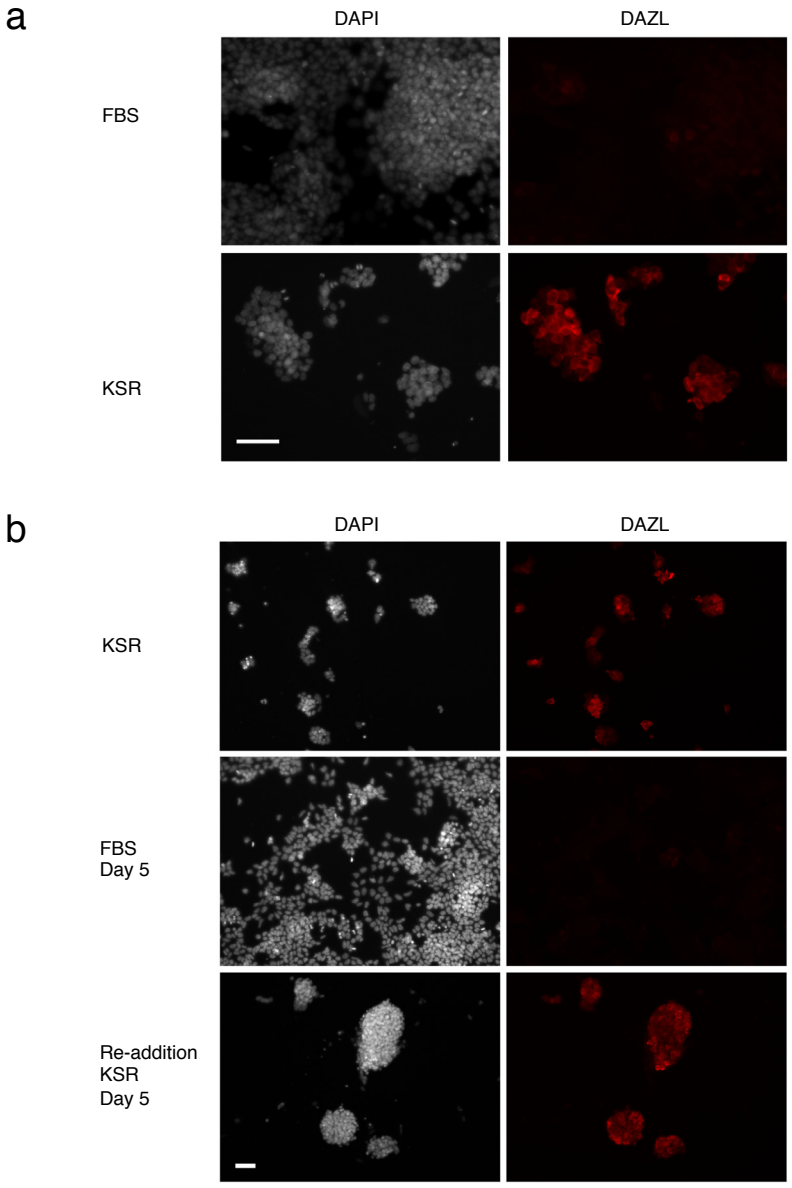
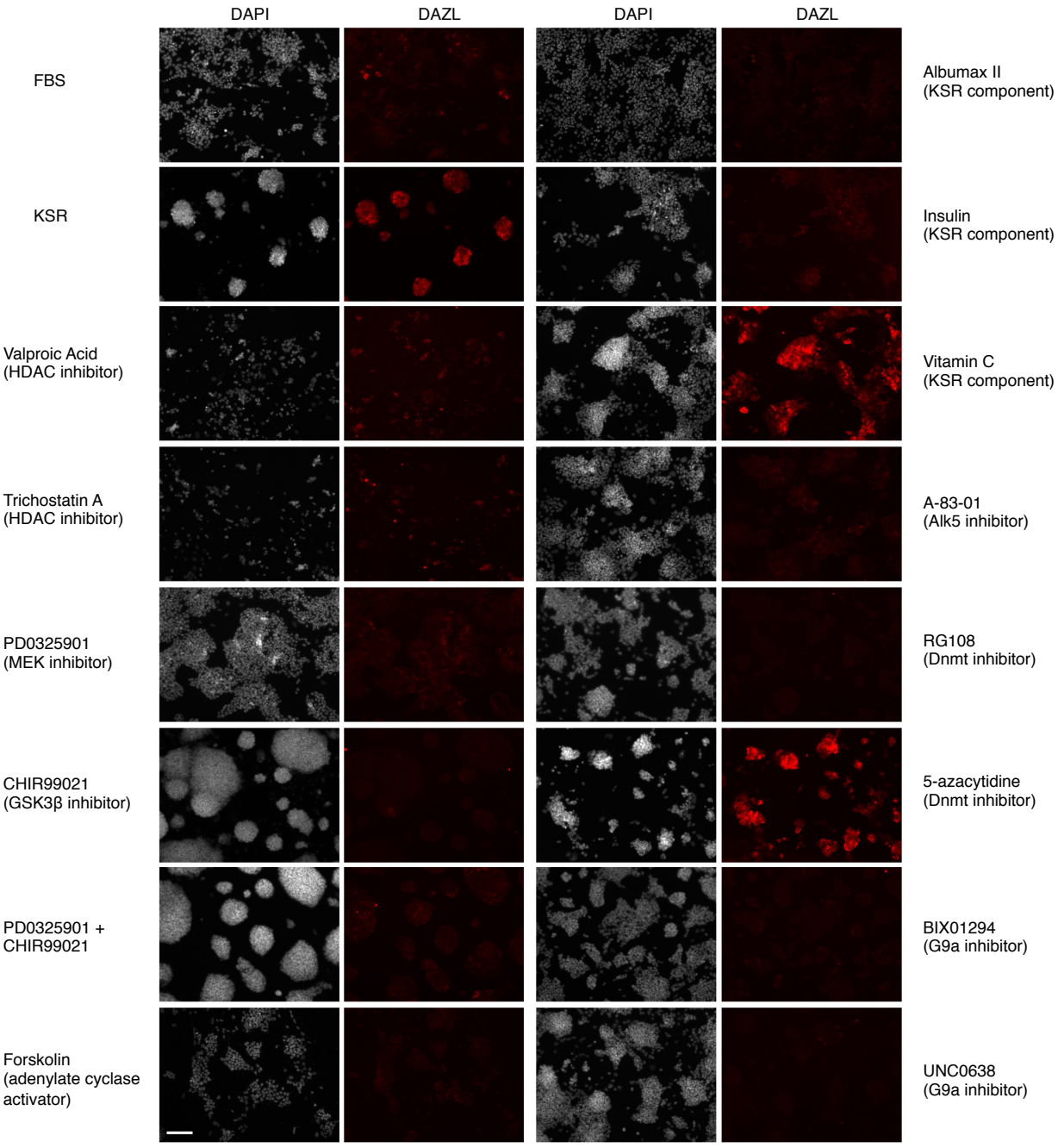
doi:10.1038/nature12362



Supplementary Figure 1: Knockout serum replacement (KSR) induces DAZL expression in ES cells. a, b, Immunofluorescence for DAZL (red). **a,** DAZL is expressed in ES cells maintained in KSR but not FBS medium. **b,** DAZL expression is reversible. ES cells maintained in KSR were transferred to FBS medium for 5 days and then returned to KSR for an additional 5 days. DAZL is decreased with FBS medium, but is re-expressed when cells are returned to KSR medium. Scale bar is 80 μm in panel a, and 100 μm in panel b.

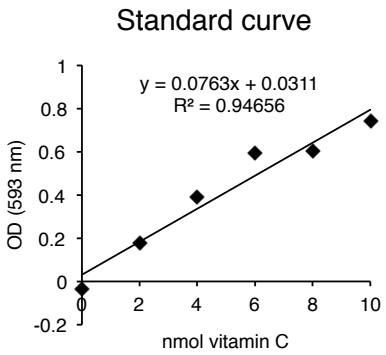


Supplementary Figure 2: Small molecule screen for factors that induce DAZL expression in ES cells. Immunofluorescence for DAZL (red). ES cells maintained in FBS medium were treated with factors for 3 - 6 days. DAZL expression is induced upon vitamin C or 5-azacytidine treatment. Scale bar is 200 µm.

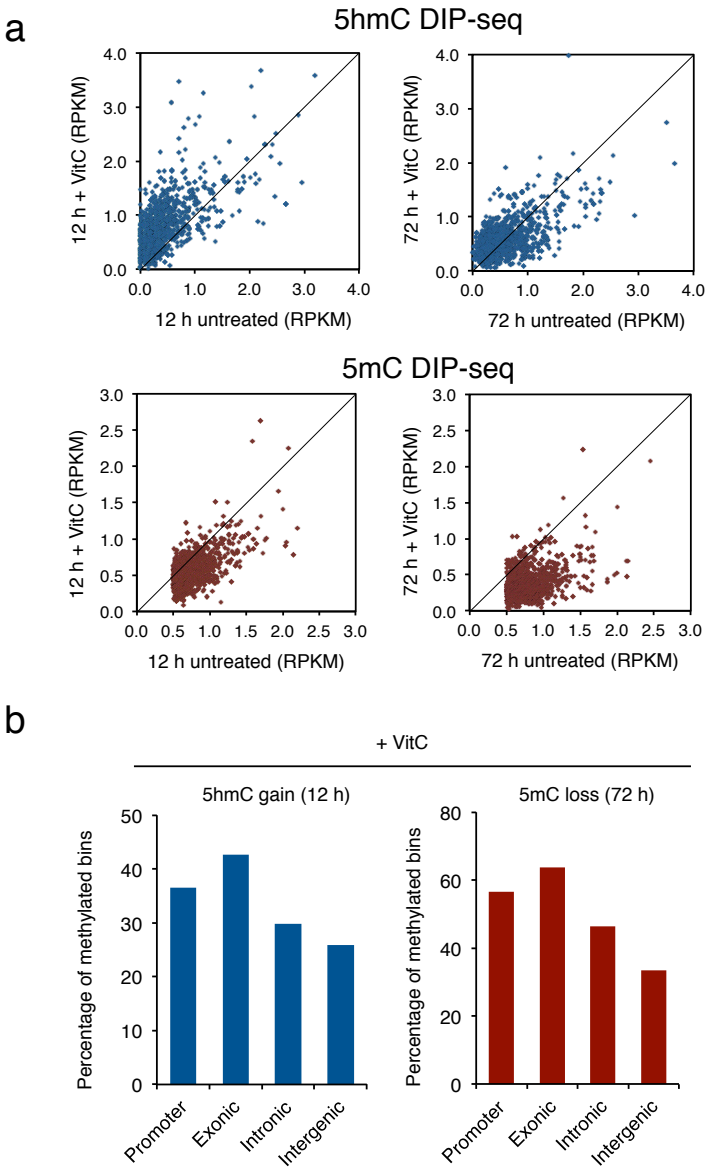
Vitamin C quantification

		Concentration of vitamin C added		
	Medium alone	2.8 µM	13.8 µM	69 µM
Calculated concentration of vitamin C (µM)	-0.03*	1.47*	10.21	46.96

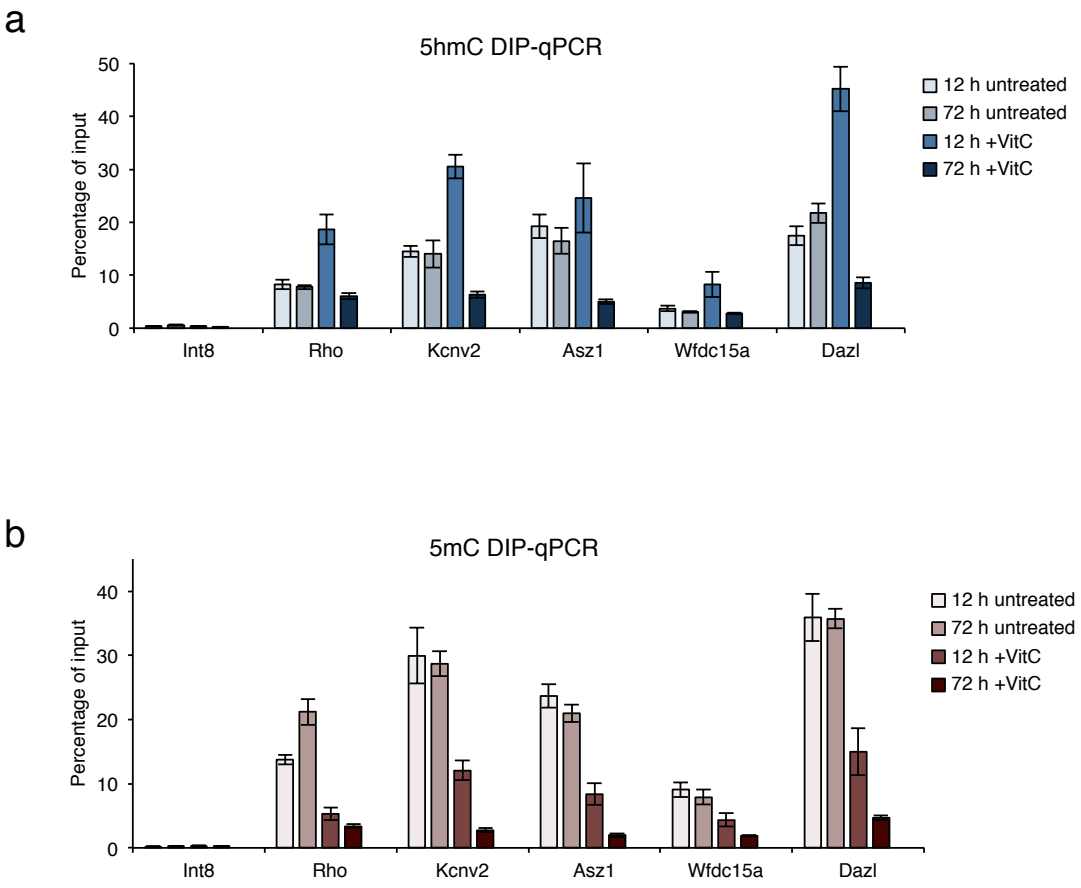
**Values are below the detection limit of the assay, which is 2 µM*



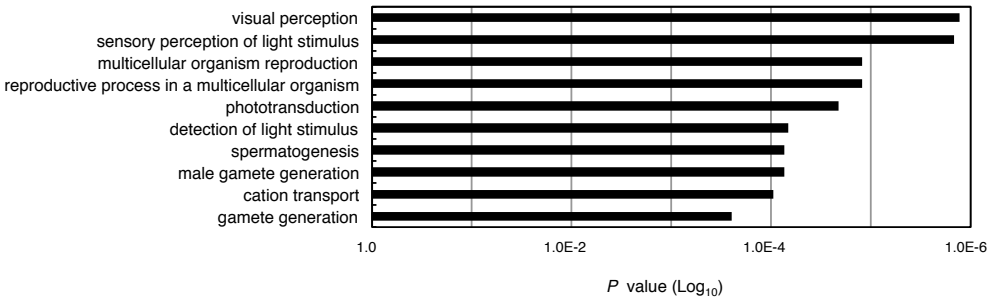
Supplementary Figure 3: 2i medium does not contain any detectable vitamin C. Vitamin C was quantified in the 2i culture medium used in this study. We confirm that there is no detectable vitamin C in the medium. Known concentrations of vitamin C were added to the medium as controls. The standard curve used to calculate vitamin C concentration is shown.



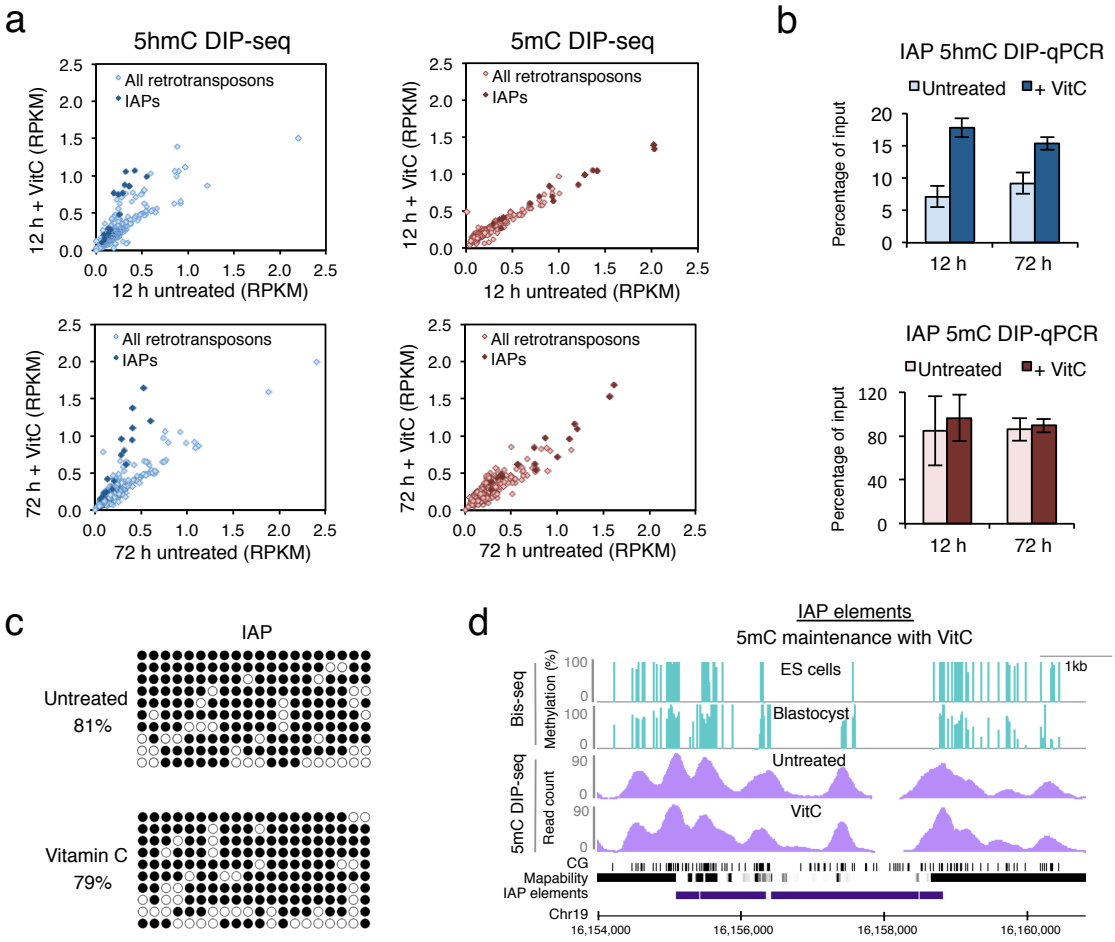
Supplementary Figure 4: Vitamin C induces a genome-wide increase in 5hmC at 12 h and decrease in 5mC at 72 h. **a,** 5mC DIP-seq RPKM values were used to identify methylated promoters in untreated ES cells (transcription start site ± 500 bp, RPKM >0.5 in both 12 and 72 h untreated). Methylated promoters were plotted comparing untreated versus vitamin C-treated at 12 and 72 h for 5hmC (blue) and 5mC (red) levels. VitC, vitamin C. **b,** Graphs show percentage of methylated bins (5mC DIP-seq RPKM >0.5) that gain 5hmC by at least twofold at 12 h (blue) or lose 5mC by at least twofold at 72 h (red) within each genomic feature after vitamin C treatment. DIP-seq genome coverage was binned using sliding 1,000 bp windows with 500 bp offset between windows.



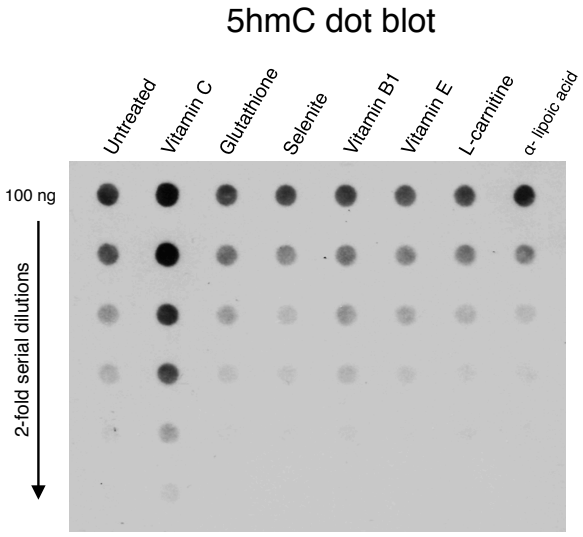
Supplementary Figure 5: Vitamin C induces a transient increase in 5hmC and a progressive decrease in 5mC at gene promoters. a, b, DIP-qPCR at 12 and 72 h after vitamin C treatment for 5hmC (a) and 5mC (b), respectively. An intergenic region on chromosome 8 (Int8) is included as a negative control. Data are expressed as percent input ± s.d., $n = 3$ technical replicates.



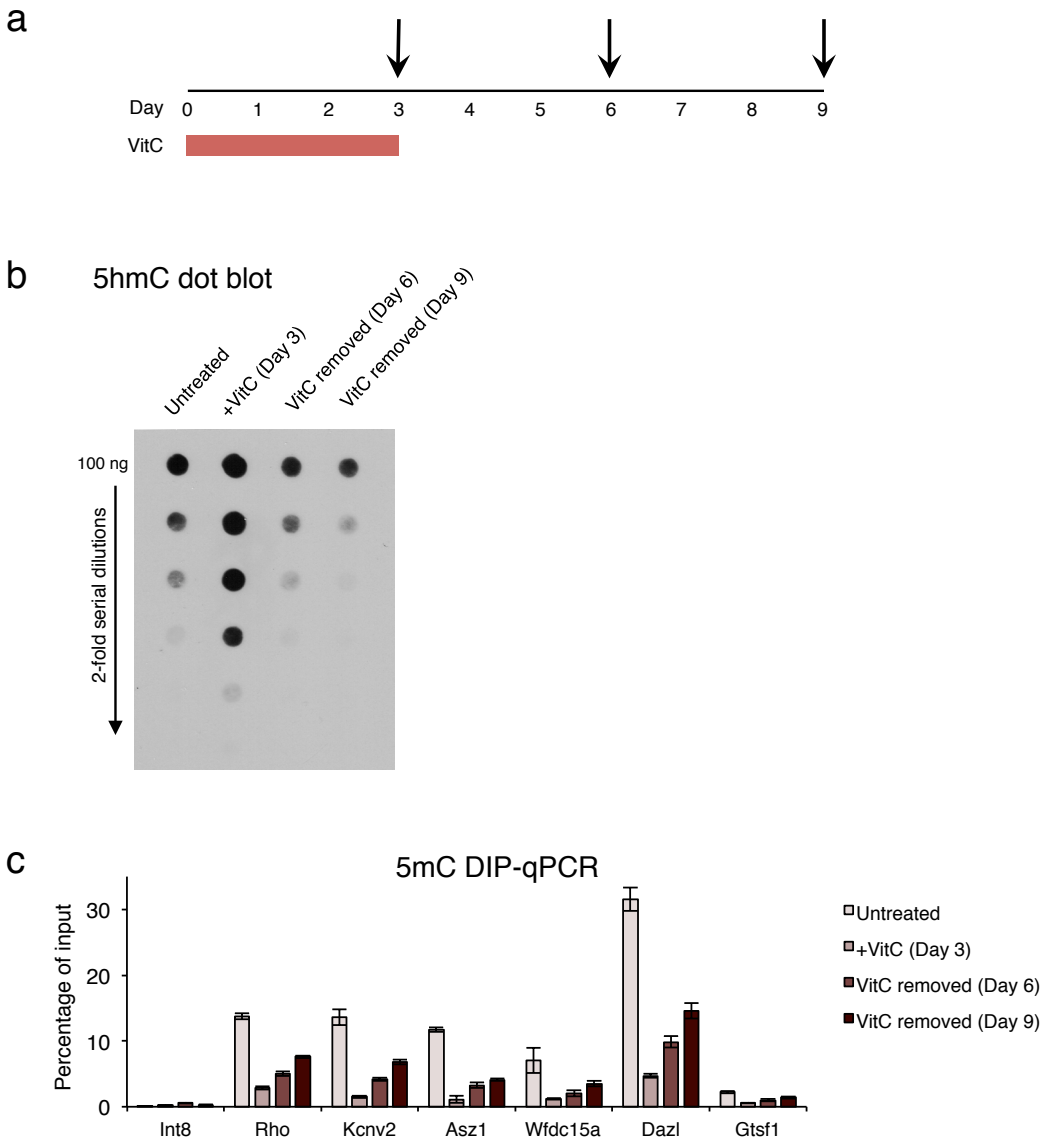
Supplementary Figure 6: Gene ontology analysis of promoters that gain 5hmC at 12 h or lose 5mC at 72 h in the presence of vitamin C. Methylated promoters that gain 5hmC at 12 h or lose 5mC at 72 h ($n = 803$) with vitamin C treatment show an enrichment for germline and vision-associated gene ontology terms.



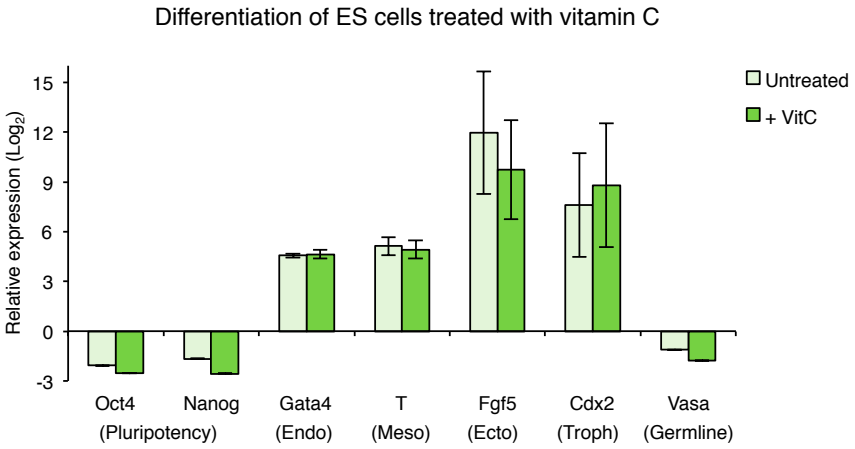
Supplementary Figure 7: IAP retrotransposons are resistant to vitamin C-induced demethylation. **a,** 5hmC and 5mC DIP-seq RPKM values at retrotransposons were plotted for untreated versus vitamin C-treated. Only the IAP subfamily of retrotransposons gains 5hmC at 12 h and maintains 5hmC at 72 h in response to vitamin C (left). The retention of 5hmC at IAPs at 72 h may account for the global increase in 5hmC observed at 72 h by immunofluorescence and dot blot (Fig. 1a, b). All retrotransposons maintain 5mC following vitamin C treatment (right). **b,** 5hmC (top) and 5mC (bottom) DIP-qPCR was performed for IAP retrotransposons at 12 and 72 h ± vitamin C treatment to validate the DIP-seq data. IAPs are resistant to vitamin C-induced demethylation despite an observed increase in 5hmC. It is likely that the observed increase in 5hmC occurs in only a small fraction of methylated CpGs within IAP elements, resulting in no detectable loss of overall methylation by DIP. Data are expressed as percent input ± s.d., $n = 3$ technical replicates. **c,** Bisulphite sequencing of IAP elements shows no significant difference in 72 h untreated and vitamin C-treated samples. Open circles, unmethylated; closed circles, methylated. **d,** Genomic region containing IAP elements that are similarly methylated in ES cells and the blastocyst and are resistant to vitamin C-induced demethylation. Bis-seq data are from ref. 13. Lack of methylation signal within IAP elements is due to poor mapability.



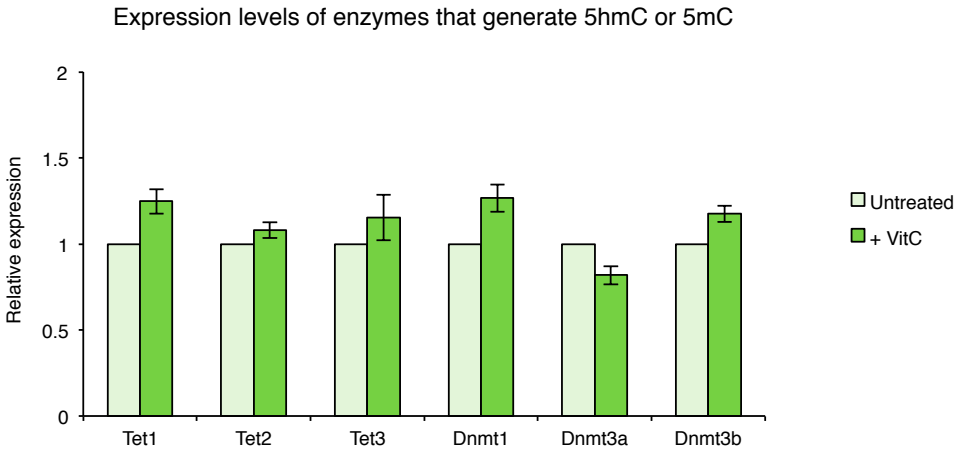
Supplementary Figure 8: Other antioxidants do not increase 5hmC levels in ES cells. ES cells treated for 24 h with vitamin C or other antioxidants, followed by global 5hmC analysis by dot blot.



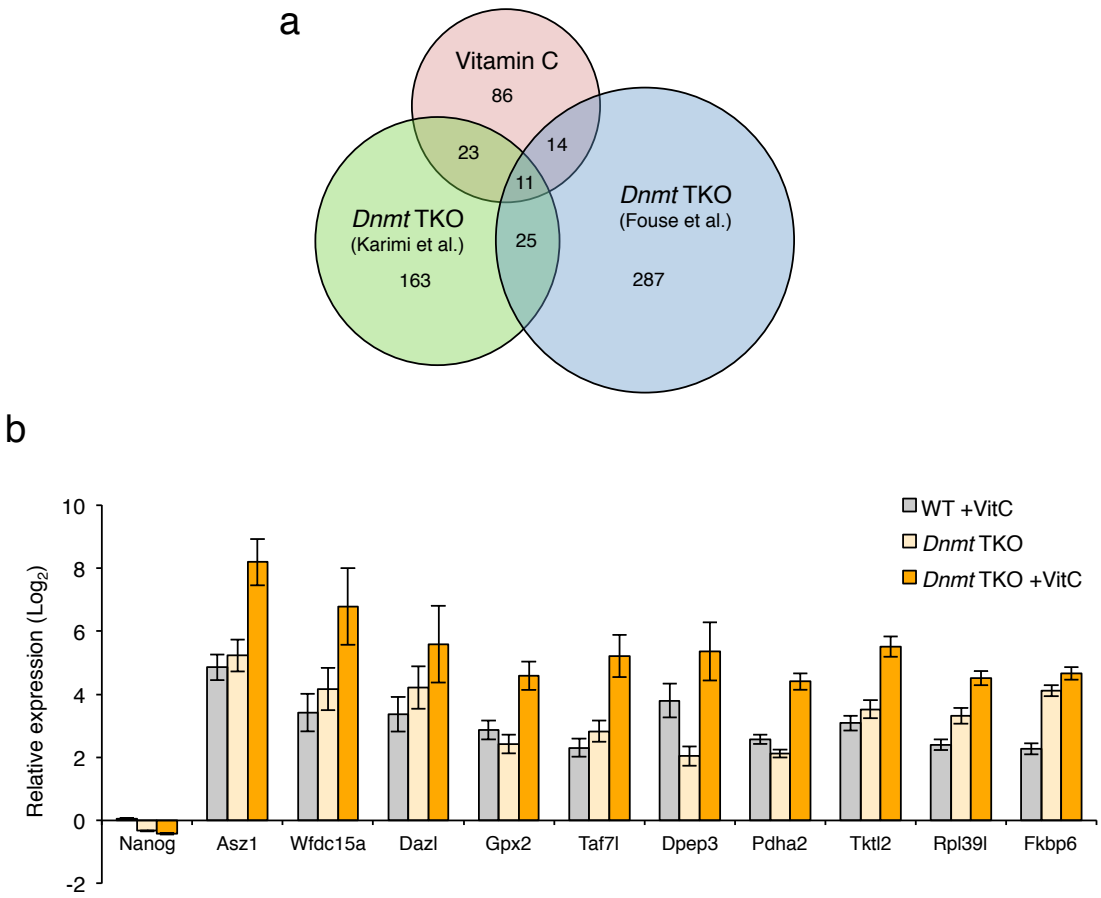
Supplementary Figure 9: The effects of vitamin C are reversible. a, Timeline of vitamin C treatment. Vitamin C was added for 3 days and then removed for 6 days. Arrows indicate time points of analysis. **b,** Dot blot for 5hmC. 5hmC is increased at day 3 of vitamin C treatment and levels return to baseline following vitamin C removal. **c,** 5mC DIP-qPCR. 5mC is reduced on day 3 of vitamin C treatment and levels increase progressively following vitamin C removal. An intergenic region on chromosome 8 (Int8) is included as a negative control. Data are expressed as percent input ± s.d., *n* = 3 technical replicates.



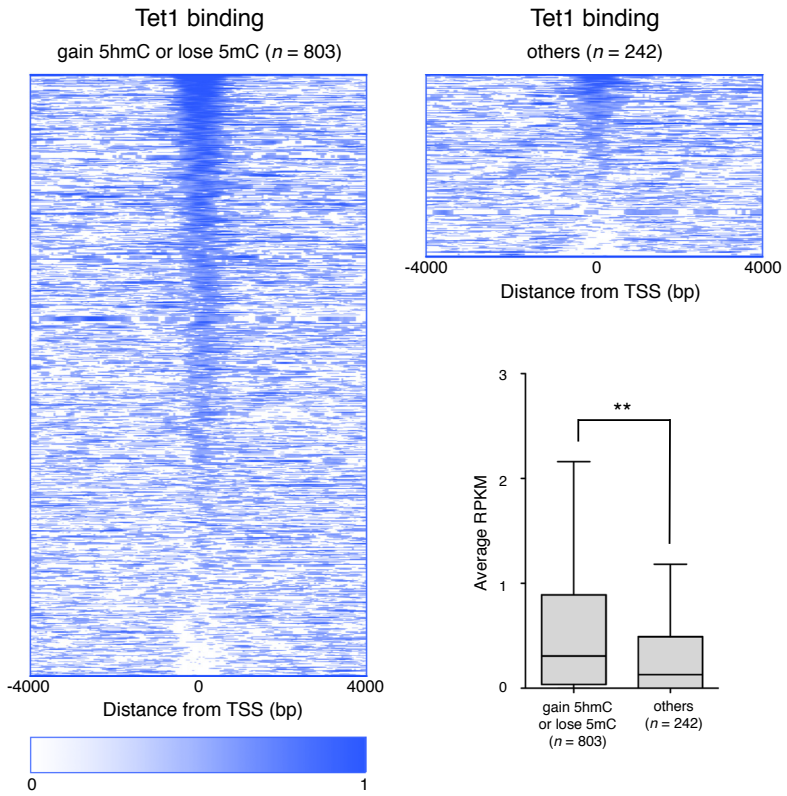
Supplementary Figure 10: ES cells treated with vitamin C show normal *in vitro* differentiation. qRT-PCR analysis of day 5 embryoid bodies generated from untreated or vitamin C-treated ES cells. Data expressed as Log_2 fold relative to untreated ES cells. ES cells treated with vitamin C down-regulate pluripotency markers and up-regulate markers of all three germ layers upon embryoid body differentiation to similar levels as untreated ES cells. Error bars represent s.e.m., $n = 3$ technical replicates. (Endo, endoderm; Meso, mesoderm; Ecto, ectoderm; Troph, trophectoderm)



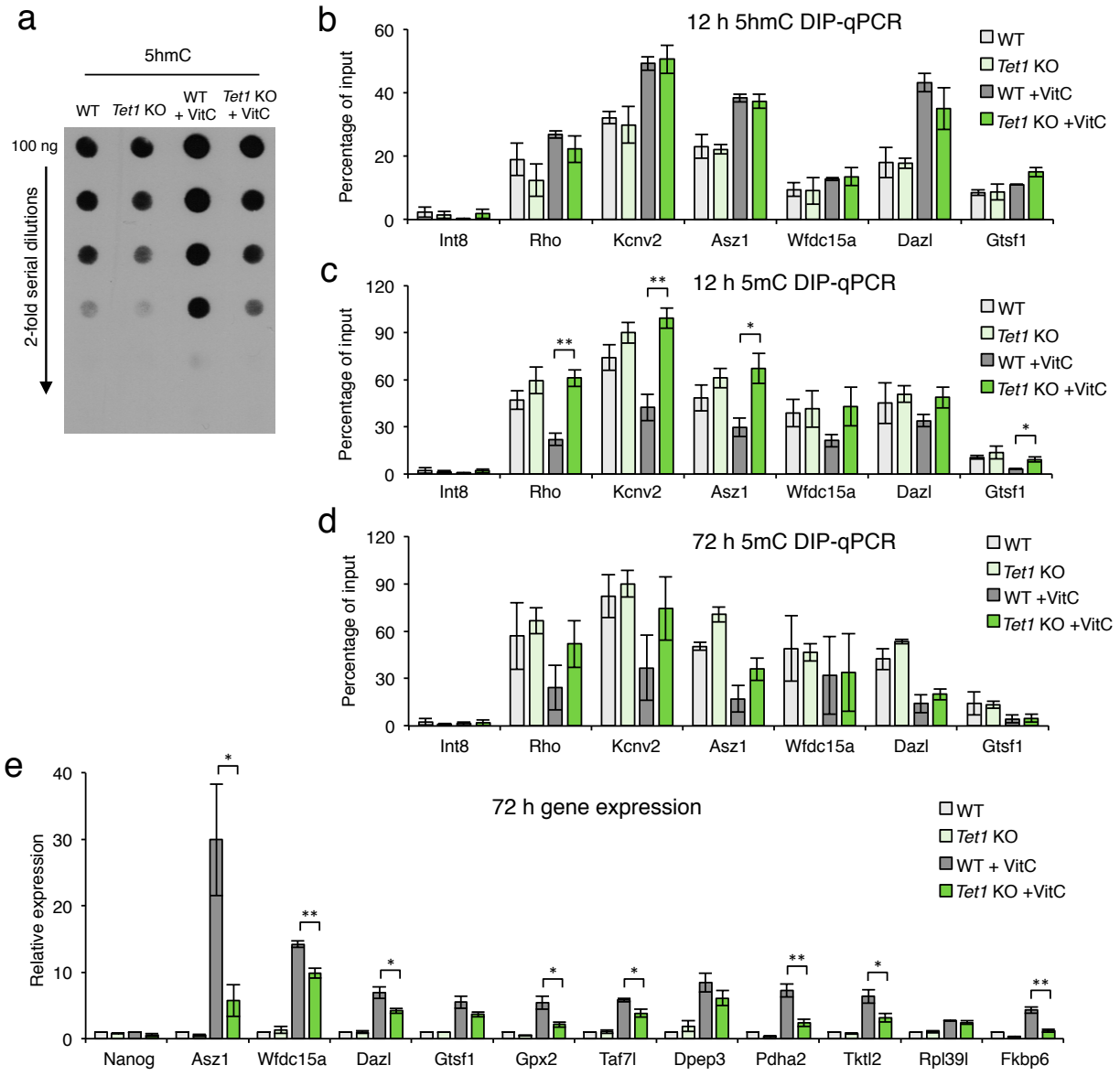
Supplementary Figure 11: Vitamin C does not alter expression of Tet or Dnmt genes. qRT-PCR analysis of ES cells treated with vitamin C for 72 h. Vitamin C-induced increase in 5hmC and decrease in 5mC cannot be explained by altered expression of Tet or Dnmt family members. Data expressed as fold relative to untreated ES cells. Error bars represent s.e.m., $n = 3$ technical replicates.



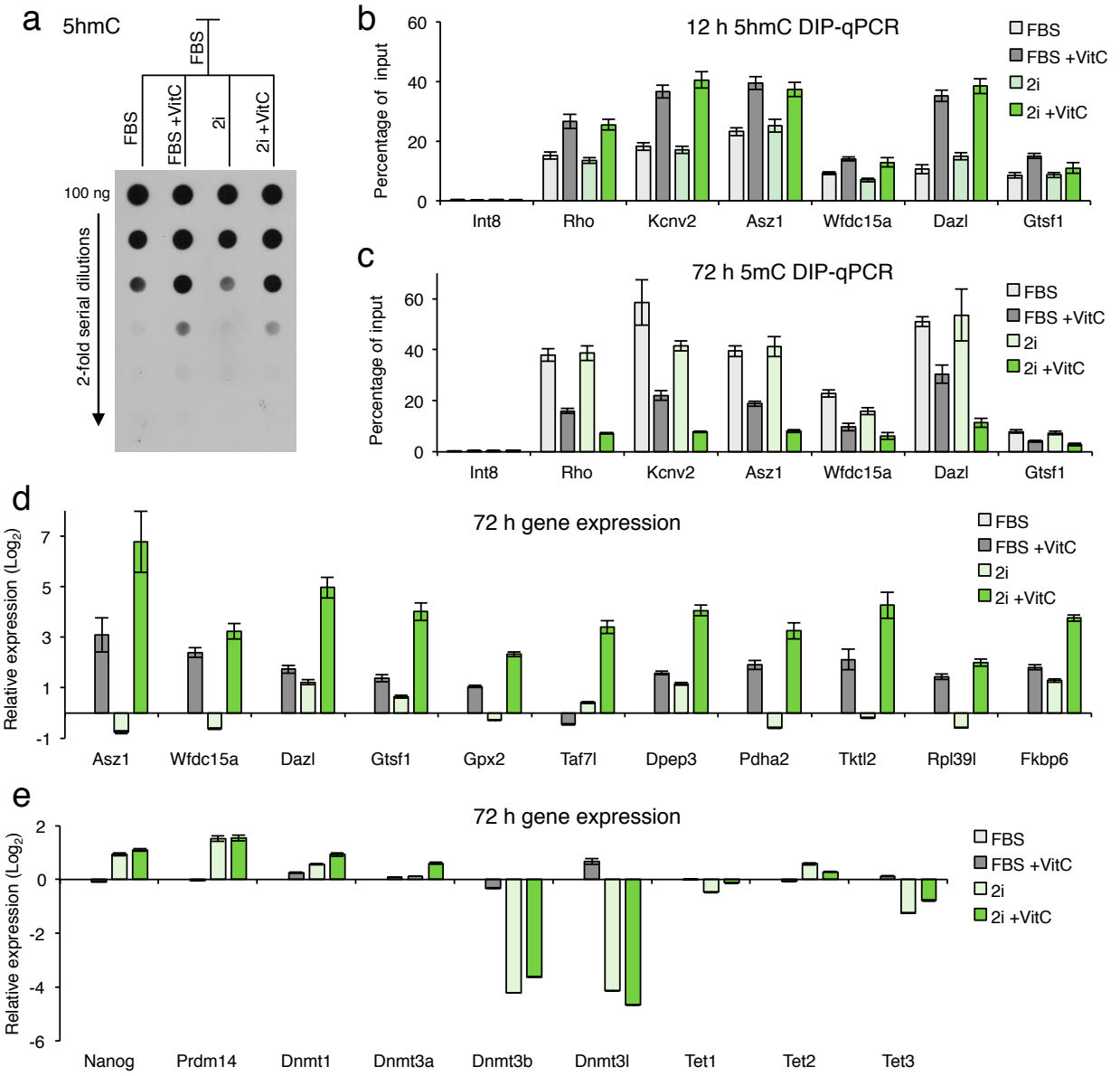
Supplementary Figure 12: Comparison of gene expression in *Dnmt* triple knockout and vitamin C-treated ES cells. **a,** 48/134 (36%) of vitamin C up-regulated genes are also up-regulated in *Dnmt* triple knockout cells. TKO, triple knockout; WT, wild-type. **b,** qRT-PCR analysis for germline genes in wild-type and *Dnmt* triple knockout cells ± vitamin C. Data are expressed as Log_2 fold relative to untreated wild-type cells. Vitamin C-induced germline genes are also up-regulated in *Dnmt* triple knockout cells. Germline genes up-regulated in *Dnmt* triple knockout cells are further induced in response to vitamin C. Nanog, whose expression is not expected to change, is included as a control. Error bars represent s.e.m., $n = 3$ technical replicates.



Supplementary Figure 13: Tet1 binding is enriched near the transcription start site (TSS) of promoters affected by vitamin C. Tet1 ChIP-seq data (C-term) analysed from a published data set (ref. 10). The heatmaps show Tet1 RPKM binding values ± 4,000 bp from the TSS for two categories: 1) methylated promoters that gain 5hmC at 12 h or lose 5mC at 72 h (n = 803) and 2) methylated promoters that do not gain 5hmC at 12 h or lose 5mC at 72 h (others) (n = 242). Methylated promoters that gain 5hmC at 12 h or lose 5mC at 72 h with vitamin C are enriched for Tet1 binding compared to promoters that do not display these vitamin C-induced changes. Box plot of Tet1 RPKM values (± 500 bp from the TSS) shows a significantly higher level of Tet1 binding at promoters that gain 5hmC at 12 h or lose 5mC at 72 h of vitamin C treatment compared to the others category (**P < 0.01, by t-test). Box plot has Tukey whiskers, a line for the median, and edges for the 25th and 75th percentiles.



Supplementary Figure 14: The effects of vitamin C are partially Tet1-dependent. **a,** Dot blot for 5hmC shows that *Tet1* knockout cells do not increase 5hmC to the same extent as wild-type cells in response to a 12 h vitamin C treatment. KO, knockout; WT, wild-type. **b - d,** DIP-qPCR for 5hmC (b) and 5mC (c, d) performed on *Tet1* knockout cells ± vitamin C. Tet1 knockout cells increase 5hmC at gene promoters to a similar level as wild-type following 12 h vitamin C treatment. Vitamin C-treated *Tet1* knockout cells show a significant retention of methylation at a subset of gene promoters compared to wild-type cells at 12 h (c), but undergo some demethylation after 72 h in vitamin C (d). An intergenic region on chromosome 8 (Int8) is included as a negative control. **e,** Germline gene expression in wild-type and *Tet1* knockout cells at 72 h of vitamin C treatment. Data are expressed relative to wild-type untreated sample. *Tet1* knockout cells show an attenuated up-regulation of germline genes upon vitamin C treatment. $n = 3$ biological replicates except for 72 h 5mC DIP-qPCR which is $n = 2$ biological replicates. Data are represented as mean ± s.e.m.*$P < 0.05$, **$P < 0.01$ by *t*-test throughout the figure.



Supplementary Figure 15: **Vitamin C and 2i promote DNA hypomethylation via distinct mechanisms.** ES cells maintained in FBS medium were cultured ± vitamin C or transferred to 2i medium and cultured ± vitamin C for 12 or 72 h. **a,** Dot blot for 5hmC at 12 h shows that ES cells grown in both FBS and 2i media increase 5hmC in response to vitamin C. **b, c,** DIP-qPCR for 5hmC at 12 h (b) and 5mC at 72 h (c) performed on ES cells grown in FBS or 2i medium ± vitamin C. Vitamin C induces a gain of 5hmC at 12 h and a loss of 5mC at 72 h in both media conditions. An intergenic region on chromosome 8 (Int8) is included as a negative control. **d,** Germline gene expression is increased at 72 h of vitamin C treatment in both media conditions. Data are expressed relative to FBS untreated sample. **e,** 2i, but not vitamin C, increases expression of naïve pluripotency genes and decreases expression of Dnmt3b and Dnmt3l after 72 h. Data are expressed relative to FBS untreated sample. The effects of vitamin C are greatest in 2i medium, likely due to the combination of enhanced Tet activity induced by vitamin C and reduced Dnmt3b and Dnmt3l expression induced by 2i medium. DIP-qPCR data are represented as mean ± s.d. and expression data are represented as mean ± s.e.m., $n = 3$ technical replicates.

Supplementary Table 5: Expression of vitamin C-induced genes in ES cells cultured in the absence of vitamin C and inner cell mass (ICM) using reads per million (RPM) from published single cell RNA-seq (ref. 23). Several vitamin C-induced genes are expressed in the ICM. Genes in bold were analysed by qRT-PCR in Fig. 4f.

Gene Name	ES cells (RPM)	ICM (RPM)
Asz1	0.00	45.59
Wfdc15a	2.48	35.95
Fkbp6	1.52	17.99
Sycp1	6.65	18.42
Rpl39l	105.42	299.56
Dazl	13.27	13.19
Gpx2	0.92	2.20
Dpep3	0.69	2.67
Pdha2	0.13	0.21
Taf7l	3.88	0.07
Tktl2	0.00	0.04

Supplementary Table 6: qRT-PCR, DIP-qPCR, and bisulphite primers.

Gene Name	Forward (5' to 3')	Reverse (5' to 3')
qRT-PCR		
Asz1	GAG TGG GCT TCT CCC AGA AA	GGT CAT TTT CCC GCT CAT TC
Cdx2	CCT GCG ACA AGG GCT TGT TTA G	TCC CGA CTT CCC TTC ACC ATA C
Dazl	CAA CTG TTA ACT ACC ACT GCA G	CAA GAG ACC ACT GTC TGT ATG C
Dnmt1	AGA ACC ACC AGG CAG ACC AC	CCC CTC TTC CGA CTC TTC CT
Dnmt3a	ACC AGC AGC TCC TCT CTG GA	CTC TTC CTT GCC ACG GTT CT
Dnmt3b	CAA TCT GCA CAG AGC CAG TC	GGC TGG AGA CCT CCC TCT TA
Dnmt3l	TCT TCC TCA TCC CCA AAG GA	GTG ACA GCA GGG TCG TCA GA
Dpep3	CGG CTA GAT CGG TGT GTG AC	AGC ACC CCC ATG GAT AGT GT
Fgf5	CTG TAT GGA CCC ACA GGG AGT AAC	ATT AAG CTC CTG GGT CGC AAG
Fkbp6	CGG CTG ATG AAA CTT GGA GA	AGG CTG GCT TGA ACA GGA AC
Gata4	CAG CCT GCC TGG ACG G	GCC TTC TGA GAA GTC ATC AAA CAT AT
Gtsf1	GAC TCC CTG GAC CCT GAA AA	GCC AAT TTG TTT GCG ACA TC
Gpx2	TGA GCT GCA ATG TCG CTT TC	CCC CAG GTC GGA CAT ACT TG
L7	AGC GGA TTG CCT TGA CAG AT	AAC TTG AAG GGC CAC AGG AA
Nanog	AGG CTT TGG AGA CAG TGA GGT GC	TAC CCT CAA ACT CCT GGT CCT TC
Oct4	AGT CTG GAG ACC ATG TTT CTG AAG T	TAC TCT TCT CGT TGG GAA TAC TCA ATA
Pdha2	AAG GGC AGG TAT TCG AAG CA	CTC GTT GGA GGT TCC CAT TC
Prdm14	GCA CAC AGG GAC AAC TCT CG	CAG TTC CCA GAA CCT TTG CC
Rpl39l	TTT AAA CTC GCC GGG AAG AG	TGT GGA ATG GGA CGA TTT TG
Sycp1	TTC ATA AAG GAG CGC ACG CG	TTC TCC ATG CTG CCT CCT GG
T	CTC TAA TGT CCT CCC TTG TTG CC	TGC AGA TTG TCT TTG GCT ACT TTG
Taf7l	TGT CAT GAC GTT GAG GAA CAG T	ATC CTT CCA AGC AGC ATT TCT
Tet1	GAA GCT GCA CCC TGT GAC TG	GAC AGC AGC CAC ACT TGG TC
Tet2	AAG CTG ATG GAA AAT GCA AGC	GCT GAA GGT GCC TCT GGA GT
Tet3	TCA CAG CCT GCA TGG ACT TC	ACG CAG CGA TTG TCT TCC TT
Tktl2	AAA GTG CCA AAG CCA CAG GT	AGC TTC ACC AAT GCC ACC TT
Ubb	GCG GTT TGT GCT TTC ATC AC	GGC AAA GAT CAG CCT CTG CT
Vasa	TGT GCC TCC CAG CTT CAG TA	TAT TCA ACG TGT GCT TGC CC
Wfdc15a	TGT GTG GAA CCC TGG ACA AC	GCC AAT GCC GTC GTT ATT TT
DIP-qPCR		
Asz1	CCT CAC TAT CGC TGC TCT CG	CGC TCG CTC AAG CTC TGA TA
Dazl	TAC AAA ATG CCC GCA GAA ATA G	CCG GAC TCA ACC TTC TCA ATG
Gtsf1	TTC CTG TGA CTG TGG CTT GC	GGA GGG TGA GCC AAA GAA AA
IAP	TAT GCC GG GGT GGT TCT CTA	TGC GGC AAA ACT TTA TTG CTT
Intergenic Chr8	AAG GGG CCT CTG CTT AAA AA	AGA GCT CCA TGG CAG GTA GA
Kcnv2	GAG TGA GGC TCA AAT ACA CGC C	TCT TCA GTT GCT CGC TCA GTT C
Rho	ACC GTA CAG CAC AAG AAG CTG C	GAA GAC CAT GAA GAG GTC AGC C
Wfdc15a	GGG AGG ACG TTT GAA TCT GC	GCA CTT CCG TTT TCC TGA CC
Bisulfite		
Asz1 (1st round)	GGT TGT TTT TGT TTT GGT TTG TAA	TAA AAT ATA ACC CCA TCA AAT TCC
Asz1 (2nd round)	TTG GTT GGT TTA ATA ATT TGA AAT A	TAA AAT ATA ACC CCA TCA AAT TCC
Dazl (1st round)	GAT TTT TGT TAT TTT TTA GTT TTT TTA GGA T	AAA ATT CTC TCA ACT AAC CTA ACT TAT TTC T
Dazl (2nd round)	GTT YGA GTT TTA TTG ATA GAT AGA TGG AT	ACT AAC CTA ACT TAT TTC TAT AAA ACC TAC
IAP (1st round)	GGY GTT GAT AGT TGT GTT TTA AGT GGT AAA T	ATT CTA ATT CTA AAA TAA AAA ATC TTC CTT A
IAP (2nd round)	GAT AGT TGT GTT TTA AGT GGT AAA TAA ATA	ATT CTA ATT CTA AAA TAA AAA ATC TTC CTT A
Kcnv2 (1st round)	AGA TTG AGG ATG TGT TTA ATA TTG G	ACT CCC TTT AAA TCT TCA ATT ACT C
Kcnv2 (2nd round)	TTT ATA AGT TTT TTG GAT GGT TAT AA	ACT CCC TTT AAA TCT TCA ATT ACT C