the abundance (Log2FC: log2 fold change) of retrotransposon RNAs (LINE, SINE and LTR Repeatmasker classes) in the indicated comparisons between cell lines and conditions. **d-e**, Overlaps between sets up retrotransposons upregulated (logFC > 0 and FDR < 0.05) in the indicated comparisons. **f**, Behaviour of selected retrotransposon RNAs between the indicated cell lines and conditions. Mean RNA abundance (CPM, counts per million mapped reads) and standard deviation between three replicates for each sample are indicated. Signalling responsive retrotransposons are upregulated in 2i in J1, TKO and 3B3l cells, i.e. regardless of the methylation state of the cell line. Methylation responsive retrotransposons are upregulated in hypomethylated (TKO) mESCs in serum relative to J1 mESCs in serum, but not in 3B3l cells in response to 2i. Retrotransposons responding to 2i conditions and to the methylation state of the cell are upregulated in 2i in J1, TKO and 3B3l cells, but the level of retrotransposon expression is affected by the methylation state of the cell (i.e. upregulated in TKO cells relative to 3B3l cells). Some of the retrotransposons identified in this analysis (RLTR13B2::EtnERV3-int, RLTR44C::RLTR44-int, RLTR10D::IAP-d-int) correspond to long terminal repeats and their cognate adjacent internal sequences that they can be found flanking in the mouse genome.

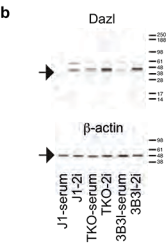
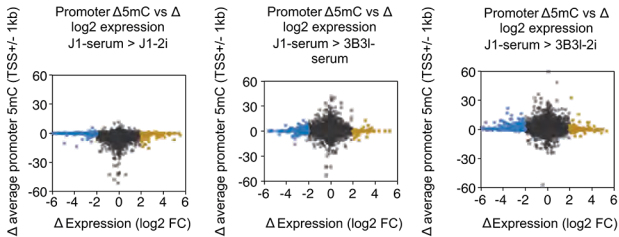
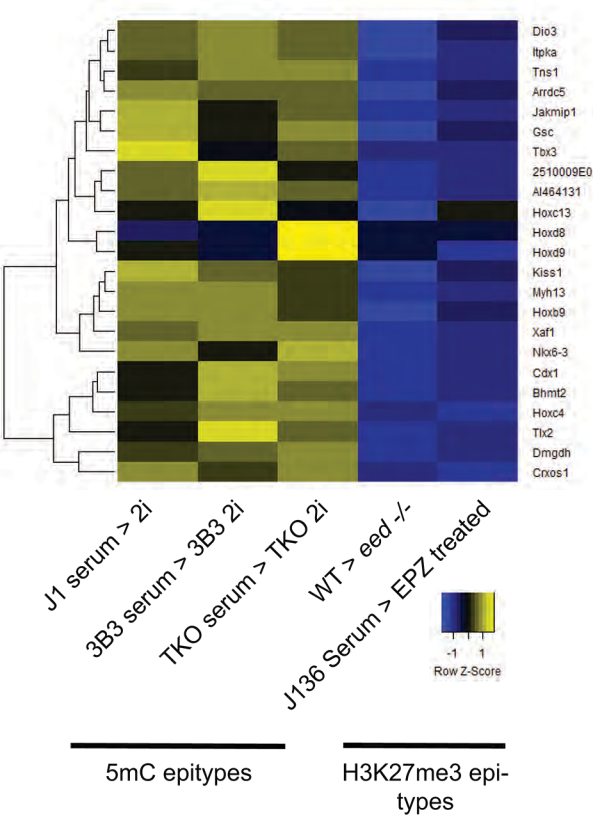


Figure S9: Analysis of relationship between gene expression changes and locus specific 5mC levels.

a, Scatter plots of change in average promoter 5mC levels against change in gene expression (log2 fold change) for indicated cell lines and culture conditions. Blue = downregulated genes, yellow = upregulated, grey = no change. **b**, Representative DigiWest Blot for Dazl and β -actin in indicated cell lines and culture conditions.

Genes with exp ↑ & H3K27me3 ↓ in
all 3 5mC epitypes Serum > 2i



Genes with exp ↓ & H3K27me3 ↑ in
all 3 5mC epitypes Serum > 2i

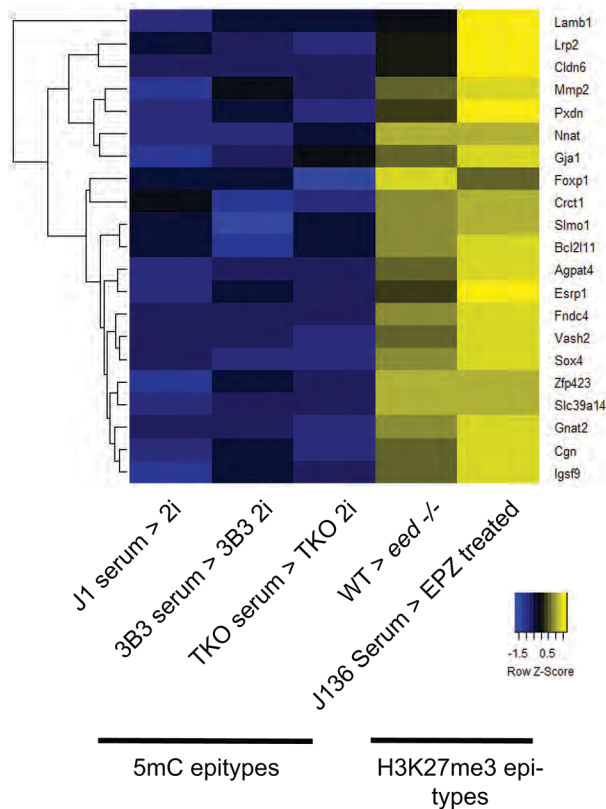


Figure S10: Z-score normalised heatmap of gene expression changes between serum to 2i of genes that display opposite expression and H3K27me3 changes: conserved across all three 5mC epitypes (J1, TKO & 3B3I – all serum to 2i) and their response to PRC2 inactivation (*eed*^{-/-} and EPZ6438 inhibition (of EZH2 activity) in serum.

By comparison cell types in which the polycomb machinery are perturbed (WT mESC in serum vs *eed*^{-/-} cells in serum) or inhibited (J136 mESC in serum vs J136 mESC treated with EPZ6438) do not show similar gene expression changes. Plots show data over serum to 2i gene expression gains/H3K27me3 losses (top) or over serum to 2i gene expression losses/H3K27me3 gains (bottom). Yellow: relative elevation in gene expression, blue: relative reduction in gene expression.

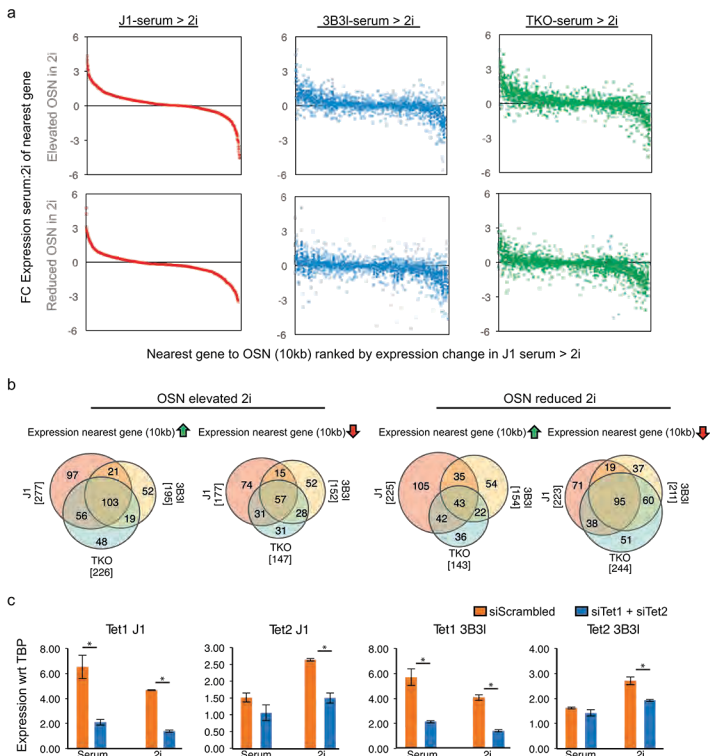


Figure S11: Relationship between, transcript levels, epigenetic marks and culture conditions in mESCs.

a, Scatterplots showing fold change gene expression 2i versus serum of the nearest gene within 10kb of an OSN site that has elevated OSN factor binding in 2i versus serum (top row), or reduced OSN factor binding in 2i versus serum (2i-serum) are similar at genes nearest an OSN gained or lost upon 2i transition; in all three cell lines. Serum and 2i specific OSN sites are taken from published datasets (see methods). **b**, Venn diagrams showing the overlap for indicated cell lines of the same gene set as in panel A. The left panel shows OSN sites that have increased binding of OSN factors in 2i, where this corresponds to an increase in gene expression of the nearest gene within 10kb (left) and where this is coincident with reduced gene expression (right). The right panel shows OSN sites that have reduced binding of OSN factors in 2i relative to serum, where gene expression of the nearest gene within 10kb either increases (left) or is reduced (right). **c**, Expression analysis of indicated genes by RT-qPCR in J1 and 3B3I mESCs in 72h serum or 48h serum + 24h 2i transfected with either scrambled siRNA (orange) or siRNAs for Tet1 + Tet2 (blue). Values represent mean \pm S.E. of 2 biological replicates, which consist of three technical replicates each with respect to (wrt) the housekeeping gene TBP (TATA Binding Protein). * $p < 0.05$ (unpaired t-test).