Supplementary Information

Naïve pluripotency is associated with global DNA hypomethylation

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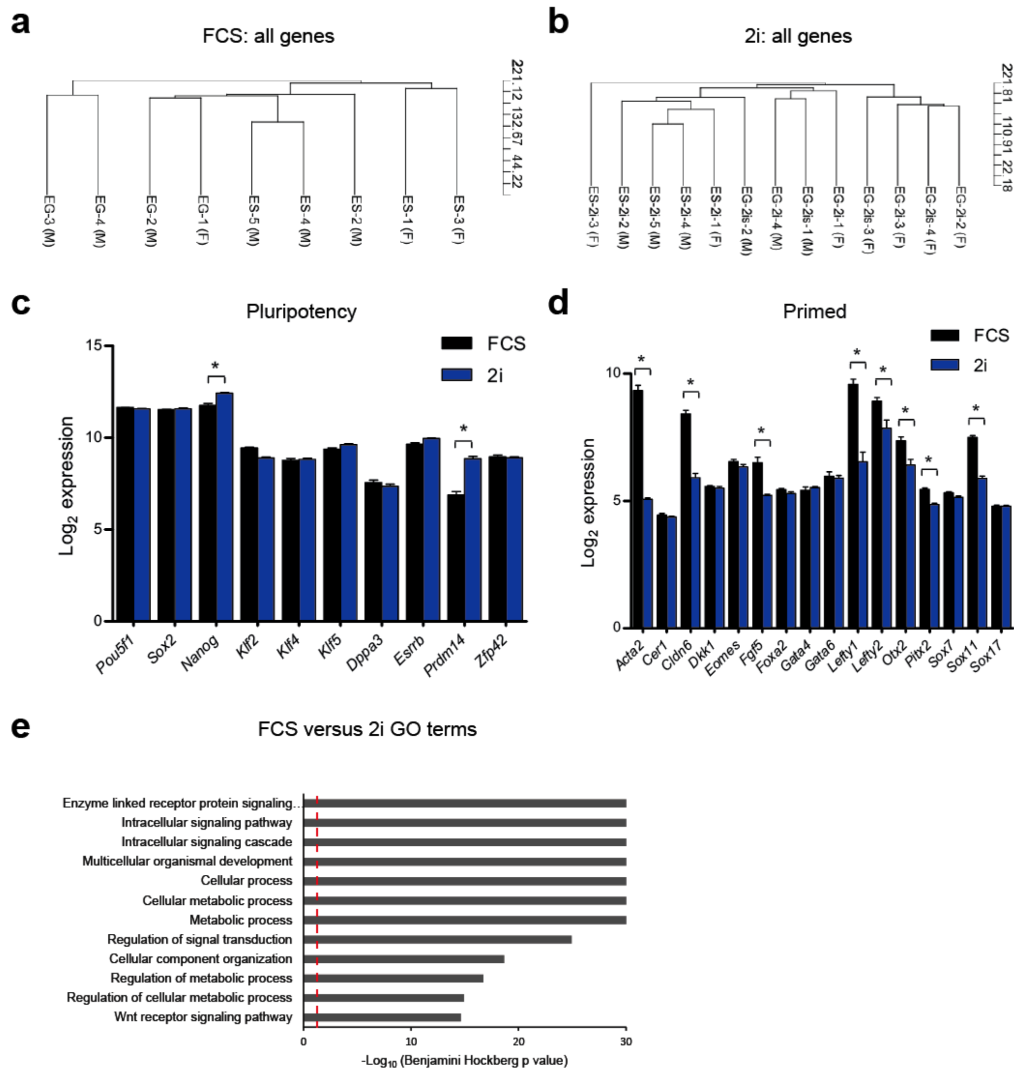
File contains Supplementary Figures 1-4 with legends, figure legend for Supplementary Table 1 and Supplementary Note (with associated references).

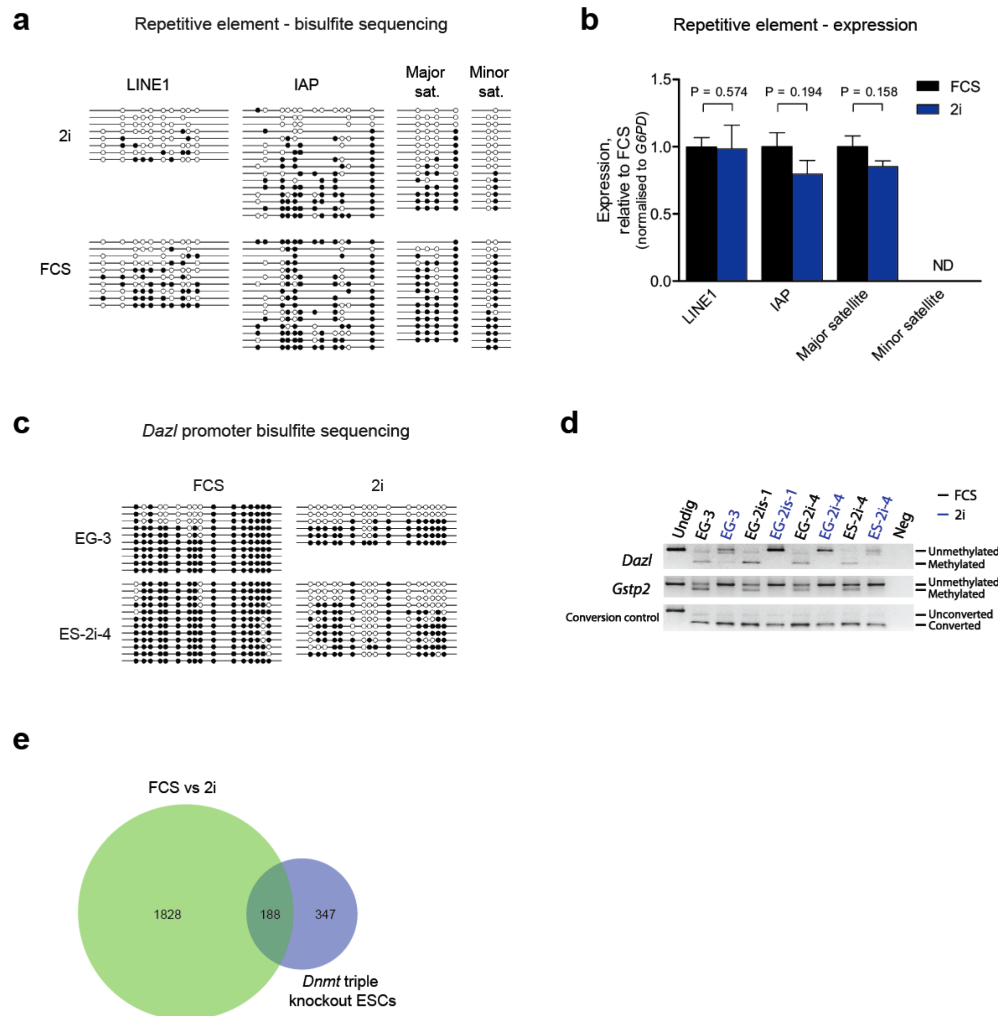
Supplementary figure 1



Supplementary Figure 1: Comparison of pluripotent cell lines in 2i and FCS

Hierarchical clustering of microarray expression data of ESC and EGC lines grown in (**a**) FCS or in (**b**) 2i conditions reveals some degree of separation between these two cell types, however clustering into two distinct groups is not observed. Microarray expression levels combining data for all cell lines depicted in **Fig. 1a** for (**c**) pluripotency markers; (**d**) differentiation markers * *FDR* < 0.05, fold change > 1.5 (see online methods for details), error bars represent standard error of mean (SEM), ≥ nine biological replicates for each condition; (**e**) Top 15 enriched gene ontology (GO) term categories for biological processes for genes significantly different between FCS and 2i conditions; red dashed line indicates $P < 0.05$, Fisher's exact test, ≥ nine biological replicates for each condition.

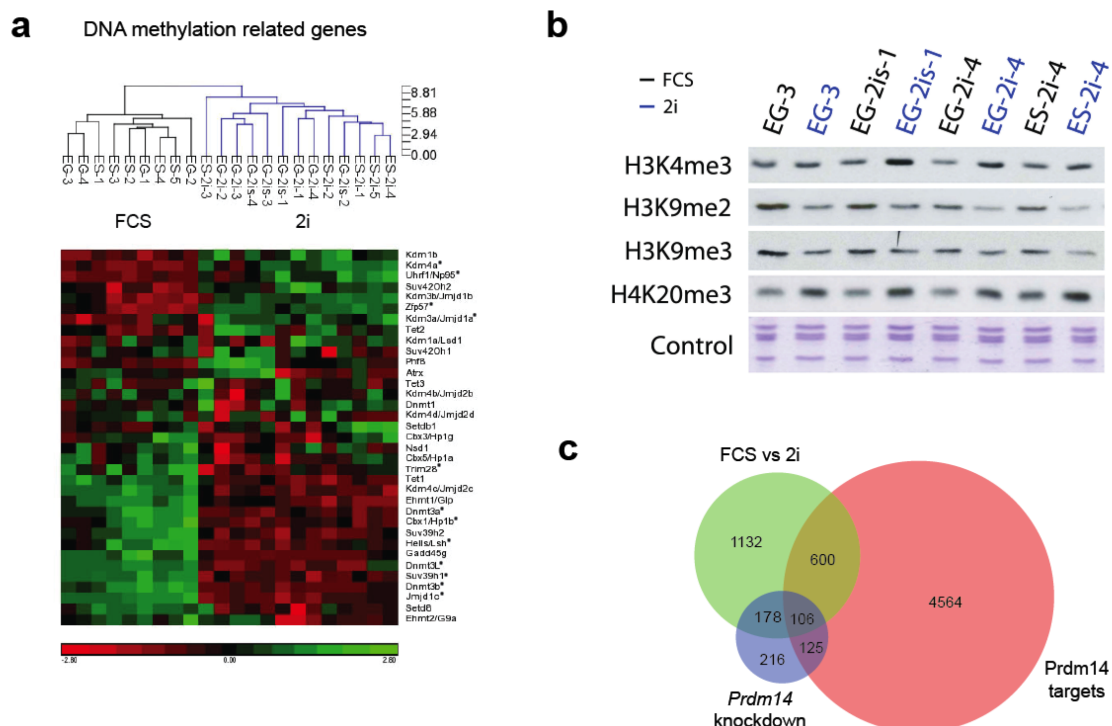
Supplementary figure 2



Supplementary Figure 2: Analysis of culture effect on DNA methylation at repetitive and single copy sequences

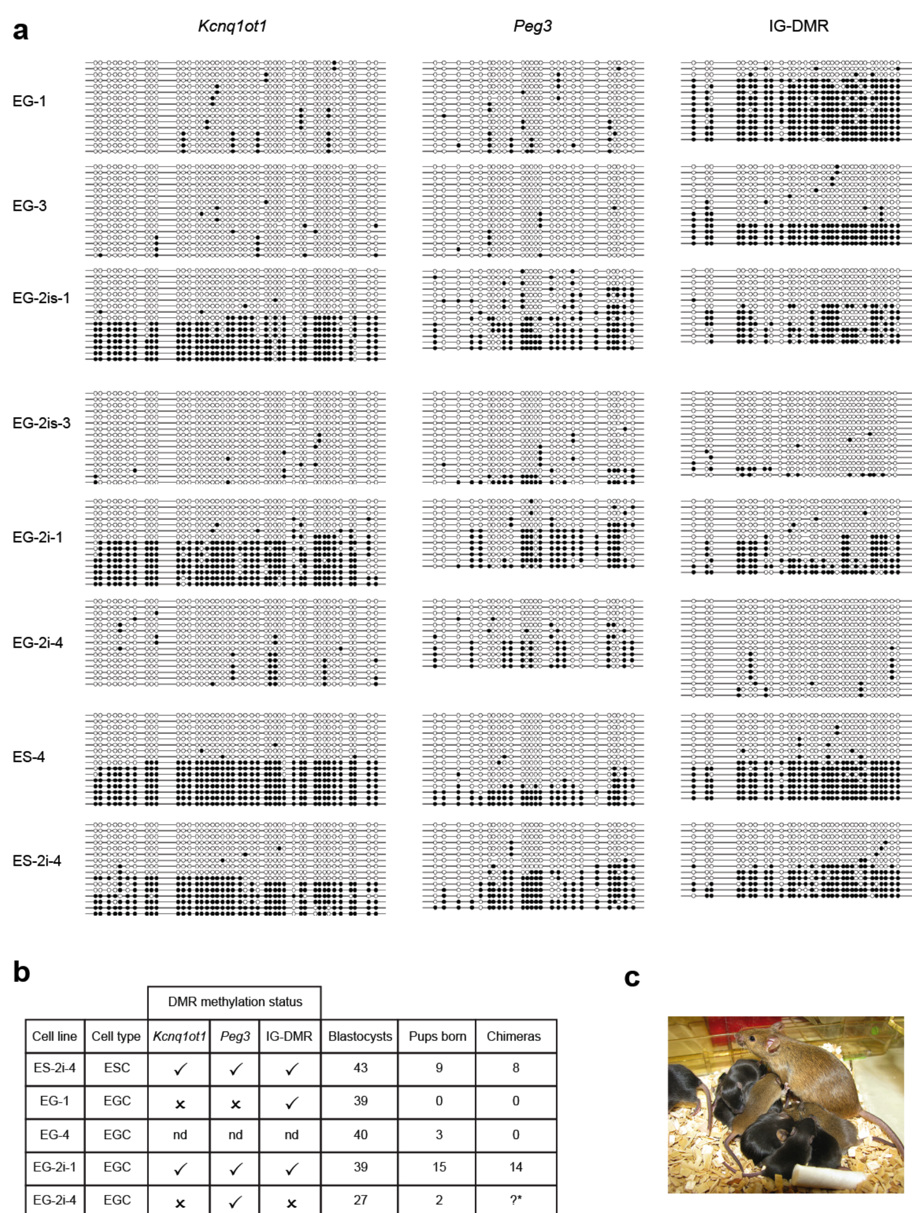
(**a**) Bisulphite analysis of repetitive elements; filled and open circles denote methylated and unmethylated CpGs, respectively; (**b**) Expression analysis of repetitive elements in ESC and EGC cultured in FCS or 2i, $\geq$ four biological replicates for each condition, unpaired t-test, error bars represent SEM. ND = not detected; (**c**) Bisulphite sequencing analysis of the *Dazl* promoter, filled and open circles denote methylated and unmethylated CpGs, respectively; (**d**) Combined bisulfite restriction analysis (COBRA) of the *Dazl* and *Gstp2* promoters showing reduced methylation in 2i across four cell lines. Methylation status was tested using Taq1 and bisulfite conversion was assessed using MseI. Undig, undigested PCR product; (**e**) Venn diagram demonstrating overlap of genes altered between FCS and 2i and genes affected in *Dnmt1, Dnmt3a* and *Dnmt3b* triple knockout ESCs[1].

Supplementary figure 3



Supplementary Figure 3: Regulation of DNA methylation in pluripotent cell lines through additional epigenetic mechanisms

(**a**) Hierarchical clustering of all cell lines based on genes with mechanistic connections to regulation of DNA methylation, with heatmap indicating relative expression levels. Asterisks (*) indicate genes regulated by Prdm14 (targets and genes affected by *Prdm14* knockdown) that are significantly different between FCS and 2i (*FDR* < 0.05, see online methods); (**b**) Analysis of abundance of histone modifications by western blot on histone extracts from ESCs and EGCs grown side-by-side in 2i and FCS. Imperial blue staining of histones shown as a loading control; (**c**) Venn diagram demonstrating overlap of genes altered between FCS and 2i, genes targeted by Prdm14 and genes affected in *Prdm14* knockdown in ESC[2].



Cell line	Cell type	DMR methylation status: *Kcnq1ot1*	DMR methylation status: *Peg3*	DMR methylation status: IG-DMR	Blastocysts	Pups born	Chimeras
ES-2i-4	ESC	✓	✓	✓	43	9	8
EG-1	EGC	×	×	✓	39	0	0
EG-4	EGC	nd	nd	nd	40	3	0
EG-2i-1	EGC	✓	✓	✓	39	15	14
EG-2i-4	EGC	×	✓	×	27	2	?*

Supplementary Figure 4: DMR methylation status and chimera forming capability of ESC and EGC lines

(**a**) Bisulphite analysis of imprinted DMRs in the ESC and EGC lines indicated; filled and open circles denote methylated and unmethylated CpGs, respectively; (**b**) Summary of ESC and EGC blastocyst injections and chimera formation.* Two pups were born, however these looked abnormal and were cannibalised at P0 before chimerism could be assessed; (**c**) Chimeric parent generated from EG-2i-1 and mixed litter of agouti and black pups demonstrating the contribution of the EG-2i-1 cells to the germline in the chimera.

Supplementary Table 1: Statistical analysis of gene expression between culture condition and between cell type

Results of limma analysis of differential expression for all genes. Benjamini-Hockberg false discovery rate (BH FDR) and log fold change (logFC) are given for comparisons between cell lines grown under FCS and 2i culture conditions and for comparisons between EGC and ESC lines. Genes in red are those with BH FDR < 0.05 and absolute fold change > 1.5.

SUPPLEMENTARY NOTE

Comparison of microarray data to existing databases

Gene ontology[3] analysis was undertaken for genes significantly different between FCS and 2i to test for enrichment of biological processes using Fisher's exact test, parent-child method. Comparisons with other published data sets[1,2] were performed by first reducing the published data to include only those genes present on the Affymetrix GeneChip Mouse Gene 1.0 ST Arrays. Binding of Prdm14 within 100 kb of a known gene was used to determine Prdm14 target genes, as per Ma et al., 2010[2]. GSEA was performed as described in online methods to test statistical enrichment.

Bisulfite conversion

Genomic DNA was isolated using the Qiagen Blood and Cell DNA Mini kit. 10 ng of DNA and no template controls were bisulfite treated according to the Sigma Imprint DNA modification kit two-step protocol. PCRs were undertaken using Qiagen HotStarTaq DNA Polymerase (94°C 5 min, followed by 35 cycles of 94°C 1 min, 56-61°C 1 min, 72°C 1 min with an additional 5 min at 72°C); primers and PCR conditions available upon request. Combined bisulfite restriction analysis (COBRA) was performed using the restriction enzyme MseI as a conversion control and Taq1 for methylation analysis. For bisulfite sequencing, PCR products were purified by agarose gel electrophoresis and the Qiagen QIAEX II Gel Extraction kit. Samples were then ligated using Promega pGEM T-easy and transformed into Invitrogen Top10 competent cells. Colony PCR was performed and products were purified followed by Sanger sequencing. Quantification tool for methylation analysis (QUMA)[4] was used for bisulfite sequence analysis. Statistical testing was undertaken in QUMA using the Mann-Whitney U-test. Bisulfite conversion rates for all clones, including repetitive elements, were > 87%.

SUPPLEMENTARY REFERENCES

1. Karimi, M. M. *et al.* DNA Methylation and SETDB1/H3K9me3 Regulate Predominantly Distinct Sets of Genes, Retroelements, and Chimeric Transcripts in mESCs. *Cell Stem Cell* **8**, 676–687 (2011).
2. Ma, Z., Swigut, T., Valouev, A., Rada-Iglesias, A. & Wysocka, J. Sequence-specific regulator Prdm14 safeguards mouse ESCs from entering extraembryonic endoderm fates. *Nat Struct Mol Biol* **18**, 120-7 (2010)
3. Carbon, S. *et al.* AmiGO: online access to ontology and annotation data. *Bioinformatics* **25**, 288–289 (2009).
4. Kumaki, Y., Oda, M. & Okano, M. QUMA: quantification tool for methylation analysis. *Nucleic Acids Res* **36**, W170–W175 (2008).