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Embryonic stem cell potency fluctuates with endogenous retrovirus activity

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Embryonic stem (ES) cells are derived from blastocyst-stage embryos and are thought to be functionally equivalent to the inner cell mass, which lacks the ability to produce all extraembryonic tissues. Here we identify a rare transient cell population within mouse ES and induced pluripotent stem (iPS) cell cultures that expresses high levels of transcripts found in two-cell (2C) embryos in which the blastomeres are totipotent. We genetically tagged these 2C-like ES cells and show that they lack the inner cell mass pluripotency proteins Oct4 (also known as Pou5f1), Sox2 and Nanog, and have acquired the ability to contribute to both embryonic and extraembryonic tissues. We show that nearly all ES cells cycle in and out of this privileged state, which is partially controlled by histone-modifying enzymes. Transcriptome sequencing and bioinformatic analyses showed that many 2C transcripts are initiated from long terminal repeats derived from endogenous retroviruses, suggesting this foreign sequence has helped to drive cell-fate regulation in placental mammals.

The zygote and its daughter cells are totipotent because they are able to develop into all embryonic and extraembryonic cell types[1,2]. The progeny of these first two daughter cells become progressively more fate-restricted as they activate distinct patterns of gene expression that first direct them towards one of three broad lineages: $Oct4^+$ $Sox2^+$ $Nanog^+$ epiblast cells that give rise to the embryo, $Gata4^+/6^+$ primitive endoderm cells that contribute to extraembryonic membranes that encase the embryo, and $Cdx2^+$ trophectoderm cells that form a large part of the placenta[3]. These early cell-fate decisions represent a major and relatively recent advance in mammalian evolution in which the placenta and extraembryonic tissues that support the intrauterine nourishment of the fetus allow development to progress further before birth. The epigenetic landscape of the zygote changes markedly during the first cell divisions. Shortly after fertilization, the oocyte maternal transcripts are replaced with newly synthesized RNAs generated by activating transcription of the zygotic genome[4–6]. The unique transcriptional profile of the zygote and its daughter cells defines a brief period when the cells are totipotent.

Mouse ES cells are isolated from the inner cell mass (ICM) of blastocysts that have already become a separate lineage from the trophectoderm[7,8]. ICM-derived ES cells are regarded as pluripotent because they have the capacity to generate tissues of the fetus, but they are extremely inefficient at colonizing the extraembryonic tissues[9]. The rare contribution of ES cells to extraembryonic tissues could be explained by contamination of ES cultures with trophectoderm or primitive endoderm-committed cells, or may occur because rare ES cells have acquired the ability to produce extraembryonic tissues in addition to embryonic tissues. This latter possibility is intriguing, because recent evidence shows that ES cell cultures are a heterogeneous mixture of metastable cells with fluctuating expression of genes such as *Zscan4*, *stella* (also known as *Dppa3*), *Nanog*, *Sox17* and *Gata6*, which could account for special attributes of individual cells[10–14].

A large number of retrotransposons are expressed when the zygotic genome is first transcribed, including the endogenous retroviruses (ERVs), long interspersed nuclear element-1 (LINE-1), and the non-autonomous short interspersed elements (SINEs)[15]. At the 2C stage, murine endogenous retrovirus with leucine tRNA primer (MuERV-L, also known as MERVL and Erv4) elements are transiently derepressed and produce 3% of the transcribed messenger RNAs[15–17]. After the 2C stage, MuERV-L retroelement expression is silenced[18,19]. We discovered that this regulated pattern of MuERV-L expression overlapped with more than 100 2C-specific genes that have co-opted regulatory elements from these foreign retroviruses to initiate their transcription. We exploited the regulated activity of these 2C virus-derived promoters to label cells, and found that both ES and iPS cell cultures contain a small but relatively constant fraction of cells that has entered into the 2C-transcriptional state. Purification of these 2C-like cells shows that they have unique developmental characteristics and efficiently produce progeny for extraembryonic and embryonic lineages.

Identification of a 2C-like state within ES cultures

To identify zygotically activated genes we performed deep RNA sequencing (RNA-seq) on mouse oocytes and 2C-stage embryos. A comparison of the transcripts in these cells identified a large number of genes and retrotransposons that became expressed in the 2C embryo, as well as numerous transcripts that were downregulated (Fig. 1a and Supplementary Table 1). The most highly activated repeat was the MuERV-L family of retroviruses and their corresponding long terminal repeat (LTR) promoters (Mt2_mm), which were activated more than 300-fold (Supplementary Table 1). Sequence alignments showed that more than 25% of the nearly 700 copies of MuERV-L elements were activated, and that 307 genes generated chimaeric transcripts with junctions to MuERV-L elements (Fig. 1a and Supplementary Table 2), including 10 that were previously described[15]. Of the 626 chimaeric transcripts generated, >90% were 5′ LTR–exon fusions that generated open reading frames (ORFs), suggesting that these LTRs had become functional promoters for

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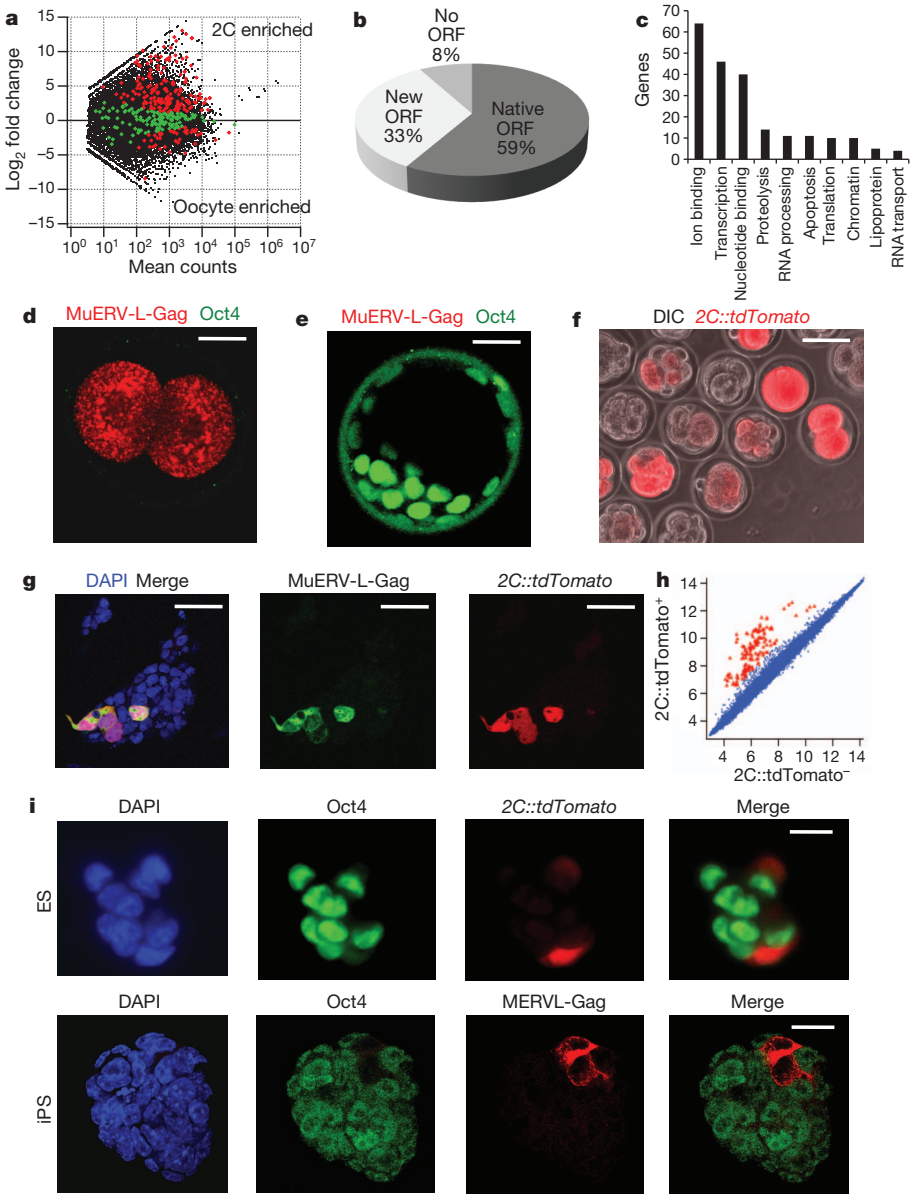

Figure 1 | The MuERV-L retrovirus and a reporter driven by its LTR marks the 2C state. **a**, Comparison of gene expression between oocytes and 2C embryos. Genes generating junctions to MuERV-L are shown in red and green, with those in red denoting significant change in expression. **b**, ORF status of predicted MuERV-L-linked chimaeric transcripts. **c**, Gene Ontology (GO) analysis of MuERV-L-linked protein-coding transcripts. The number of genes from the ten most enriched GO categories are shown. **d**, **e**, 2C (**d**) and blastocyst (**e**) embryos were mixed and immunostained with MuERV-L-Gag and Oct4 antibodies. Scale bars, 20 μm. **f**, Zygotes were injected with the *2C::tdTomato* transgene, and allowed to develop *in vitro* for 48 h before imaging. DIC, differential interference contrast. Scale bar, 50 μm. **g**, 2C::tdTomato$^+$ ES cells express MuERV-L-Gag protein, as detected by immunofluorescence. DAPI, 4′,6-diamidino-2-phenylindole. Scale bars, 50 μm. **h**, Microarray analysis of 2C::tdTomato$^+$ and 2C::tdTomato$^-$ cells. Red indicates genes with a greater than fourfold change in expression. **i**, 2C::tdTomato$^+$ MuERV-L-Gag$^+$ ES and iPS cells lack Oct4 protein, as determined by immunofluorescence. Scale bars, 20 μm.

protein-coding genes (Fig. 1b and Supplementary Fig. 1a). The most significantly enriched Gene Ontology categories representing these chimaeric proteins were regulation of transcription, ion binding, translation, nucleotide binding and mRNA transport (Fig. 1c). Two notable transcription factors that used alternate MuERV-L-LTR promoters were *Gata4* and *Tead4*, which are important for the specification of primitive endoderm and trophectoderm, respectively[20–22].

Because more than 300 of the nearly 700 copies of the MuERV-L endogenous retroviruses still encode Gag viral protein, we stained 2C and early blastocyst embryos to confirm that viral Gag was expressed and developmentally regulated. We found that 2C embryos express Gag but lack the pluripotency marker Oct4, whereas blastula cells lack Gag but express Oct4 (Fig. 1d, e). Thus, MuERV-L activity is developmentally regulated and these retroviral promoters have been co-opted by many cellular genes to impose tight control over their expression.

Next we asked whether it was possible to use the regulatory sequences from MuERV-L elements to label 2C cells. We cloned the MuERV-L 5′ LTR, the primer-binding site, and a portion of the *gag* gene upstream of the red fluorescent protein tandem dimeric Tomato (tdTomato). We injected fertilized eggs with the *2C::tdTomato* construct and monitored the expression of tdTomato during culture *in vitro*. tdTomato expression was highest in arrested zygotes and 2C embryos and became downregulated at the morula stage (Fig. 1f and Supplementary Movie 1). Notably, when we introduced the *2C::tdTomato* construct into ES cells and selected for clonal stable integrants, we found several colonies that contained 1–5 cells that were strongly labelled with tdTomato among cells lacking expression of the reporter (Fig. 1g). We also found that rare ES cells expressed *MuERV-L* mRNA and Gag protein, and that these overlapped with 2C::tdTomato$^+$ cells (Fig. 1g and Supplementary Fig. 1b, c). The

correspondence between the *2C::tdTomato* reporter and MuERV-L expression was further confirmed by immunoblotting, and electron microscopy imaging of viral epsilon particles encoded by MuERV-L within the endoplasmic reticulum of tdTomato$^+$ cells but not tdTomato$^-$ cells (Supplementary Fig. 1d, e). Thus, MuERV-L expression is restricted *in vivo* to 1–4-cell-stage embryos and is reactivated within a small subpopulation of ES cells derived from blastocysts.

To characterize the unexpected *2C::tdTomato*-labelled cells within ES cultures, we sorted tdTomato$^+$ and tdTomato$^-$ cells and performed microarray and mRNA sequencing analyses (Fig. 1h and Supplementary Tables 3–5). tdTomato$^+$ cells expressed 55-fold higher levels of MuERV-L transcripts than tdTomato$^-$ cells, but the vast majority of other retrotransposons were unaffected (Supplementary Table 3). Notably, tdTomato$^+$ cells had 165 transcripts activated more than fourfold, and no genes repressed more than fourfold compared with tdTomato$^-$ cells (Fig. 1h, Supplementary Table 4 and Supplementary Fig. 2a–f). Among the genes that were highly enriched in tdTomato$^+$ cells, several were previously shown to be restricted to the 2–4-cell stage of development, including *Zscan4*, *Tcstv1/3*, *Eif1a*, *Gm4340* (also known as *Thoc4*), *Tdpoz1–5* and *Zfp352* (refs 23–25). In total, 525 genes that were enriched in 2C::tdTomato$^+$ cells were also activated at the 2C stage, including 52 genes that generated chimaeric transcripts linked to MuERV-L elements (Supplementary Tables 6 and 7).

A hallmark of the ICM and ES cells is their expression of Oct4, Sox2 and Nanog, whereas totipotent 2C embryos do not express Oct4 (Fig. 1d, e). We found that 2C::tdTomato$^+$ cells within ES cultures also lacked Oct4, Sox2 and Nanog (Fig. 1i and Supplementary Fig. 1f). The reduction in Oct4, Sox2 and Nanog protein labelling occurred despite no changes in their mRNA levels, suggesting that the regulation is occurring post-transcriptionally (Fig. 1h–i and Supplementary Fig. 2g). In summary, *2C::tdTomato* labels a subset of ES cells that share transcriptional and proteomic features of 2C embryos and display markedly different patterns of pluripotency markers from most ES cells in culture.

ES cells cycle in and out of the 2C state

We considered the possibility that the expression of the *2C::tdTomato* reporter and MuERV-L-Gag protein in sporadic cells within ES cultures might arise from contamination with trophectoderm or primitive endoderm. To exclude this possibility, we examined iPS cells derived from mouse fibroblasts because they should not be contaminated with cells from blastocyst embryos. Similar to ES cells, we found that sporadic iPS cells express the MuERV-L-Gag protein and lack Oct4 (Fig. 1i). Thus, the heterogeneity within ES cultures is a property that is shared with iPS cell cultures and is unlikely to arise from a cell contaminant.

Next we examined whether the 2C::tdTomato$^+$ cells represent a stable cell population or whether ES cells transition in and out of this 2C-like state. We used a Cre/*loxP* fate-mapping strategy to indelibly mark cells that had expressed 2C genes (Supplementary Fig. 3a–c). We generated a transgenic mouse line using the MuERV-L regulatory elements driving expression of a tamoxifen-inducible Cre recombinase (*2C::ERT2-Cre-ERT2*; Supplementary Fig. 3a). These mice were then mated with Cre-responsive reporter lines (*ROSA::LSL-tdTomato* and *ROSA::LSL-LacZ*; Supplementary Fig. 3b). ES cell lines were derived from double-positive transgenic blastocysts (Supplementary Fig. 3c). After addition of 4-hydroxytamoxifen (4HT) to the ES cultures we detected nuclear Cre expression in MuERV-L-Gag$^+$ cells (Supplementary Fig. 3d). When ES cultures were grown for 2–6 days with 4HT we found a steady increase in the percentage of reporter-positive cells (Fig. 2a). Remarkably, over extended passages nearly every ES cell activated the reporter (Fig. 2b), demonstrating this transient state is regularly entered by ES cells.

To monitor the kinetics of the interconversion between 2C::tdTomato$^+$ and 2C::tdTomato$^-$ cells we performed flow cytometry to collect tdTomato$^+$ and tdTomato$^-$ cells. When these purified subpopulations were cultured we found that tdTomato$^+$ cells produced tdTomato$^-$ cells and vice versa (Fig. 2c). Within 24 h nearly 50% of the tdTomato$^+$ cells convert to tdTomato$^-$, independently of the starting percentages of the two different cell populations (Fig. 2c and data not shown). Under hypoxic conditions (5% O_2), the percentage of cells expressing the *2C::tdTomato* reporter was decreased, which could be reversed by shifting the cultures back to 20% O_2 (Fig. 2d). We also found that growing cells for 48 h in 'ground-state' media conditions (2i media[26]) reduced but did not eliminate the presence of tdTomato$^+$ cells relative to media containing knockout serum replacement, suggesting

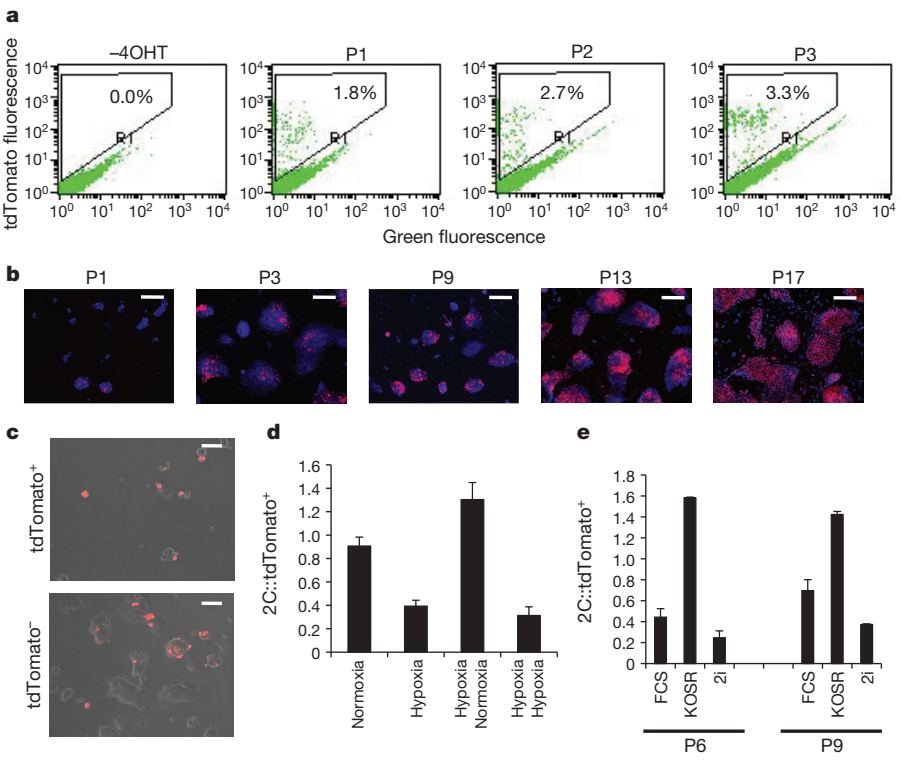

Figure 2 | ES cells enter the 2C state regularly, but remain in the state transiently owing to cell intrinsic and extrinsic factors. **a**, FACS analysis of *2C::ERT2-Cre-ERT2*, *ROSA::LSL-tdTomato* ES cells at increasing passage (P) in the presence of 4HT. The percentage of tdTomato$^+$ cells is indicated. **b**, *2C::ERT2-Cre-ERT2*, *ROSA::LSL-LacZ* ES cells were cultured in the presence of 4HT, and at increasing passage, cells were fixed and immunostained with anti-β-galactosidase antibodies and counterstained with DAPI. Scale bars, 50 μm. **c**, 2C::tdTomato$^+$ and 2C::tdTomato$^-$ cells were collected by FACS and plated before imaging 48 h later. Scale bars, 50 μm. **d**, *2C::tdTomato* ES cells were cultured in 20% O_2 (normoxia) or 5% O_2 (hypoxia) for 48 h, or 48 h sequentially, and the percentage of tdTomato$^+$ cells was determined by FACS. Error bars represent s.d., $n = 3$ **e**, *2C::tdTomato* ES cells at the indicated passage were cultured in media containing 15% fetal calf serum (FCS), 20% knockout serum replacement (KOSR) or N2B27 media containing 3 mM glycogen synthase kinase 3β (GSK3β) and mitogen-activated protein-kinase kinase (MEK) inhibitors (2i) for 48 h before counting the percentage of 2C::tdTomato$^+$ cells by FACS. Error bars represent s.d., $n = 3$.

extrinsic and intrinsic mechanisms regulate the MuERV-L and 2C gene network (Fig. 2e).

The 2C–ES switch is regulated by histone modification

After activation of the zygotic genome in mouse development, histone deacetylation and histone H1 synthesis lead to the formation of repressive chromatin that is thought to limit the broad pattern of transcription present in 2C embryos[27,28]. Using indirect immunofluorescence, we found that tdTomato$^+$ cells had markedly higher levels of active histone marks, including methylation of histone 3 lysine 4 (H3K4) and acetylation of H3 and H4 (Supplementary Fig. 4a), a finding confirmed using immunoblot analysis of sorted cell populations (Fig. 3a). This type of chromatin mirrors that found in 2C embryos[28]. Next we tested whether tdTomato$^+$ cells had different levels of DNA methylation compared with non-labelled ES cells. We found that the MuERV-L sequences were hypomethylated in tdTomato$^+$ cells compared with tdTomato$^-$ cells. In contrast to the MuERV-L sequences, intracisternal A-type particle retroviruses were highly methylated in both tdTomato$^+$ and tdTomato$^-$ cells, suggesting the altered pattern of methylation was not uniform across the genome (Supplementary Fig. 4b). In summary, these data suggest that as ES cells (re)enter into the 2C state, their chromatin and DNA is altered to favour transcription in a way that mirrors the 2C embryo.

We previously demonstrated that MuERV-L and 2C-specific genes were increased in mutant ES cells lacking the histone lysine-specific demethylase gene *Kdm1a* (also known as *LSD1*)[29]. To test whether other proviral co-repressors and histone-modifying enzymes also influence 2C-specific gene expression we profiled the transcriptome of ES cells with homozygous mutations in the KRAB (Kruppel-associated box)-associated transcriptional repressor *Kap1* and the H3K9 histone methyltranferase *G9a*[29–31]. We found that MuERV-L and several 2C genes were significantly upregulated in *Kdm1a*, *Kap1* and *G9a* mutant ES cells (Fig. 3b, Supplementary Fig. 5b, c and Supplementary Table 7). These findings were confirmed using *in situ* hybridization and immunofluorescence microscopy (Fig. 3c and Supplementary Fig. 5d). Treatment of *2C::tdTomato* ES lines with the histone deacetylase inhibitor trichostatin A also increased the number of tdTomato$^+$ cells fourfold (Fig. 3d). To understand better how 2C gene regulation is controlled when chromatin repressors are acutely eliminated we used a stably integrated *2C::tdTomato* ES line that is homozygous for a floxed allele of *Kdm1a* and contains a *Cre-ERT* transgene that can be activated with 4HT. Within 24 h of deleting *Kdm1a* we found a tenfold increase in tdTomato$^+$ cells that was steadily maintained (Fig. 3e). In addition, fluorescence-activated cell sorting (FACS)-purified tdTomato$^-$ cells more rapidly became tdTomato$^+$ in the absence of *Kdm1a* (Fig. 3f), and stayed in this state longer (Supplementary Movies 2 and 3). These findings suggest that Kdm1a, Kap1, G9a and histone deacetylases all contribute to the repression of 2C genes in ES cells, and that they function by altering the equilibrium between the 2C::tdTomato$^+$ and 2C::tdTomato$^-$ states.

2C-like ES cells have expanded fate potential

Because 2C-like cells within ES cultures express high levels of 2C-restricted genes found in totipotent blastomeres and reduced levels of pluripotency-associated proteins, we reasoned that this subpopulation of ES cells might have distinct functional characteristics. We tested whether *2C::tdTomato*$^+$ cells have acquired the ability to produce extraembryonic tissues, a characteristic that ES cells lack. We used FACS to collect tdTomato$^+$ and tdTomato$^-$ cells from a *2C::tdTomato* ES line, and injected four cells into morula-stage embryos. The tdTomato$^-$ cells contributed exclusively to the ICM of all five chimaeric blastocysts analysed (Fig. 4a). By contrast, the tdTomato$^+$ cells contributed to the trophectoderm (in four out of five chimaeric embryos) in addition to the ICM (in three out of five chimaeric embryos) (Fig. 4a). To track the fate of the *2C::tdTomato* ES cells later in development, we injected blastocysts with tdTomato$^+$ or

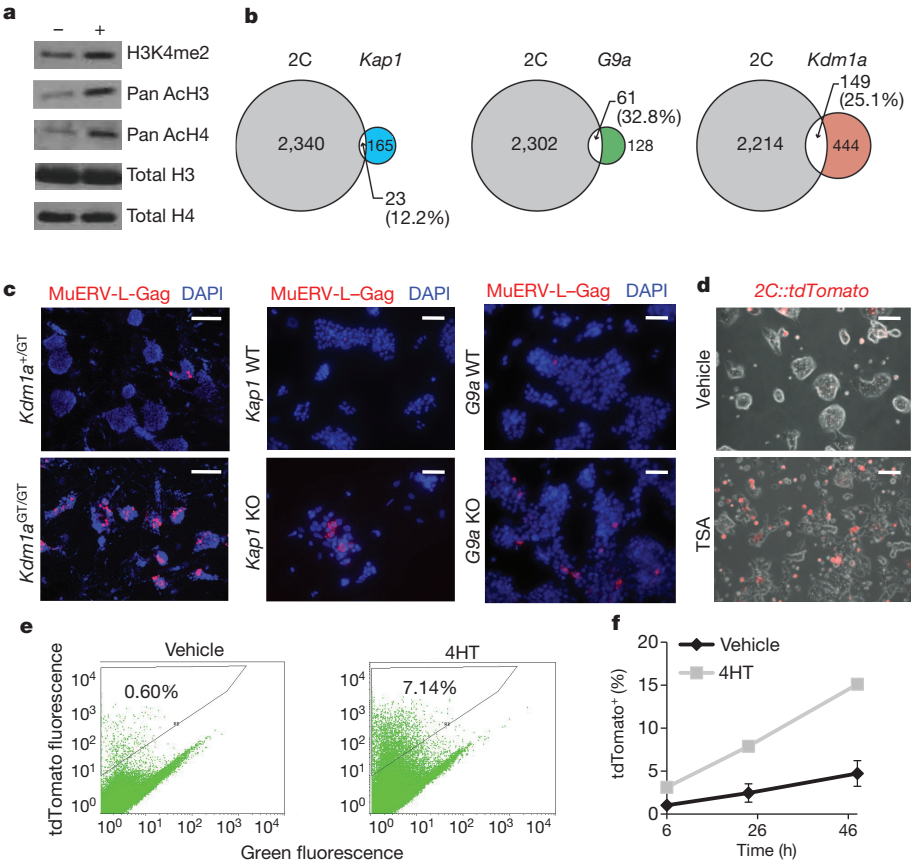

Figure 3 | The 2C state is associated with an active epigenetic signature and is antagonized by repressive chromatin-modifying enzymes. **a**, 2C::tdTomato$^+$ (+) and 2C::tdTomato$^-$ (−) cells were collected by FACS and subjected to immunoblot analysis with indicated antibodies. H3K4me2, histone H3 dimethyl Lys 4; AcH3, acetylated histone H3. **b**, Pairwise comparisons of the number of genes activated in *Kap1*, *G9a* and *Kdm1a* mutant ES cells compared with genes activated in 2C embryos. **c**, ES cell lines homozygous for mutant alleles of *Kdm1a*, *Kap1* and *G9a*, and corresponding wild-type (WT) ES lines were immunostained with MuERV-L-Gag antibodies and counterstained with DAPI. GT, gene trap; KO, knockout. Scale bars, 50 μm. **d**, *2C::tdTomato* ES cells were treated with 40 nM trichostatin A (TSA) for 24 h before imaging. Scale bars, 50 μm. **e**, *Kdm1a*$^{fl/fl}$; *Cre-ERT* ES cells containing a stably integrated *2C::tdTomato* transgene were treated with vehicle or 4HT and subject to FACS analysis to determine the percentage of tdTomato$^+$ cells. **f**, *2C::tdTomato*; *Kdm1a*$^{fl/fl}$; *Cre-ERT* ES cells were treated with 4HT or vehicle for 24 h, then passaged for 72 h before collecting tdTomato$^-$ cells by FACS. The percentage of tdTomato$^+$ cells was plotted after increasing hours in culture. Error bars represent s.d., $n = 3$.

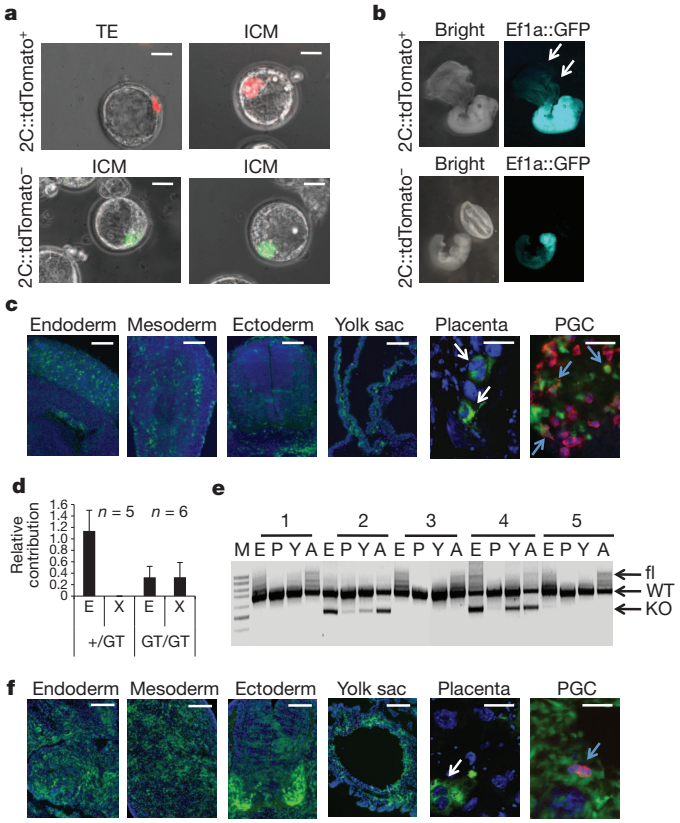

Figure 4 | Activation of the 2C state is associated with expanded potency in chimaeric embryos towards extraembryonic lineages. **a**, 2C::tdTomato$^+$ or 2C::tdTomato$^-$ cytomegalovirus (CMV)–GFP ES cells were injected into morula-stage embryos, which were then grown *in vitro*. The resulting blastocysts were imaged to visualize the position of injected cells in either the trophectoderm (TE) or ICM. Scale bars, 20 μm. **b**, 2C::tdTomato$^+$ or 2C::tdTomato$^-$, Ef1a::GFP$^+$ cells were injected into blastocysts that were then implanted into pseudopregnant females to generate chimaeric embryos. Arrows indicate 2C::tdTomato$^+$, GFP$^+$ cells contributing to the yolk sac and placenta. Bright denotes bright-field microscopy. **c**, 2C::tdTomato$^+$, Ef1a::GFP$^+$ cells contribute to embryonic endoderm, mesoderm, ectoderm, yolk sac, placental tissues (including giant trophoblast cells, white arrows) and primordial germ cells (PGCs, colabelled with anti-Ddx4 antibody in red, blue arrows). Scale bars, 500 μM (endoderm, mesoderm, ectoderm and yolk sac) and 50 μm (placenta and PGCs). **d**, Heterozygous (+/GT) or homozygous (GT/GT) *Kdm1a*-β-geo gene-trap ES cells were injected into wild-type blastocysts that were implanted into pseudopregnant females. (β-geo is a fusion of β-galactosidase and neomycin-resistance genes.) Embryonic (E) and extraembryonic (X) tissues were separated from chimaeric embryos, and subject to semiquantitative PCR with β-geo primers to determine the relative contribution of the injected cells to these lineages. Error bars represent s.e.m. **e**, A 1:1 mixture of *Kdm1a*$^{fl/fl}$ control and *Kdm1a* knockout (KO) ES cells were co-injected into wild-type blastocysts. At embryonic day 12.5, chimaeric embryonic (E) tissue was separated from placenta (P), yolk sac (Y) and amnion (A) and subject to PCR to detect the floxed (fl) and knockout alleles of the injected cells relative to wild-type alleles of the resident injected embryo. M denotes PCR marker. **f**, *Kdm1a*$^{GT/GT}$, Ef1a::GFP$^+$ cells contribute to embryonic endoderm, mesoderm, ectoderm, yolk sac, placental tissues (including giant trophoblast cells, white arrow) and primordial germ cells (PGCs, colabelled with anti-Ddx4 antibody in red, blue arrow). Scale bars, 500 μM (endoderm, mesoderm, ectoderm and yolk sac) and 50 μm (placenta and PGCs).

tdTomato$^-$ cells that were pre-infected with a lentivirus encoding green fluorescent protein (GFP) from a constitutively active Ef1a promoter (Ef1a::GFP). tdTomato$^-$ GFP$^+$ cells contributed exclusively to embryonic tissues, whereas tdTomato$^+$ GFP$^+$ cells contributed to embryonic endoderm, ectoderm, mesoderm, the germ lineage as well as the yolk sac and placenta (Fig. 4b, c and Supplementary Fig. 6a, b). The extraembryonic contribution of the tdTomato$^+$ GFP$^+$ cells included giant trophoblast cells of the placenta (Fig. 4c). Thus, the developmental potential of 2C::tdTomato$^+$ cells includes embryonic plus extraembryonic tissues in contrast to most ES cells in culture, which are restricted to generating only embryonic cell types.

We next examined whether *Kdm1a* mutant ES lines, which contain higher frequencies of 2C::tdTomato$^+$ cells, also had increased potency in mouse chimaera assays. As expected, *Kdm1a* heterozygous ES cells contributed exclusively to embryonic tissues (in five out of five chimaeric embryos) but never to extraembryonic tissues (Fig. 4d). By contrast, *Kdm1a* homozygous mutant ES cells generated both embryonic (in four out of six chimaeric embryos) and extraembryonic (in five out of six chimaeric embryos) tissues (Fig. 4d). To confirm the increased potential of *Kdm1a* mutant ES cells, we used a competition chimaera assay. We co-injected a 1:1 mixture of control *loxP*-flanked (floxed) *Kdm1a*$^{fl/fl}$ and homozygous *Kdm1a* knockout ES cells into five wild-type blastocysts. PCR was then used to detect the appearance of *Kdm1a*$^{fl/fl}$ or knockout cells in dissected tissues. We detected *Kdm1a*$^{fl/fl}$ ES cells in the embryonic tissues and amnion, but not the yolk sac or placenta (Fig. 4e). By contrast, *Kdm1a* mutant ES cells contributed to embryonic tissues, the amnion, yolk sac and placental tissues, including giant trophoblast cells and primordial germ cells (Fig. 4e, f). Thus, the artificial activation of 2C genes achieved with removing *Kdm1a* is associated with expanded fate potential.

We have shown that *2C::tdTomato*$^+$ cells within ES cultures have increased potency, but it is unclear whether entrance into this state is essential for their long-term pluripotency. To test this possibility, we performed serial depletion of 2C-like ES cells by genetic ablation with diptheria toxin (DTA). We generated ES lines by crossing *2C::ERT2-Cre-ERT2* mice (Supplementary Fig. 3a) with a Cre-responsive DTA allele (*ROSA::LSL-DTA*) and treated the ES line with 4HT for 20 passages (Supplementary Fig. 7a). We found that these 2C-depleted ES cultures were still capable of generating high contribution chimaeras (Supplementary Fig. 7b), although their differentiation was biased towards mesoderm and ectoderm lineages *in vitro* (Supplementary Fig. 7c). These data suggest that occasional entry into the 2C-like state might help to preserve the broad embryonic fate potential of ES cells.

Discussion

In mammalian development, the zygote and its daughter cells progress from totipotent cells capable of generating an entire mouse to more lineage-restricted inner and outer cells of the morula capable of generating embryonic or extraembryonic lineages, respectively. A key transcriptional feature of the totipotent cells is the onset of zygote genome activation in which the embryo switches from a maternal to a zygotic transcriptome. To mark cells at this early stage of embryonic development, we generated a reporter with the regulatory elements from the endogenous retrovirus MuERV-L, which is highly restricted to the zygote/2C stage. Surprisingly, we found that rare ES and iPS cells expressed the reporter. When we characterized these cells, we found that they lacked expression of the pluripotency proteins Oct4, Sox2 and Nanog. Instead, these rare cells expressed a large number of genes restricted to the 2C stage, and most importantly, were capable of forming both embryonic and extraembryonic lineages (Fig. 5a, b). Our studies identify a rare 2C-like cell in ES cultures that has expanded fate potential.

Although it is unclear how MuERV-L and other 2C genes regulate potency, several lines of evidence indicate that 2C-like cells are required for the health and maintenance of ES cultures. First, we found that nearly all cells enter into the 2C-like state over increasing passage. Second, when we depleted 2C-like ES cells from cell cultures we found that their differentiation characteristics were altered to generate more ectoderm and mesoderm derivatives. Third, functional studies of the *Zscan4* gene, found adjacent to a full-length MuERV-L element and highly enriched within 2C::tdTomato$^+$ cells, have shown that it is required for the maintenance of telomeres within ES cultures[14]. Another important question that remains is whether the selection of

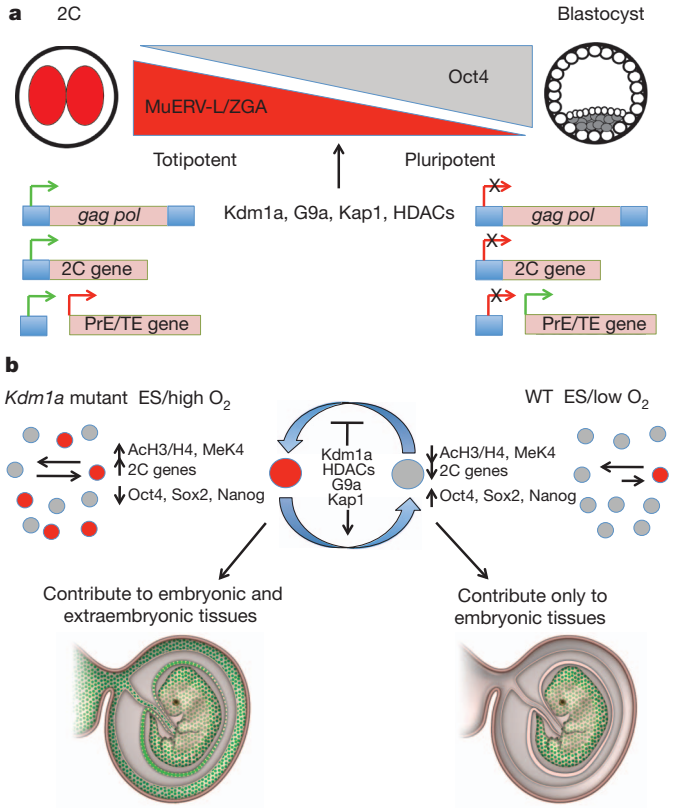

Figure 5 | Model of the role of the MuERV-L-LTR-linked 2C gene network in regulating embryonic potency. a, During zygote genome activation, a network of genes that use MuERV-L-LTRs as promoters is activated. This stage correlates with a period in which blastomeres are totipotent. As development progresses, the MuERV-L-LTR-linked 2C gene network is silenced by chromatin repressors, as the ICM segregates from the trophectoderm and primitive endoderm (PrE). HDACs, histone deacetylases. **b**, During the derivation of ES cells from blastocysts, a rare transient population of cells marked by the *2C::tdTomato* reporter expresses high levels of 2C genes and low levels of pluripotency markers. In mouse chimaera assays, these cells contribute to embryonic and extraembryonic tissues (shown in green). Increasing the oxidative tension of ES cell cultures or deletion/inhibition of repressive histone-modifying enzymes alters the equilibrium between the 2C and ES states.

these special ES cells can be used for practical purposes, such as reprogramming somatic nuclei. This idea is supported by the finding that 2C genes are not properly activated in cloned embryos, and that reprogramming efficiency is enhanced by inhibition of histone deacetylases and Kdm1a, which repress the 2C state[32–34]. Thus, overexpression of one or several MuERV-L-linked 2C genes or inhibition of other 2C gene repressors may be useful strategies to facilitate reprogramming. This possibility is supported by the recent finding that forced Zscan4 expression in fibroblasts enhances their iPS cell reprogramming efficiency[35].

Transposable elements are a major driving force of evolution. Our findings support the notion that the co-option of retrotransposable elements by cellular genes can act as an evolutionary mechanism for coordinately linking the expression of many genes[15,29,36]. Transposon sequences have recently been shown to have a crucial role in rewiring gene regulatory networks in ES cells and in the endometrium that contributed to the evolution of pregnancy in mammals[37,38]. It has also been speculated that ERVs were involved in the evolution of the placenta by providing fusogenic envelope genes adapted for formation of the syncytiotrophoblasts[39]. We suggest that endogenous retroviruses, which are found in all placental mammals[40], may have had an equally important gene regulatory role in early mammalian development, by contributing to the specification of cell types and leading to the formation of placental tissues.

METHODS SUMMARY

2C::tdTomato was created by digesting the MuERV-L-LTR-Gag clone 9 (ref. 29) with MluI and HindIII, resulting in MuERV-L-LTR 1-730, and was ligated into pcDNA3 hygro tdTomato with the cytomegalovirus (CMV) promoter removed. To generate *2C::tdTomato* ES cells, *Kdm1a*$^{fl/fl}$; *Cre-ERT* ES cells were transfected with *2C::tdTomato* using Lipofectamine 2000 (Invitrogen) and selected with 150 μg ml^{-1} hygromycin for 7 days. Colonies containing tdTomato^{+} cells were then picked and expanded. *2C::ERT2-Cre-ERT2* was generated by replacing tdTomato with an ERT2-Cre-ERT2 insert using EcoR1 and Not1 sites. DNA was linearized with Mlu1 and AvrII sites before injection into embryos to generate transgenic mice. The resulting mice were mated with *ROSA::LSL-tdTomato* mice (JAX 007905), *ROSA::LSL-DTA* mice (JAX 010527) or *ROSA::LSL-LacZ* mice (gift from D. Anderson laboratory), and ES lines were derived using standard procedures. *Kdm1a*$^{GT/GT}$, *Kdm1a*$^{fl/fl}$, *Kap1* and *G9a* mutant ES cells were described previously[29–31]. RNA-seq from oocytes and 2C embryos was performed by lysing litters of embryos (5–10 embryos) in prelude direct lysis buffer (Nugen), and amplifying RNA using the ovation RNA-seq system (Nugen) before library construction using the Tru-seq RNA sample prep kit (Illumina). Microarray, quantitative PCR with reverse transcription (qRT–PCR), immunostaining and chimaeric mouse injections were performed as described[29]. All animal experiments we performed in accordance with the Salk Institute Institutional Animal Care and Use Committee guidelines.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions T.S.M. designed and performed all experiments with assistance from W.D.G., S.D., D.B. and K.L. under the supervision of S.L.P. D.T. generated *Kap1* mutant ES cells and H.M.R. and D.T. provided mRNA-seq data from these cells. A.F. and O.S. generated and provided iPS cell lines and lentivirus constructs. T.S.M., W.D.G. and S.L.P. wrote the manuscript.

Author Information Microarray and RNA-seq files have been submitted to the NCBI Gene Expression Omnibus database under accession GSE33923. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.L.P. (pfaff@salk.edu).

METHODS

RNA-seq. For RNA-seq analysis of early stage embryos, three independent litters of superovulated oocytes or naturally fertilized superovulated oocytes were collected and lysed directly in 2 µl of prelude direct lysis buffer (Nugen). RNA was then subject to amplification using the ovation RNA-seq system (Nugen). Amplified complementary DNA was fragmented using Covaris, and single-end (oocytes) or paired-end (2C embryos) libraries were then constructed using the mRNA-seq sample prep kit (Illumina) or Tru-seq RNA library construction kit (Illumina) starting with end repair. Sequencing was performed on either a Illumina genome analyzer (oocytes) or a Hi-Seq (2C embryos). A total of 72-base pair (bp) single-end reads (oocytes) or 100-bp paired-end reads (2C embryos) were aligned to the mouse genome using Bowtie, allowing up to three mismatches per alignment and up to 20 alignments per read, filtering out any read aligning in more than 20 locations. To compare the oocyte data with the 2C data, read lengths were cut down to 72 bp (from the 3′ end). The oocyte data had an average of 33 million alignments per sample, whereas the 2C data had an average of 49 million alignments per sample. Read counts were quantified using a custom gene reference based on the University of California, Santa Cruz (UCSC) database. At each gene locus, all isoforms belonging to a single gene were fused into one transcript containing all exons from each isoform. Counts aligning in several locations were counted as a fraction of their total number of alignments at each location. Differential expression testing was performed with DESeq[41]. Genes with adjusted *P* values less than 0.05 were marked as significant. Chimaeric transcripts were identified using the spliced alignment data produced by Tophat. Tophat identifies exons based on alignment pileup, and it follows by aligning previously unaligned reads across potential splice junctions. We split the junction information into two lists, the left and right side of each junction, and compared with both the UCSC knowngene database for mm9 and the RepeatMasker database, also from the UCSC database. Only junctions that hit an exon of a known model on one end and a repeat element on the other were retained. GO analysis was performed using the David Bioinformatic Resource (http://david.abcc.ncifcrf.gov/)[42].

For RNA-seq of 2C::tdTomato^{+} and 2C::tdTomato^{-} cells, and *Kdm1a*, *Kap1* and *G9a* knockout ES cells, sample libraries were prepared from 500 ng–5 µg of total RNA using the mRNA-seq sample prep kit (Illumina) or Tru-seq RNA library construction kit. Library samples were amplified on flow cells using cluster generation kit (Illumina) and then sequenced using consecutive 36 cycle sequencing kit on the genome analyzer (Illumina) or 100-bp paired-end reads on the Hi-Seq (Illumina). Raw sequence data was then aligned to the mouse genome using the short read aligner Bowtie and the default setting (two mismatches per 25 bp and up to 40 genomic alignments) (http://bowtie-bio.sourceforge.net/index.shtml). Reads per kilobase of exon model per million mapped reads (RPKM) values were also determined by Bowtie. For repetitive sequences, we aligned sequencing reads to the Repbase database using Bowtie (http://www.girinst.org/repbase/index.html). Differential expression was determined using DESeq as described earlier. To compare gene expression in *G9a*, *Kdm1a* and *Kap1* mutant ES cells, we identified upregulated genes by combining previously identified upregulated genes[29–31] and our own DESeq analysis.

ES culture and generation of *2C::tdTomato* and *2C::ERT2-Cre-ERT2* ES lines. The derivation and culture of *Kdm1a*$^{GT/GT}$ and *Kdm1a*$^{fl/fl}$; *Cre-ERT* ES cells were described previously[29]. The *2C::tdTomato* construct was created by digesting the MuERV-L-LTR-Gag clone 9 in pGL3 basic with MluI and HindIII, resulting in MuERV-L-LTR 1-730, and was ligated into pcDNA3 hygro tdTomato digested with MluI and HindIII (to remove the CMV promoter). To generate *2C::tdTomato* ES cells, *Kdm1a*$^{fl/fl}$; *Cre-ERT* ES cells were transfected with *2C::tdTomato* using Lipofectamine 2000 (Invitrogen) and selected with 150 µg ml^{-1} hygromycin for 7 days. Colonies containing tdTomato^{+} cells were then picked and expanded. *2C::tdTomato* ES cells were also derived from a transgenic mouse generated by pronuclear injection of the *2C::tdTomato* ES line. *2C::ERT2-Cre-ERT2* was generated by replacing tdTomato with an ERT2-Cre-ERT2 insert using EcoR1 and Not1 sites. DNA was linearized with Mlu1 and AvrII sites before injection into embryos to generate transgenic mice. The resulting mice were mated with *ROSA::LSL-tdTomato* mice (JAX 007905), *ROSA::LSL-DTA* mice (JAX 010527) or *ROSA::LSL-LacZ* mice (gift from D. Anderson laboratory), and ES cell lines were derived using standard procedures.

Kap1 and *G9a* mutant ES cells were described previously[30,31]. To recombine and delete the *Kdm1a*, *Kap1* and *G9a* floxed alleles, cells were treated with 1 µM 4HT for 24 h. Cells were collected at a minimum of 48 h later to allow for loss of residual protein. To activate the *2C::ERT2-Cre-ERT2* transgene, cells were maintained in 1 µM 4HT and fed daily. tdTomato^{+} and tdTomato^{-} cells were counted using a FACScan and sorted using a FACSDiVA. For differentiation assays, ES cells were grown in suspension in the absence of leukaemia inhibitory factor as described[29].

Immunofluorescence staining and microscopy. ES and iPS cells were plated on gelatinized glass coverslips on primary mouse embryonic fibroblasts. Cells were fixed with 4% paraformaldehyde (PFA) for 10 min, followed by washing with PBS-T (0.05% tween). Cells were blocked in PBS-T containing 3% BSA for 10 min and stained with primary antibody for 1 h at room temperature. Antibodies used: mouse anti-Kap1 (Abcam, 1:1,000); mouse anti-Oct3/4 (Santa Cruz, 1:500); rabbit anti-MuERV-L-Gag (gift from T. Heidmann laboratory, 1:2,000); rat anti-E-cadherin (Abcam, 1:500); rabbit anti-Pan-acetylated histone H3 (Upstate, 1:1,000); rabbit anti-Pan-acetyl H4 (Upstate, 1:1,000); and rabbit anti-H3K4me2 (Abcam, 1:1,000). After washing three times for 10 min with PBS-T, cells were stained with secondary antibody (1:1,000 anti-mouse, -rat or -rabbit IgG Alexa fluor 488, 55 or 647) for 1 h at room temperature and washed again three times with PBS-T. Coverslips were stained with DAPI in PBS for 5 min before inverting onto slides in mounting medium. Cells were then imaged using either an Olympus FV1000 confocal microscope and a ×60 oil objective, or a Zeiss Axioskop 2 epifluorescence microscope and ×40 objective. Quantification of histone stains was performed with Fluoview. Preimplantation embryos were stained as described with minor modifications. Embryos were fixed in 4% PFA for 30 min and permeabilized in 0.25% Triton for 20 min, before blocking in 10% FBS for 1 h in 0.1% Triton-PBS. Primary antibodies were incubated overnight at 4 °C in blocking buffer. Subsequent washes and secondary antibody incubations were at room temperature in 0.1% Triton-PBS.

***In situ* hybridization.** A MuERV-L probe was generated by PCR from mouse ES cDNA using the forward primer 5′-CCATCCCTGTCATTGCTCA-3′ and reverse primer 5′-CCTTTTCCACCCCTTGATT-3′, and cloned into the PCR2.1 TOPO vector. A digoxigenin (DIG)-labelled probe was prepared using *in vitro* transcription with the T7 polymerase. ES cell samples were fixed in 4% PFA, digested for 2 min with proteinase K, washed with PBS, acetylated and hybridized with denatured probe overnight at 68 °C. After washing with 5× saline sodium citrate (SSC) and 0.2× SSC, DIG-labelled probe was visualized using an anti-DIG antibody coupled to alkaline phosphatase.

Immunoblotting. Whole cell extracts were prepared by pelleting ES cells at 200*g* and resuspending in 1:5 volume of 1% NP40 lysis buffer containing 10 mM Tris, 150 mM NaCl and 1× protease inhibitors. To solubilize histones, extracts were also sonicated using a bioruptor on the high setting for 10 min. Ten to fifty micrograms of total protein in LDS sample buffer (Invitrogen) was then loaded onto a 4–12% NuPage gel (Invitrogen), electrophoresed at 200 V for 60 min, and transferred to nitrocellulose membranes at 30 V for 90 min. Membranes were blocked in PBS-T containing 5% non-fat dry milk. Primary antibodies were incubated overnight at 4 °C. Antibodies used: rabbit anti-GAPDH (Santa Cruz, 1:1,000); rabbit anti-MuERV-L-Gag (gift from T. Heidmann laboratory, 1:1,000); anti-Pan-AcH3 (Upstate, 1:1,000); anti-Pan-AcH4 (Upstate, 1:1,000); anti-H3K4me2 (Abcam, 1:500); anti-H4 (Novus, 1:1,000); and anti-H3 (Novus, 1:500). After washing extensively with PBS-T, secondary antibodies (anti-rabbit or -mouse horseradish peroxidase (HRP) conjugate, 1:10,000 dilution) were incubated for 1 h at room temperature. After washing extensively with PBS-T and water, blots were developed using ECL plus detection system (Amersham).

Bisulphite sequencing. ES cells were lysed in tail lysis buffer (0.1 M Tris, pH 8.5, 5 mM EDTA, 0.2% SDS and 0.2 M NaCl) containing proteinase K (Roche) for 1 h at 55 °C, followed by treatment with DNase-free RNase for 30 min at 37 °C. DNA was then sonicated briefly and purified using Qiagen PCR purification columns. Bisulphite conversion of genomic DNA was carried out using the epitect bisulphite kit (Qiagen). Bisulphite-converted DNA was then PCR-amplified using accuprime Taq polymerase (Invitrogen) followed by TOPO TA cloning (Invitrogen). At least 10 individual clones per primer pair were sequenced (Eton Bio). Primer sequences were described previously[29].

qRT–PCR. For quantitative PCR with reverse transcription (qRT–PCR) analysis, first-strand cDNA was generated from up to 5 µg total RNA using Superscript III (Invitrogen) and polydT or random hexamer priming. qPCR was performed using SYBR green master mix (Applied Biosystems) in 96-well dishes in triplicate and repeated with at least two biological replicates with similar results. Standard curves were generated for each primer pair (described previously[29]) and expression levels were plotted relative to *Gapdh* (in arbitrary units).

Microarray. Total RNA was prepared from *2C::tdTomato*$^{+}$ and *2C::tdTomato*$^{-}$ cells using RNEasy kits (Qiagen). Labelling of 100 ng of total RNA was performed using the whole transcript sense target labelling assay kit (Affymetrix) before hybridization to genechip mouse gene 1.0 ST arrays. Probeset normalization and summarization were prepared using robust multichip analysis (RMA) in expression console (Affymetrix).

Mouse chimaera assay. ES cells were injected into either embryonic day (E) 2.5 or E3.5 C57Bl/6J embryos, and cultured *in vitro* or implanted into pseudopregnant

females. For PCR assays, dissected tissues were placed in lysis buffer (1% SDS, 150 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) containing proteinase K overnight at 55 °C. DNA was then isolated by phenol–chloroform extraction and ethanol precipitation, followed by PCR analysis with primers designed to amplify the β-geo cassette or the wild-type *Kdm1a* foxed allele. For embryo imaging, chimaeric mice were collected between E9.5 and E12.5 and fixed with 4% PFA for 2 h, washed extensively in PBS overnight, incubated in 30% sucrose for 4 h, and frozen on dry ice in OCT. Cryosections were then taken and stained with DAPI before imaging.

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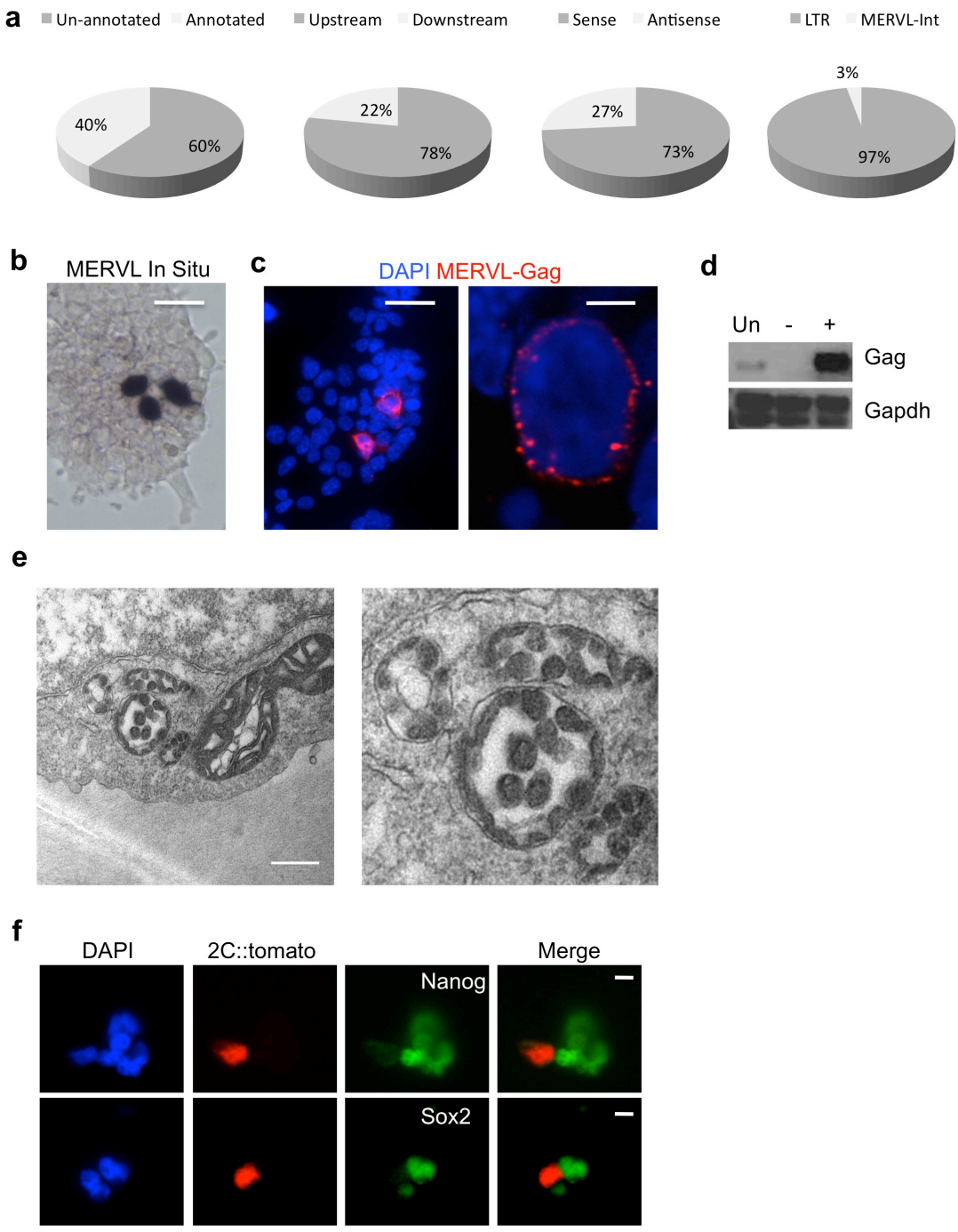
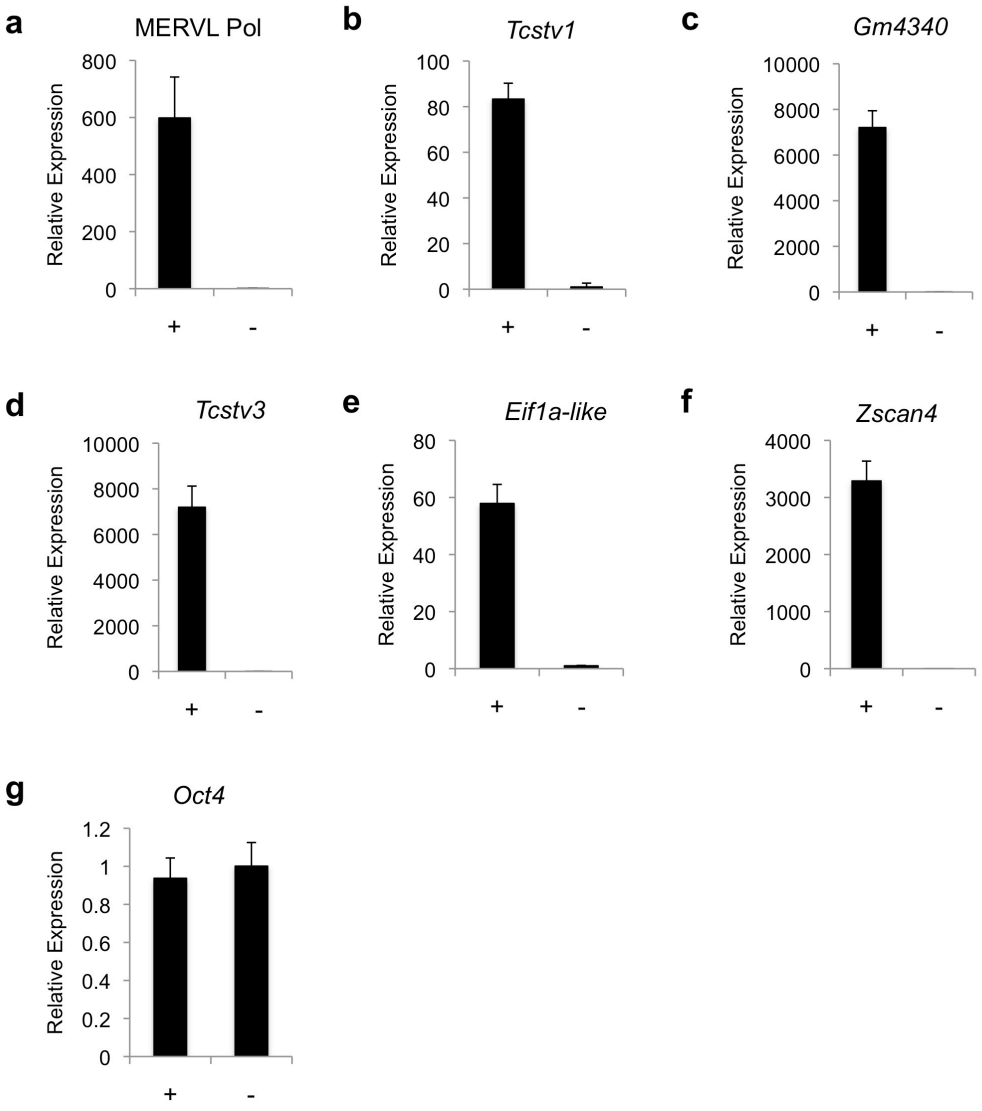
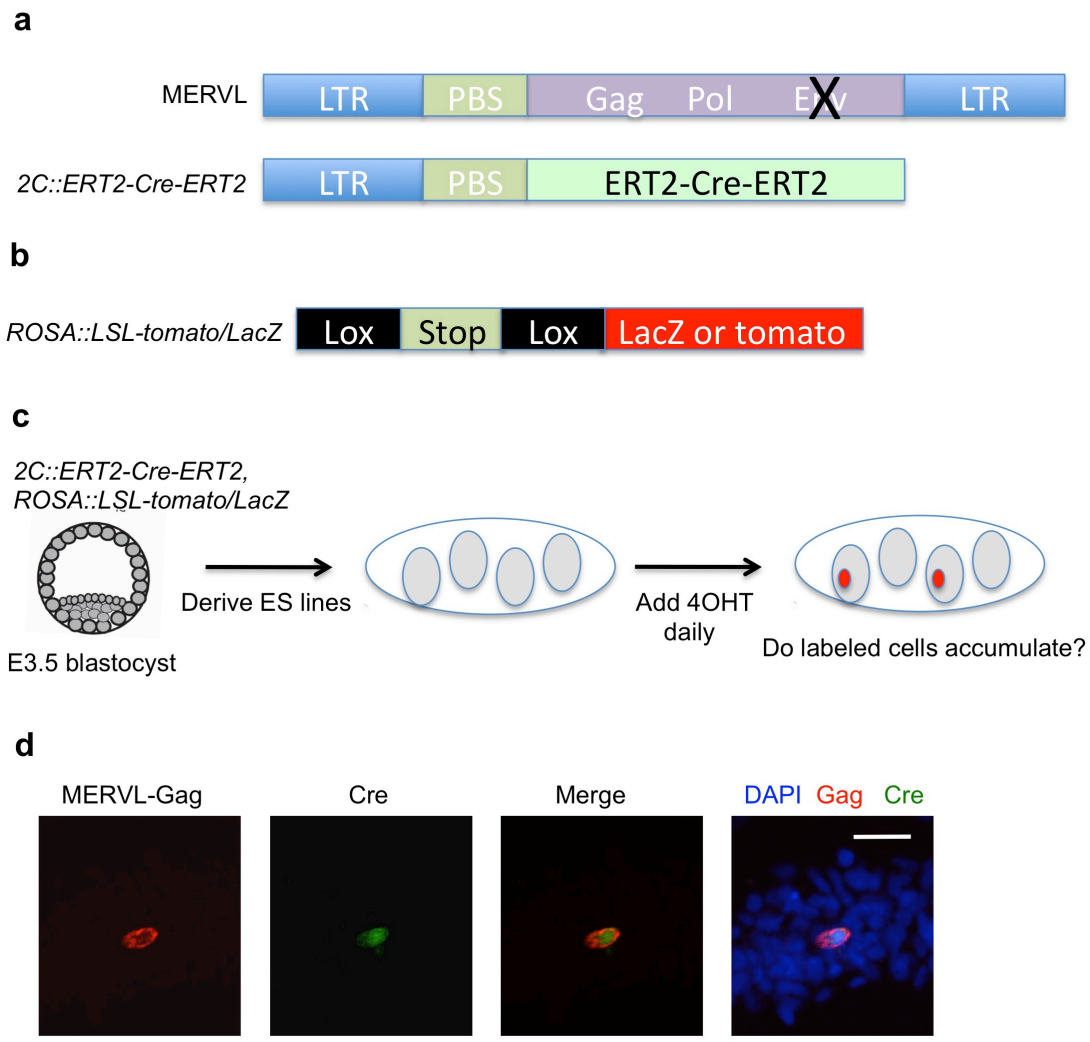


Supplementary Figure 1. a, MERVL-linked chimeric transcripts were grouped according to whether the transcripts were previously annotated, whether the MERVL portion of the transcript was the upstream or downstream portion of the chimera, whether the LTR was sense or antisense to the gene, and whether the MERVL portion of the chimera was derived from the LTR or part of

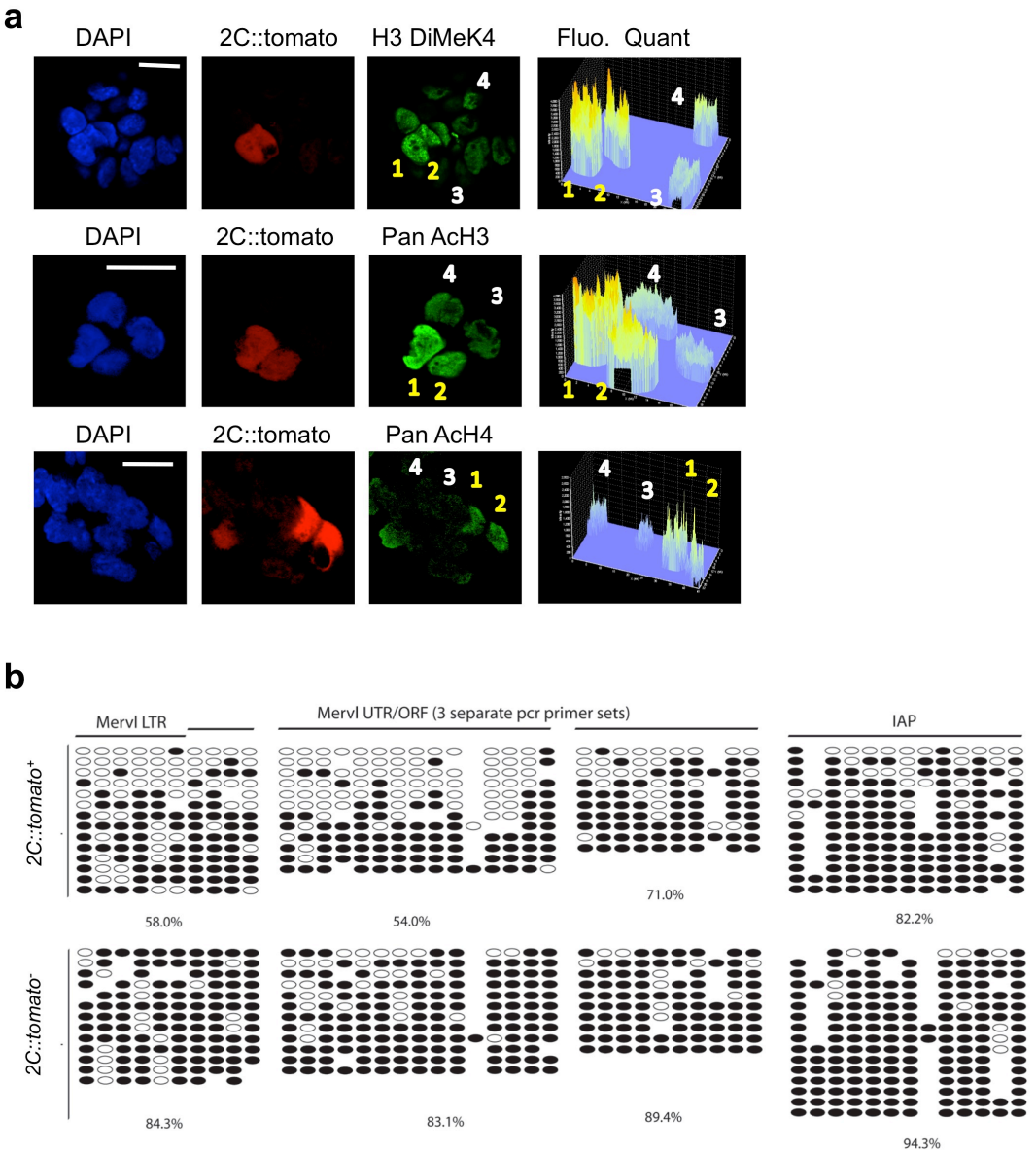
the viral internal sequence (-int). **b-c,** MERVL expression was visualized in wild type mouse ES cells by **(b)** in-situ hybridization (Scale bar 10µm**)** and **(c)** immunofluorescence microscopy with antibodies recognizing MERVL-Gag protein (ES colony left; Scale bar 10µm, single ES cell, right, Scale bar 2µm). **d,** Unsorted (Un) *2C::tomato* ES cultures or *2C::tomato* $^{+}$ and $^{-}$ cells collected by FACS were subject to immunoblots with indicated antibodies to demonstrate accuracy of the reporter. **e,** Transmission electron microscope image of a *2C::tomato*$^{+}$ ES cell collected by FACS. Epsilon particles encoded by MERVL fill the lumen of the endoplasmic reticulum. Scale bar 500nm. **f,** *2C::tomato* ES cells were immunostained with Sox2 and Nanog antibodies and counterstained with DAPI, demonstrating that *2C::tomato*$^{+}$ cells express low levels of pluripotency markers. Scale bar 10µm.



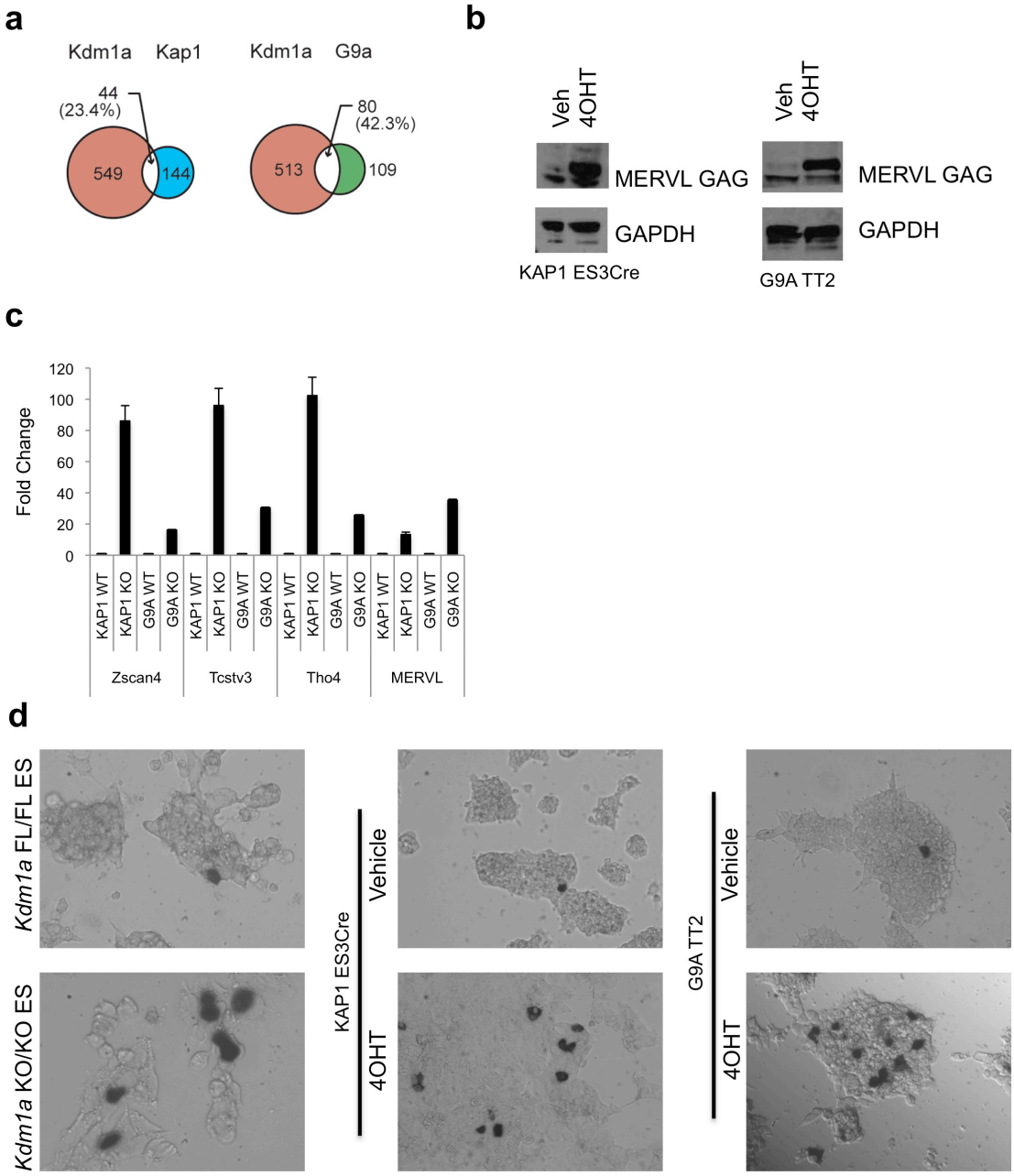
Supplementary Figure 2: a-g The relative expression of the indicated genes/retroelements (compared with *Gapdh*) was determined using QRT-PCR on *2C::tomato*$^{+}$ and $^{-}$ cells collected by FACS. Error bars represent s.d., n=3.



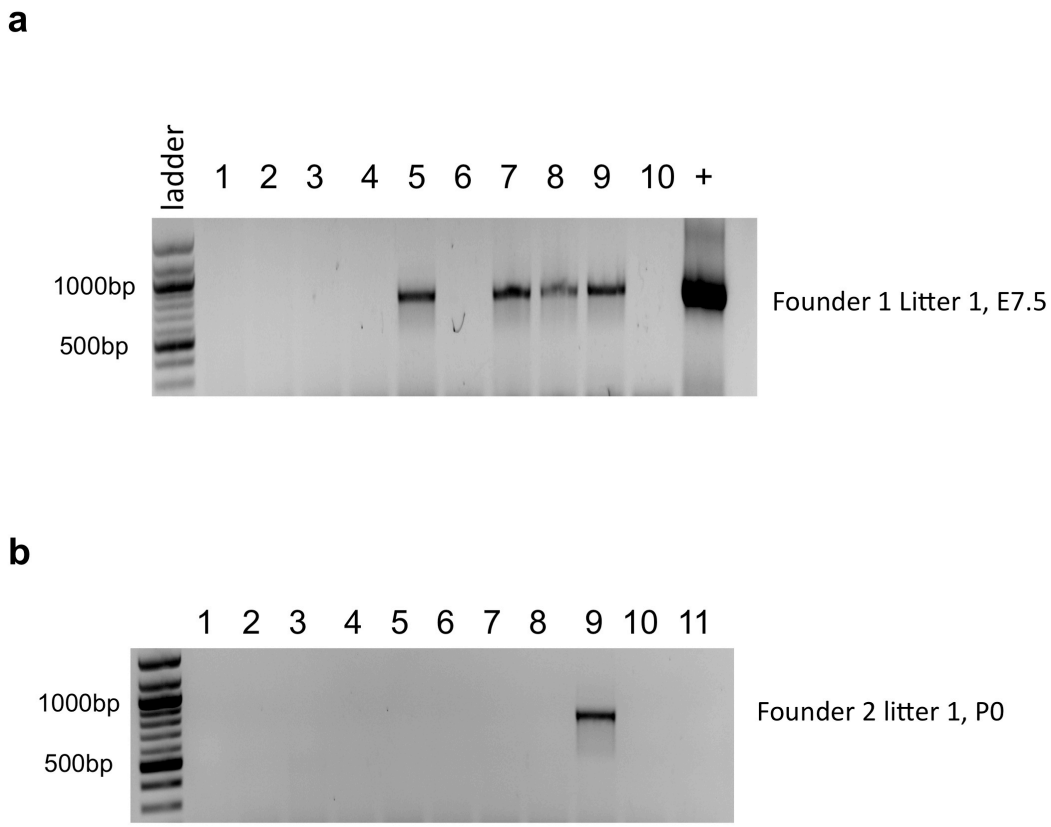
Supplementary Figure 3: **a,** Schematic of a typical MERVL retro-element and the *2C::ERT2CreERT2* transgene. **b,** Schematic of ROSA::LSL-LacZ/tomato transgene. **c,** Schematic of fate mapping strategy to observe activation of 2C genes within a population of ES cells. **d** *2C::ERT2CreERT2* ES cells were treated with 4OHT for 2 hours and immunostained with MERVL-Gag and Cre recombinase antibodies as indicated and counterstained with DAPI. Scale bar 25µm.



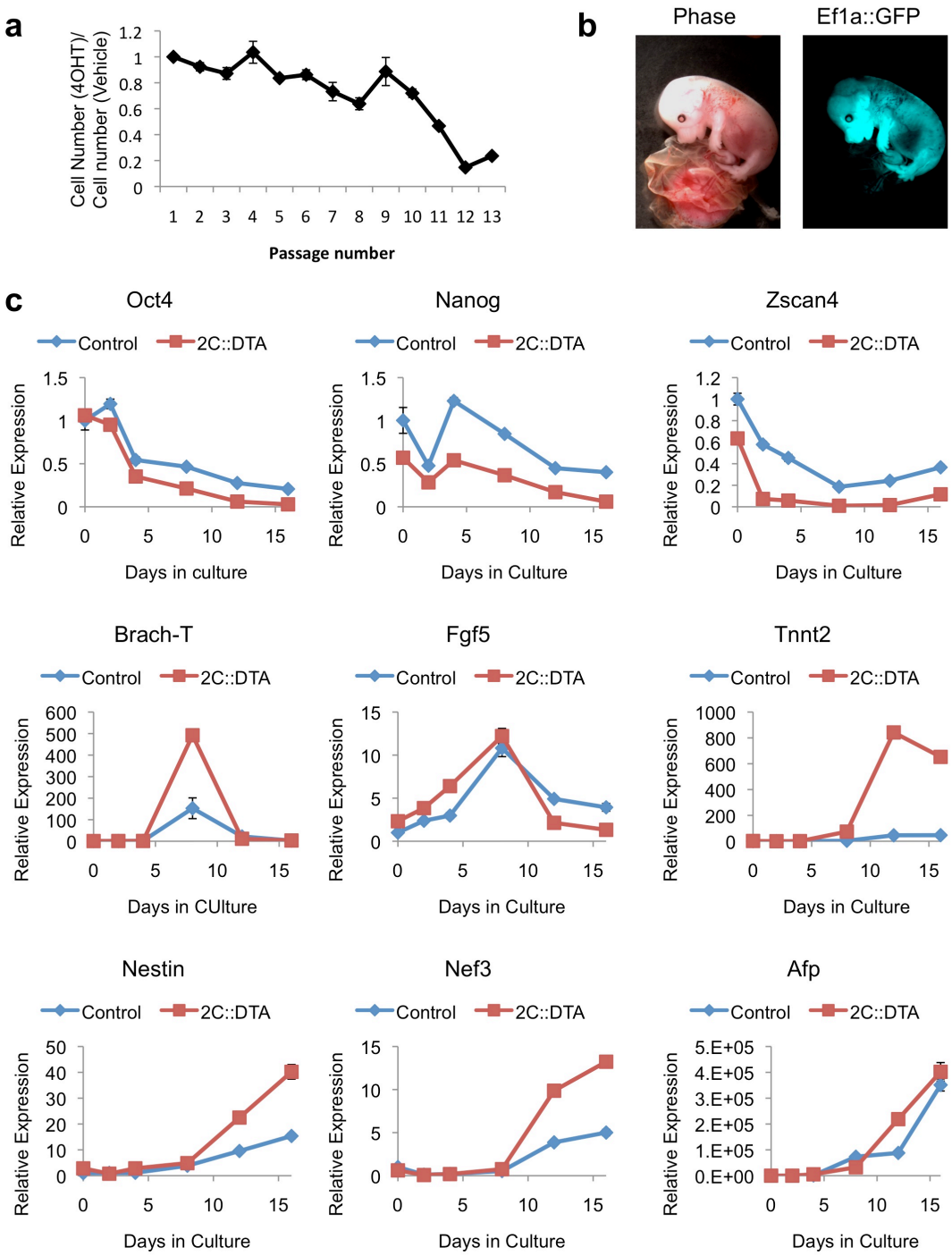
Supplementary Figure 4: a, *2C::tomato* ES cultures were immunostained with histone modification-specific antibodies and fluorescent secondary antibodies as indicated. The fluorescence intensity was quantified for two tomato^{+} cells (1, 2 yellow) or 2 tomato^{-} cells (3, 4 white) in each image using Fluoview. Scale bar 20µm. **b,** *2C::tomato* $^{+}$ and $^{-}$ cells were collected by FACS and subjected to bisulfite sequencing analysis to determine the methylation status of MERVL and IAP retroviruses. The percentage of methylated cytosines for each primer set is shown.



Supplementary Figure 5: a, Pair-wise comparison of re-activated genes in *Kdm1a, Kap1, and G9a* mutant ES cells. **b,** *Kap1* ES3Cre ES cells or *G9a* TT2 ES cells were treated with vehicle or 4OHT to delete *Kap1* and *G9a*, respectively, and cell extracts were subject to immunoblot with the indicated antibodies. **c.** The fold change in expression of the indicated 2C genes in *Kap1* and *G9a* KO ES cells was determined by QRT-PCR. Error bars represent s.e.m., n=3 **d,** In situ hybridization using a MERVL probe was performed on *Kdm1a, KAP1*, and *G9A* mutant ES cell lines and corresponding wild type ES lines.



Supplementary Figure 6: a-b, ES cells derived from *2C::tomato* transgenic mice were subject to FACS to collect tomato^{+} cells, and these cells were immediately injected into wild type blastocysts to generate chimeric mice. Chimeric founder mice were then mated with wild type mice and E7.5 (a) or P0 (b) litters (from two separate founders) were subject to genotyping PCR with a tomato primer set. These data indicate that *2C::tomato*$^{+}$ cells contribute to the germ lineage.



Supplementary Figure 7: a, 2C::ERT2-Cre-ERT2, ROSA LSL::DTA ES cells were cultured in the presence or absence of 4OHT, and the relative cell numbers were plotted at each passage. Error bars represent s.e.m., n=3. **b,** *2C::ERT2CreERT2*, *LSL::DTA* ES cells were treated with 4OHT for 20 passages prior to injection into blastocyst stage embryos which were implanted into

pseudopregnant females. Prior to injection (at passage 18), cells were infected with a lentivirus expressing an Ef1a::GFP reporter. Chimeric embryos were harvested at E13.5 and visualized using fluorescence microscopy. **c,** *2C::ERT2CreERT2*, *LSL::DTA* ES cells treated with vehicle (control) or 4OHT (2C::DTA) for 20 passages were grown in suspension in the absence of Lif to induce differentiation. Samples were harvested at indicated time points for triplicate QRTPCR analysis using the indicated primers and *Gapdh* to normalize. Expression was plotted relative to control sample at the day 0 time point. Error bars represent s.d., n=3.

Supplementary Movie Legends

Supplementary Movie 1: The *2C::tomato* reporter is restricted to the zygote and 2C/4C stage. The *2C::tomato* reporter was injected into fertilized eggs, which were then developed *in vitro* for 24 hours before imaging overnight.

Supplementary Movies 2: *2C::tomato* is transiently expressed in ES cultures. *2C::tomato* (-) cells were collected by FACS and plated. After 2 hours, live imaging was performed overnight to visualize the appearance of *2C::tomato* (+) cells.

Supplementary Movies 3: Entrance into the *2C::tomato* (+) state is more rapid in *Kdm1a* mutant ES cells. *2C::tomato* (-) cells were collected by FACS in cells depleted with *Kdm1a* (using Cre-mediated excision) and plated. After 2 hours, live imaging was performed overnight to visualize the appearance of *2C::tomato* (+) cells.