Intracellular α-ketoglutarate maintains the pluripotency of embryonic stem cells

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The role of cellular metabolism in regulating cell proliferation and differentiation remains poorly understood. For example, most mammalian cells cannot proliferate without exogenous glutamine supplementation even though glutamine is a non-essential amino acid. Here we show that mouse embryonic stem (ES) cells grown under conditions that maintain naive pluripotency are capable of proliferation in the absence of exogenous glutamine. Despite this, ES cells consume high levels of exogenous glutamine when the metabolite is available. In comparison to more differentiated cells, naive ES cells utilize both glucose and glutamine catabolism to maintain a high level of intracellular a-ketoglutarate (αKG). Consequently, naive ES cells exhibit an elevated αKG to succinate ratio that promotes histone/DNA demethylation and maintains pluripotency. Direct manipulation of the intracellular αKG/succinate ratio is sufficient to regulate multiple chromatin modifications, including H3K27me3 and ten-eleven translocation (Tet)-dependent DNA demethylation, which contribute to the regulation of pluripotency-associated gene expression. In vitro, supplementation with cell-permeable αKG directly supports ES-cell self-renewal while cell-permeable succinate promotes differentiation. This work reveals that intracellular αKG/succinate levels can contribute to the maintenance of cellular identity and have a mechanistic role in the transcriptional and epigenetic state of stem cells.

Mouse ES cells can be maintained in two medium formulations: a serum-free medium reported to support a cellular phenotype that mimics ‘naive’ epiblast cells of the inner cell mass (containing GSK-3β and MAPK/ERK inhibitors (2i)/leukaemia inhibitory factor (LIF), hereafter 2i/L); or a serum-based medium that supports the proliferation of a more committed ES cell phenotype (serum/LIF, hereafter S/L)++1. To characterize ES cell metabolism, we investigated whether cells cultured in these two media have different requirements for glucose and/or glutamine. ES cells cultured in either medium proliferated at equivalent rates when glucose and glutamine were abundant and cells cultured with or without 2i were unable to proliferate in the absence of glucose (Extended Data Fig. 1a, b). In contrast, cells cultured in 2i/L, but not S/L, proliferated robustly in the absence of exogenous glutamine (Fig. 1a and Extended Data Fig. 1c). Likewise, four newly derived ES-cell lines (ESC-1–4) exhibited convincing glutamine-independent proliferation in 2i/L medium while retaining features of pluripotent cells, including ES-cell-like morphology, reactivity to alkaline phosphatase (AP) and the ability to form teratomas (Fig. 1b, c and Extended Data Fig. 1d). Cells cultured in 2i/L medium alone could also proliferate in the absence of exogenous glutamine (Extended Data Fig. 1e).

This effect was not due to differences in medium nutrient formulations as supplementing S/L medium with the GS-3β and MAPK/ERK inhibitors present in 2i also enabled glutamine-independent proliferation while maintaining ES cell morphology and markers of pluripotency (Fig. 1d, e). An alternative ES-cell medium containing BMP4 and LIF added to the same serum-free formulation as in 2i/L++1 failed to support glutamine-independent growth (Fig. 1f). Likewise, epiblast stem cells (EpiSCs) could not proliferate in the absence of exogenous glutamine (Extended Data Fig. 1f, g). However, the ability to undertake glutamine-independent growth was not limited to embryonic pluripotency; fibroblast-derived induced pluripotent cells (iPSCs) were also able to proliferate in glutamine-free 2i/L medium (Extended Data Fig. 1h). These results indicate that the GSK-3β and MAPK/ERK inhibitors in 2i-containing medium are both necessary and sufficient to enable proliferation of pluripotent cells in the absence of exogenous glutamine.

The fact that cells proliferated in the absence of exogenous glutamine in 2i/L medium, albeit at a slower rate than cells cultured in glutamine-replete medium (Extended Data Fig. 1i), indicates that these cells must be capable of de novo glutamine synthesis. Indeed, chemical inhibition of glutamine synthase was sufficient to block proliferation of cells in glutamine-free 2i/L medium (Extended Data Fig. 1j). Likewise, addition of cell-permeable dimethyl-α-ketoglutarate (DM-αKG), a precursor for glutamine synthesis, was sufficient to enable glutamine-independent proliferation in both S/L and 2i/L conditions (Extended Data Fig. 1k), suggesting that the supply of precursors for glutamine synthesis determines the ability of ES cells to proliferate in the absence of glutamine.

In support of this model, cells cultured in 2i/L preserved larger intracellular pools of glutamate after glutamine withdrawal than cells cultured in S/L (Fig. 1g). These results suggest that 2i/L cells can generate glutamate from αKG, a precursor for glutamine synthesis. Indeed, αKG significantly increased glutamine synthesis in 2i/L cells (Extended Data Fig. 1h).

**Figure 1 | 2i is necessary and sufficient to confer glutamine independence.**

a–f, Growth curves and representative images of ES cells grown in the absence of glutamine (Q). a, b, Growth curves of ESC-V19 cells (a) and V6.5 ES-cell lines (ESC–1–4) (b) cultured in glutamine-free S/L or 2i/L medium. c, e, Phase images showing ESC–1 cells cultured in glutamine-free 2i/L (c) or S/L/2i (e) medium for 3 days. Top, brightfield (BF); bottom, AP staining. Scale bars, 500 μm. d, Growth curve of ESC-V19 cells in glutamine-free S/L or S/L/2i medium. f, Growth curve of ESC-V19 cells cultured without glutamine in two serum-free media formulations containing N2 and B27 supplements, 2i/L, and BMP4/L. g, Intracellular glutamate levels 8 h after addition of medium with or without glutamine. a.u., arbitrary units. Data are presented as the mean ± standard deviation (s.d.) of triplicate wells from a representative experiment.

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glutamate (and glutamine) from carbon sources other than glutamine itself.

Despite their different growth requirements, cells cultured in both S/L and 2i/L consumed high levels of glucose and glutamine and excreted similar levels of lactate, consistent with the metabolic profile of most proliferating cells, including cancer cells and pluripotent cells (Fig. 2a). Oxidation of glucose and glutamine via the mitochondrial tricarboxylic acid (TCA) cycle provides a critical source of the biosynthetic precursors required for cell proliferation. With the exception of αKG, steady-state levels of TCA cycle metabolites were reproducibly diminished in ES cells cultured in 2i/L (Fig. 2b).

In most cells, glutamine is catabolized to αKG to support TCA cycle anaplerosis (Fig. 2c). ES cells grown in S/L medium exhibited high levels of αKG and malate (Fig. 2d). In contrast, a substantial fraction of these metabolites failed to become labeled with glutamine in ES cells grown in 2i/L. Instead, there was a rapid labeling of these three metabolite pools from [U-13C]glucose (Fig. 2e). Quantification of metabolite fluxes revealed that although the flux of glutamine-derived carbon through αKG was similar in both conditions, glutamine entry through malate was significantly diminished in cells cultured in 2i/L, indicating that the entry of glutamine-derived αKG into the TCA cycle is repressed by culture in 2i/L (Fig. 2f). Instead, KG/succinate ratio was robustly elevated by 2i/L in every ES-cell line tested (Fig. 2a). Cellular αKG/succinate ratios have been implicated in the regulation of the large family of αKG-dependent dioxygenases14. As Jumonji C (JmjC)-domain-containing histone demethylases and the Tet family of DNA demethylases comprise a major subset of these enzymes, the elevated ratio of αKG/succinate observed in cells grown in 2i/L medium could have important implications for the regulation of chromatin structure.

Since αKG was largely derived from glutamine metabolism (Fig. 2d), we tested whether glutamine deprivation affected histone lysine methylations known to be regulated in part by αKG-dependent demethylases15. Cells cultured in glutamine-free medium exhibited increases in trimethylation and decreases in monomethylation on H3K9, H3K27, H3K36 and H4K20, whereas H3K4 methylations remained unchanged (Fig. 3b). DM-αKG reversed the increase in H3K27me3 and H4K20me3 observed in glutamine-deficient medium (Extended Data Fig. 3a), confirming that these changes could be accounted for by the decline in glutamine-dependent or derived from [U-13C]glucose (13C-Glc) (e) over time (0–12 h). Mean ± standard error of the mean (s.e.m.) of three independent experiments are shown. f, g, Glutamine (f) and glucose (g) flux through αKG and malate pools. Mean ± s.e.m. of flux calculated for three independent experiments (shown in d, e) are shown. *P < 0.05, **P < 0.005, ***P < 0.0005. P values were determined by unpaired two-tailed Student’s t-tests.

Figure 2 | 2i/L alters glucose and glutamine utilization. a, Analysis of glucose uptake (left), glutamine uptake (centre) and lactate secretion (right). b, Intracellular metabolite levels. Bars show mean of n = 4 (a) or n = 3 (b) replicate wells ± s.d. from representative experiments. c, Schematic of the TCA cycle including entry points for glucose- and glutamine-derived carbons. Isotope tracing was performed for metabolites shown in red. d, e, Fraction of each metabolite labelled by 13C derived from [U-13C]glutamine (13C-Gln) (d) or derived from [U-13C]glucose (13C-Glc) (e) over time (0–12 h).
In ES cells, ‘bivalent domains’ are developmentally regulated genomic regions characterized by the colocalization of H3K4me3 and H3K27me3 (refs 17–19). Recent genome-wide analysis of H3K27me3 in S/L- and 2i/L-cultured ES cells revealed that H3K27me3 was specifically depleted at bivalent domain gene promoters in 2i/L-cultured cells11. Our data suggest that the observed increase in αKG might promote αKG-dependent H3K27me3 demethylation in 2i/L ES cells. Indeed, cells cultured in 2i/L exhibited a greater increase in H3K27me3 at bivalent domain promoters when incubated with the H3K27me3 demethylase inhibitor GSK-J4 than cells cultured in S/L (Fig. 3e and Extended Data Fig. 3b, c). The average fold change across the 14 bivalent promoters tested showed a highly significant increase in 2i/L-cultured ES cells compared with S/L-cultured ES cells (Fig. 3e). Similarly, two independent cell lines with mutations in the Jumonji domain of the H3K27me3 demethylase JMD3 (JMJD3<sup>3<sub>V/A</sub></sup> and JMJD3<sup>3<sub>V/A-2</sub></sup>) (Extended Data Fig. 4a–c) demonstrated increases in H3K27me3 levels relative to control lines that were significantly elevated in cells cultured in 2i/L, reflecting enhanced demethylation at these loci in ES cells cultured in 2i/L (Fig. 3f). Furthermore, treatment with GSK-J4, but not the inactive isomer GSK-J5, increased the αKG/succinate ratio in cells cultured in 2i/L (Fig. 3g). These results indicate that 2i/L rewires glutamine metabolism to maintain αKG pools favouring active demethylation of a variety of histone marks.

In addition to reduced H3K27me3 at bivalent domain promoters, cells cultured in 2i/L exhibit DNA hypomethylation<sup>7,9</sup>. Incubating cells with ascorbic acid, a cofactor for αKG-dependent dioxygenases, activates Tet-dependent gene expression and promotes DNA demethylation<sup>20</sup>. Therefore, we tested whether αKG treatment could exert similar effects (Extended Data Fig. 5a). Total DNA methylation was reduced in cells cultured with cell-permeable αKG (Extended Data Fig. 5b) and treatment with αKG, but not succinate, induced expression of inner-cell-mass- and germline-associated genes previously identified as targets of Tet-mediated activation (Extended Data Fig. 5c).<sup>20,21</sup> The effects of αKG persisted upon extended passaging (Extended Data Fig. 5d) and were largely-abrogated in <i>Tet1/Tet2</i> double-knockout ES cells (Extended Data Fig. 5e). These results suggest that intracellular αKG production may stimulate the activity of multiple αKG-dependent dioxygenases to regulate coordinately the epigenetic marks characteristic of naïve pluripotency.

To test whether modulation of the αKG/succinate ratio can influence pluripotent cell fate decisions, we performed colony-formation assays with S/L-cultured ES cells in the presence of αKG or succinate. Colonies formed in S/L medium supplemented with DM-αKG (S/L + DM-αKG) had brighter AP staining and retained the compact colony morphology typical of undifferentiated ES cells (Fig. 4a). Although the total number of colonies was similar in all three conditions, the S/L + DM-αKG cells contained more than double the number of fully undifferentiated colonies compared with S/L only and S/L + DM-succinate (Fig. 4b). As a further test of the ability of αKG to promote maintenance of ES cells, we used a knock-in Nanog–green fluorescent protein (GFP) reporter line<sup>22</sup> and found that αKG was sufficient to enhance Nanog expression in a dose-dependent manner (Fig. 4c and Extended Data Fig. 6). These results support the conclusion that αKG promotes the self-renewal of ES cells in vitro.

These data demonstrate that the cellular αKG/succinate ratio contributes to the ability of ES cells to suppress differentiation. The rewiring of cellular metabolism by inhibitors of αKG-3β and MAPK/ERK signalling results in a reprogramming of glucose and glutamine metabolism; in turn, this leads to accumulation of αKG and favours demethylation of repressive chromatin marks such as DNA methylation and H3K9me3, H3K27me3 and H4K20me3 (see Supplementary Discussion). Future studies will investigate the mechanisms through which these inhibitors influence the nuclear/cytosolic accumulation of αKG derived from glucose and glutamine. While we cannot rule out chromatin-independent effects of αKG supplementation on ES cells, our results support the notion that chromatin in pluripotent ES cells is responsive to alterations in intracellular metabolism. Indeed, recent clonal analysis
of pluripotent cells revealed that DNA methylation is highly dynamic, balancing the antagonistic processes of removal and addition. Together, these results suggest that continued elucidation of these interconnections between signal transduction and cellular metabolism will shed important light on stem cell biology, organismal development and cellular differentiation.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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METHODS

Cell lines. ESC-1–4 lines are V6.5 ES cells derived from C57BL/6 JAX 06-00064 and 129S/Vyae JAX 001904. Tet1/2 double-knockout ES cells22, V19 ES cells (ESC- V19) and OKS iPSCs23 were a gift from R. Jaenisch. All cells were routinely tested for mycoplasma contamination. Mice were maintained at The Rockefeller University. All animal procedures were designed following National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee at The Rockefeller University.

Cell culture. Maintenance media for ES cells were as follows: serum/LIF (S/L) maintenance medium contained Knockout DMEM (Gibco) supplemented with 15% ES-cell-qualified FBS (Gemini), penicillin/streptomycin (Life Technologies), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Life Technologies), 10 mM β-mercaptoethanol (Invitrogen 0050128DJ) containing either 20 mM [U-13C]glucose or 2 mM [U-13C]glutamine (Invitrogen A14430-01) and glutamine- and glucose-free Neurobasal medium (Life Technologies 21103-049) with or without 2 mM glutamine. With the exception of 15% dialysed FBS (Gemini 100-108) in S/L experimental medium, all other supplements were equivalent to maintenance media (S/L or 2i/L). For growth curves without glucose, 13C Isotope tracing experiments and 13C labelling experiments, media contained a 1:1 mix of glucose-free DMEM (Life Technologies 11960-051) and Neurobasal medium (Life Technologies 21103-049) with or without 2 mM glutamine. For experiments and the average flux for each metabolite was shown. Flux experiments represent the average of three independent experiments; all other experiments were performed independently at least twice and a representative experiment is shown. Protein labelling. ES cells were plated at 7.5 × 105 cells per 6-well plate into experimental medium (S/L or 2i/L) containing 0.1% unenriched [1,3-14C]-glucose (Perkin Elmer NEC942V290UC) or [1,3-13C]-glutamine (Perkin Elmer NEC45 179005-01) and dialysed FBS (Gemini 100-108) in 2i/L conditions this included dialysed FBS) with or without individual metabolites. Cells were maintained at 37°C for 30 min. Samples were analysed using an Agilent 7890A GC coupled to 5975C mass selective detector. The GC was operated in splitless mode with constant helium gas flow at 1 ml min⁻¹. One microlitre of derivatized metabolites was injected onto an HP-SMS column and the GC oven temperature ramped from 60°C to 290°C over 25 min. Peaks representing compounds of interest were extracted and integrated using MassHunter software (Agilent Technologies) and then normalized to both the internal standard (d5-2HG) peak area and the protein content of duplicate samples as determined by a BCA protein assay (Thermo Scientific). Ions used for quantification of metabolite levels are as follows: d5-2HG m/z 354; 2kG, m/z 304; aspartate, m/z 334; glutamate, m/z 336; malate, m/z 335; and succinate, m/z 247. All peaks were manually inspected and verified relative to known spectra for each metabolite. For isotope tracing studies, experiments were performed as described earlier using glucose- and glutamine-free DMEM: NB media base supplemented with [13C]-glucose (Sigma) and [13C]-glutamine (Gibco) or the [13C]versions of each metabolite, [U-13C]glucose or [U-13C]glutamine (Cambridge Isotope Laboratories). Enrichment of 13C was assessed by quantifying the abundance of the following ions: 2kG, m/z 304–315; aspartate, m/z 334–346; glutamate, m/z 363–377; and malate, m/z 335–347. Correction for natural isotope abundance was performed using Isco software24. Flux was calculated as the product of the first order rate constant of the kinetic labelling curve and relative metabolite pool size (normalized to mean S/L values for each experiment)25. The flux from glucose- and glutamine-derived carbons was calculated for each of three independent experiments and the average flux for each metabolite was shown. Flux experiments represent the average of three independent experiments; all other experiments were performed independently at least twice and a representative experiment is shown. Glucose, glutamine and lactate measurements. Glucose, glutamine and lactate levels in culture medium were measured using a YSI 7100 multichannel biochemistry analyser (YSI Life Sciences). Fresh medium was added to 12-well plates of sub-confluent cells and harvested 48 h later. Changes in metabolite concentrations relative to fresh media were normalized to the protein content of each well. These experiments were performed independently at least two times. Growth curves. ES cells or EpSCs were plated in maintenance medium at a concentration of 375,000 cells per 12-well plate. The following day cells were washed with PBS and media were changed to EpiSC maintenance medium supplemented with 6 mM JAK inhibitor (Calbiochem) for five passages. Analysis was performed on passage 7 EpSCs. GSK-J4 and GSK-J5 were purchased from Tocris Bioscience. Teratocarcinoma formation. ESC-1 cells were plated in maintenance medium at a concentration of 2.5 × 105 cells per T25 dish. The following day medium was changed to 2i/L experimental medium with or without glutamine. 72 h later, 1 × 105 cells were harvested from each group and mixed 1:1 with experimental medium (without glutamine) plus Matrigel Basement Membrane Matrix (BD) or experimental medium alone and injected into the flanks of recipient SCID mice aged 8–12 weeks (NOD scid gamma JAX 005557 purchased from Jackson Laboratories). All conditions produced tumours in 4–8 weeks. Mice were euthanized before tumour size exceeded 1.5 cm in diameter. Tumours were excised and fixed in 4% paraformaldehyde overnight at 4°C. Tumours were paraffin-embedded and sections were stained with haematoxylin and eosin according to standard procedures by Histoserv. Growth curves. ES cells or EpSCs were plated in maintenance medium at a concentration of 375,000 cells per 12-well plate. The following day cells were washed with PBS and media were changed to experimental media (for S/L conditions this included dialysed FBS) with or without individual metabolites. Cells were counted each day using a Beckman Coulter Multisizer 4. All growth curves were performed independently at least twice.

ChIP. Native ChIP assays (histones) were performed with approximately 6× 10⁵ ES cells per experiment. Cells were subject to hypotonic lysis and treated with micrococcal nuclease to recover mono- to tri-nucleosomes. Nuclei were lysed by brief sonication and dialysed into N-ChIP buffer (10 mM Tris pH 7.6, 1 mM EDTA, 0.1% Triton X-100, 1 mM proteinase K). Soluble material was incubated overnight at 4°C after addition of 0.5–1 μg of antibody to 25 μl protein A Dynal magnetic beads (Invitrogen), with 5% kept as input DNA. Magnetic beads were washed, chromatin was eluted and ChIP DNA was dissolved in 10 mM Tris pH 8 for quantitative PCR reactions (see later). Three separate ChIP experiments were performed on replicate biological samples. The data shown are the qRT–PCR values (n = 3).

ChIP-qPCR. Primers are listed below. All qPCR was performed using an Applied Biosystems StepOnePlus system and Power SYBR Green PCR master mix. ChIP samples were diluted 1:100 in H₂O and 0.5 μl was used per reaction. ChIP-qPCR signals were calculated as fold enrichment relative to control. Primers were as follows: Gapdh, forward, 5'-CGGACGACACAGGGTACAGTT-3', reverse, 5'-GGGATCTCAACAGGATTTGA AA-3'; Pax9, forward, 5'-AGGTTGAGGACGACTAAGG-3', reverse, 5'-ATC AACCGGAGGTATGCAAAG-3'; Fox1, forward, 5'-TGGCCAGGGACATACCA GT-3', reverse, 5'-GGCGGAGGGAACACATGTA-3'; Hexa2, forward, 5'-CC AGTAAAGATGTTGTTG-3'; reverse, 5'-TGAAGGTCCTTCTTTGACT-3'; Hexa9, forward, 5'-TTCACCTCTTGGTGGGACG-3'; reverse, 5'-AGGTTGC TTGCGTCTTCATCA-3'; E1x1, forward, 5'-GGCGAGGTACGTCCTGGG-3', reverse, 5'-GAGGGTGGTTTCTTTAGTGGGG-3'; E1x2, forward, 5'-TGAGAACCGGCTTCGTTTGC-3'; Fg5, forward, 5'-GGATCTC TGTGCTCCTGGG-3'; reverse, 5'-GGGCTTGTTAAGGGCGACATTT-3'; E1x3, forward, 5'-TGAAGAACGTCGCTTCTTC-3'; reverse, 5'-GATA GAGCCAGAAGCCAGACCCCC-3'; E1x4, forward, 5'-TCAAGCTCTCAGTTGCTC-3'; reverse, 5'-GTGCTGCTCGGTGAGG-3': Ctr, forward, 5'-CAAAGTCCTGCTTGTCCGCC-3'; reverse, 5'-GGGCAACAAGGTGGTTTCACA-3'; Lhx5, forward, 5'-AACCCCTTGAGCGACAGGCGGG-3'; reverse, 5'-GTTGCGGCTGGAGGAAGAA-3'; Sox17, forward, 5'-GTCCTCCCATGATGTCTCTCCTG-3'; reverse, 5'-AGAGCGAGACGACACAGC-3'; reverse, 5'-GTTGCGGCTGGAGGAAGAA-3'; reverse.
Two gRNAs targeting exon 17 of mouse Jmjd3 were as follows: forward, 5′-GGGAGGATCTGAAACTGGC-3′; reverse, 5′-GGCTGCGGTGACGAGGTGG-3′; gRNA #2, forward, 5′-GGTTCTCGAGAGGGGCGACG-3′; reverse, 5′-CCGAGGATCTGAAACTGGC-3′.

PCR. RNA was isolated using the RNeasy kit (Qiagen). After DNase treatment, 1–2 μg RNA was used for cDNA synthesis using the First-Strand Synthesis kit (Invitrogen). Quantitative RT–PCR analysis was performed in biological triplicate using an ABI Prism 7000 (Applied Biosystems) with Platinum SYBR green. All data were generated using cDNA from three wells for each condition. Primers used were as follows: Pou5f1, forward, 5′-CTTTTGACTCAGGAT-3′; reverse, 5′-TTCTACA-3′. All data were generated using cDNA from three wells for each condition. Western blot analysis. Lysates were extracted in 1× Laemmli buffer, separated by SDS–PAGE and transferred to Immobilon PVDF (Millipore) membranes. Membranes were blocked in 5% milk prepared in phosphate-buffered saline (PBS) plus 0.1% Tween 20 (PBS-T), incubated with primary antibodies overnight at 4°C and with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h the next day. After ECL application (Millipore), imaging was performed using Lumagamer LAS-3000 (FujiFilm). The following antibodies were used for western blotting: H3 (Abcam 1791), H3K4me3 (Active Motif 39159), H3K4me1 (Millipore 07-436), H3K9me1 (gift from T. Jenuwein), H3K9me3 (Active Motif 39161), H4 (Abcam 0158), H4K20me1 (Abcam 9051), H4K20me2 (Millipore 07-463), H3K27me1 (Millipore 07-448), H3K27me2 and H3K27me3 (Millipore 07-449), H3K36me3 (Abcam 9050) and H3K36me2 (Millipore 07-548). All antibodies were used at a dilution of 1:1,000. H3K27me3 antibody was used for ChIP-qPCR, Cell Signaling 9733B.

Self-renewal assays. ES cells free from feeder MEFs were plated at 100 cells per well in 6-well plates coated with 20 μg ml−1 mouse laminin (Stemgent 06-0002) in maintenance S/L medium. The next day, media were changed to S/L experimental medium containing dimethyl-α-ketogluconate (4 mM, Sigma 349631), dimethyl-succinate (4 mM, Sigma W239607) or DMSO vehicle control. Four days later, cells were washed with PBS and stained for alkaline phosphatase using Vector Red Western blot analysis. Lysates were extracted in 1× Laemmli buffer, separated by SDS–PAGE and transferred to Immobilon PVDF (Millipore) membranes. Membranes were blocked in 5% milk prepared in phosphate-buffered saline (PBS) plus 0.1% Tween 20 (PBS-T), incubated with primary antibodies overnight at 4°C and with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h the next day. After ECL application (Millipore), imaging was performed using Lumagamer LAS-3000 (FujiFilm). The following antibodies were used for western blotting: H3 (Abcam 1791), H3K4me3 (Active Motif 39159), H3K4me1 (Millipore 07-436), H3K9me1 (gift from T. Jenuwein), H3K9me3 (Active Motif 39161), H4 (Abcam 0158), H4K20me1 (Abcam 9051), H4K20me2 (Millipore 07-463), H3K27me1 (Millipore 07-448), H3K27me2 and H3K27me3 (Millipore 07-449), H3K36me3 (Abcam 9050) and H3K36me2 (Millipore 07-548). All antibodies were used at a dilution of 1:1,000. H3K27me3 antibody was used for ChIP-qPCR, Cell Signaling 9733B.

Statistical comparisons were made using unpaired two-tailed Student’s t-tests or two-way ANOVA with appropriate post-test (determined using GraphPad Prism) as indicated. Experiments were performed with three or four replicates as is the standard in the field. Variation is shown as s.d., s.e.m. or 95% confidence intervals as indicated in figure legends.
Extended Data Figure 1 | Pluripotent stem cells can proliferate in the absence of glutamine when cultured in 2i/LIF medium. a, Doubling time of ESC-V19 cells cultured in S/L or 2i/L. b, Growth curve of ESC-1 cells cultured in S/L or S/L/2i medium devoid of glucose. c, Samples of S/L (left) and 2i/L (right) media with and without glutamine were analysed by GC–MS. Representative chromatograms of the total ion count reveal a clear glutamine (Q) peak in +Q media (grey) and no detectable glutamine in −Q media (red). d, Teratoma formation from ES cells grown in 2i/L medium without glutamine for 3 days. Representative images of haematoxylin and eosin staining reveal neural tissue (ectoderm), hepatocytes and pancreatic acinar cells (endoderm) and smooth muscle (mesoderm). Scale bar, 200 μm. e, Growth curve of ESC-1 cells grown in glutamine-free 2i/L medium. f, Gene expression analysis confirms that EpiSCs, which represent post-implantation pluripotency, were generated from ESC-1 cells by culture with Fgf and activin A. Transcript levels were assessed by quantitative real-time polymerase chain reaction with reverse transcription (qRT–PCR), normalized to Gapdh and expressed as a ratio of values of mouse ES cells cultured in 2i/L medium. g, Growth curve of EpiSCs cultured in serum-free epiblast medium (serum-free medium containing FGF and activin A (Fgf/ActA)) with or without glutamine. h, Growth curve of an iPSC line derived from fibroblasts using Oct3/4 (O), Klf4 (K) and Sox2 (S) cultured in glutamine-free S/L or 2i/L medium. i, Doubling time of ESC-1 cells cultured in 2i/L medium in the presence and absence of glutamine. j, Growth curve of ESC-V19 cells cultured in glutamine-free 2i/L medium in the presence or absence of 1 mM methylsulphoxide (MSO). k, ESC-V19 cells grown in glutamine-free S/L (left) or 2i/L (right) medium with or without 4 mM DM-αKG. For growth curve experiments, cells were seeded on day 0 in complete medium and then were changed to experimental medium on day 1 (indicated by red arrow). Data are presented as the mean ± s.d. of triplicate wells from a representative experiment.
Extended Data Figure 2 | Mouse ES cells cultured with 2i demonstrate altered glucose and glutamine utilization. a, 2i enables glutamate synthesis from glucose-derived carbons. ESC-1 cells cultured in S/L, S/L/2i or 2i/L medium were incubated with medium containing [U-13C]glucose for 4 h and the fraction of glutamate containing glucose-derived carbons is shown. b, ESC-1 cells were cultured for 4 h in glutamine-free S/L or 2i/L medium containing [U-13C]glucose and the total amount of glutamate labelled by glucose-derived carbons is shown. c, Incorporation of 14C derived from [U-14C]glucose (14C-glc) (left) or derived from [U-14C]glutamine (14C-gln) (right) into total cellular protein after 48 h incubation. P < 0.05 for 14C-glc, P = 0.1 for 14C-gln, calculated by unpaired two-tailed Student's t-test. Data are presented as the mean ± s.d. of triplicate wells (a, b) or ± s.e.m. of quadruplicate wells (c) from a representative experiment.
**Extended Data Figure 3 | Regulation of histone methylation in 2i/LIF cells.**

**a.** Western blot analysis of ESC-1 cells grown in glutamine-free 2i/L medium for 24 h with supplementation as indicated.

**b.** H3K27me3 ChIP-qPCR of ESC-1 cells cultured in S/L (b) or 2i/L (c) medium with or without 30 μM GSK-J4 for 5 h. Data are presented as the mean ± s.e.m. of triplicate samples. *P < 0.05 by unpaired Student’s two-tailed t-test.
Extended Data Figure 4 | Generation of JMJD3 mutant cells. a, Schematic of targeting strategy for guide RNAs (gRNAs) to mouse Jmjd3 exon 17. gRNA sequences are highlighted in blue. b, Representative sequences from two clones used in this study. Sanger sequencing revealed indels as shown in schematic. Red dashes, deleted bases; red bases, insertions. gRNA is highlighted in blue and protospacer adjacent motif (PAM) sequences are identified in green. Predicted cut site is indicated by red triangle. Location of in-frame downstream stop is indicated on the right. c, An example chromatogram for clone JMJD3Δ/Δ−2 showing single-base-pair insertions at the predicted Cas9 cleavage site.
Extended Data Figure 5 | αKG increases Tet activity in mouse ES cells.

**a.** Simplified schematic of the reaction mechanism of Tet1/2 enzymes. **b.** Relative per cent 5-methylcytosine (% 5-mC) in ESC-1 cells cultured in S/L medium with or without DM-αKG for 24 h. Each data point represents a sample from triplicate wells of a representative experiment. **c.** Gene expression in ESC-1 cells cultured with DM-αKG or DM-succinate for 3 days. **d.** Gene expression in ESC-1 cells cultured in S/L medium with or without DM-αKG for four passages. **e.** Gene expression in wild-type or Tet1/Tet2 double-knockout (KO) mouse ES cells cultured with DM-αKG or DM-succinate for 72 h. qRT-PCR data (c–e) was normalized to actin or Gapdh and samples were normalized to the control group. Oct3/4 is not expected to change and is included as a control. Data are presented as the mean ± s.e.m. of triplicate wells.
Extended Data Figure 6 | αKG increases Nanog expression.

a, Representative histogram of GFP intensity of Nanog–GFP cells treated with or without DM-αKG for 3 days. Grey represents background staining. 
b, ESC-1 cells were cultured in S/L medium with DM-αKG for four passages and then switched to medium containing the indicated amounts of DM-αKG (0.5–4 mM) or vehicle control (S/L) for 3 days. GFP expression (M.F.I.) was determined by fluorescence-activated cell sorting (FACS). Data are presented as the mean ± s.d. of triplicate wells from a representative experiment.