SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

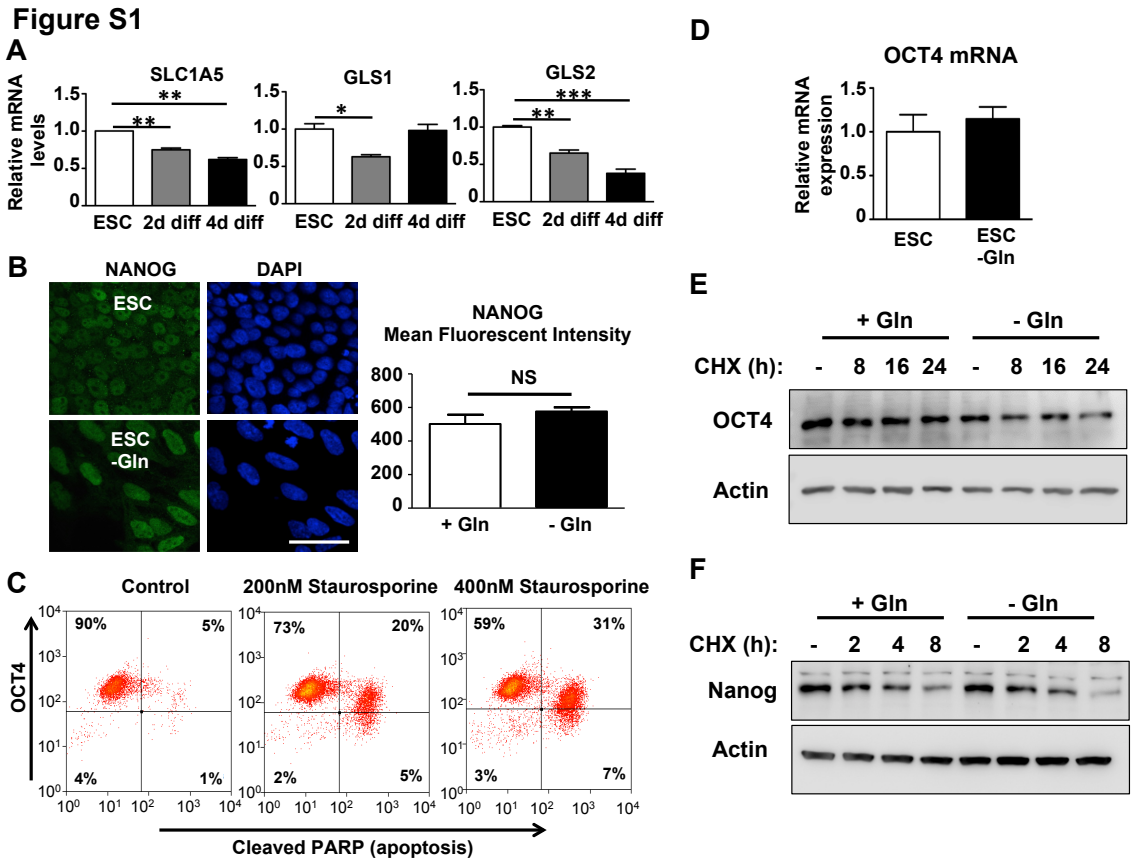

Figure S1. Influence of glutamine withdrawal on OCT4 and NANOG degradation. Related to Figure 1.

(**A**) Gene expression time course during spontaneous differentiation in response to bFGF withdrawal and no conditioning of the cell culture medium. The sodium-dependent neutral amino acid transporter SLC1A5 is responsible for the uptake of glutamine and its expression decreases upon differentiation. At the same time, glutaminase 2 (GLS2), which catalyzes the conversion of glutamine into glutamate, is highly expressed in hESCs but its expression decreases upon differentiation. mRNA expression of glutaminase 1 (GLS1) is not significantly different between hESCs and spontaneously differentiating cells (N=3/group). (**B**) Quantification of NANOG immunofluorescence reveals no difference in mean fluorescence intensity between hESCs grown with or without glutamine for 4d (n=40 cells/group). Scale bar, 20μm. (**C**) Human ESCs were treated with staurosporine for 1.5h as a positive control for apoptosis and then stained for OCT4 and cleaved PARP. A distinct cleaved PARP positive population can be observed, confirming that the cleaved PARP antibody can detect apoptosis in hESCs. (**D**) OCT4 mRNA levels were not altered in response to 4d of glutamine withdrawal. n=4/group. (**E-F**) Time course of OCT4 and NANOG protein levels after cycloheximide (CHX) treatment in cells grown for 2d with or without glutamine. In line with Figure 1E, OCT4 degradation is enhanced by glutamine withdrawal. In contrast, NANOG half-life is not altered by glutamine withdrawal. *P < 0.05, **P < 0.01, ***P < 0.001; Unpaired Student's t-test for comparison between 2 groups, ANOVA with post hoc Dunnett's test for comparing gene expression to the control ESC group in panel A. Error bars show s.e.m.

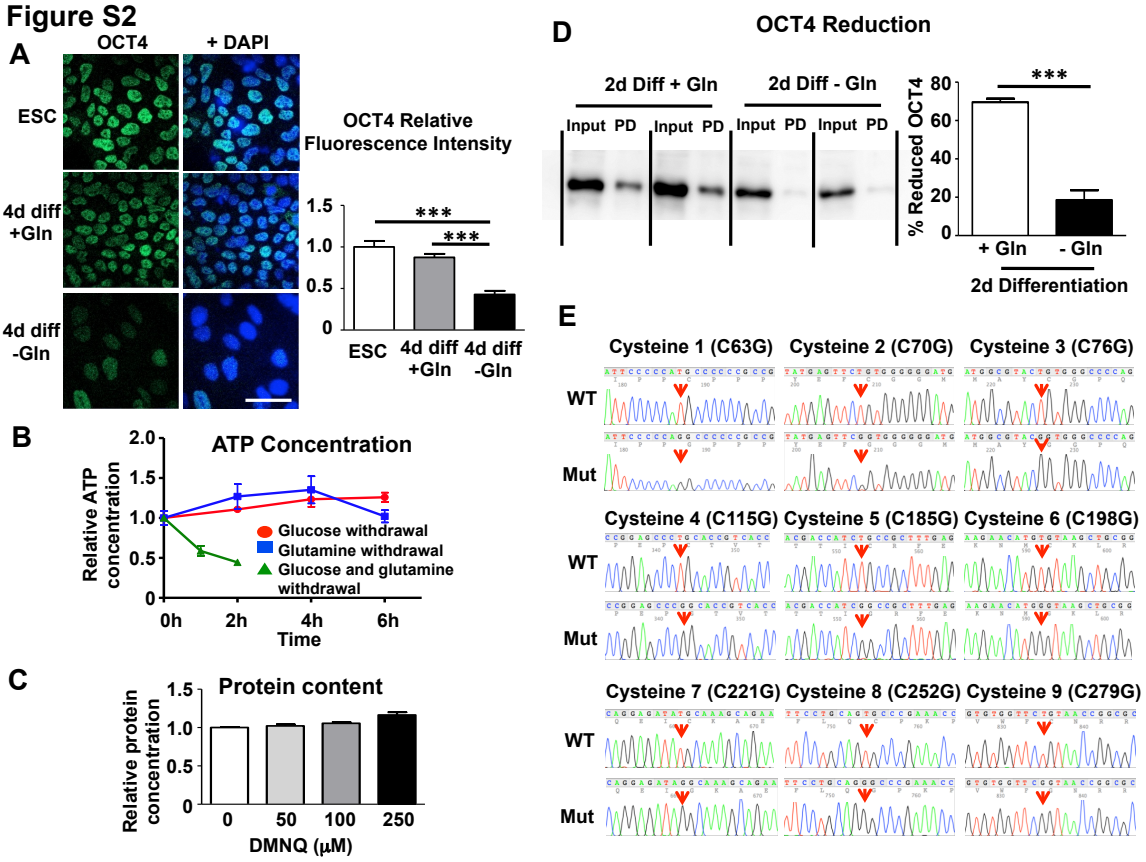

Figure S2. Oxidation of OCT4 is enhanced during spontaneous differentiation and sequencing chromatograms confirming site-directed mutagenesis of cysteines into glycines. Related to Figures 1, 2, and 3.

(**A**) Glutamine withdrawal enhances OCT4 disappearance during spontaneous differentiation in response to unconditioned medium and bFGF withdrawal. Scale bar, 20μm. (**B**) ATP levels are maintained for at least 6h if hESCs are grown with only glucose or only glutamine. In contrast, if both glucose and glutamine are absent from the medium, ATP levels decrease rapidly and cell death increases after 2h. ATP levels are normalized to protein content (n=3-4/group). (**C**) Cells do not show obvious signs of cell death in response to the doses of DMNQ used as protein levels are similar under all treatment conditions. n=4/group. (**D**) Protein lysates of hESCs undergoing spontaneous differentiation were incubated with maleimide-biotin to label reduced cysteine groups. Pulldown of biotin-labeled proteins is followed by an immunoblot for OCT4. For each sample, total nuclear extract (Input) and pulled down (PD) or reduced OCT4 are shown side by side. The percentage of reduced OCT4 is calculated after measuring the relative band intensities. During spontaneous differentiation in the absence of glutamine, OCT4 is almost completely oxidized. n=5/group. (**E**) Sequencing chromatograms confirming 1 base changes leading to a substitution of cysteine by glycine. Red arrows indicate the mutated base. The triplets TGC and TGT encoding cysteine were converted into GGC and GGT respectively, leading to the replacement of cysteine by glycine. ***P < 0.001; Unpaired Student's t-test for comparison between 2 groups, ANOVA with post hoc Dunnett's test for comparing gene expression to the control ESC group in panel A. Error bars show s.e.m.

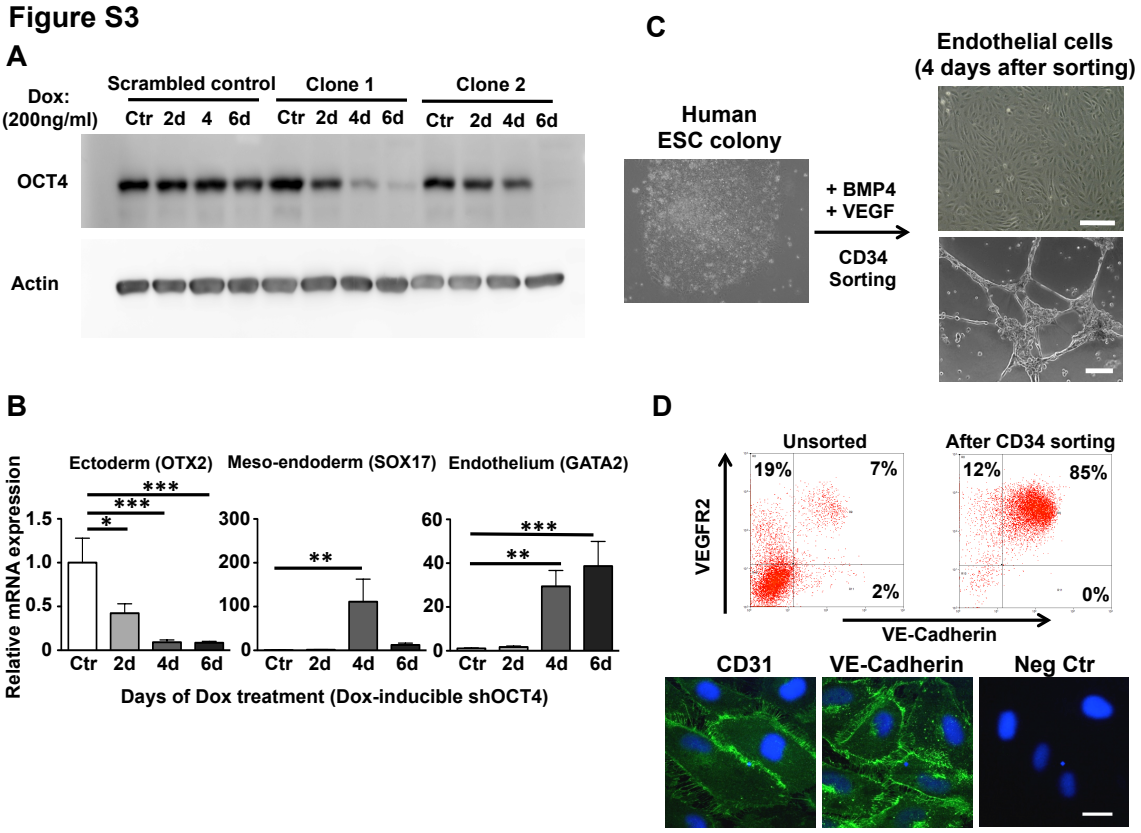

Figure S3. Characterization of stable inducible shOCT4 cell lines and characterization of hESC endothelial differentiation. Related to Figure 4.

(A) Immunoblot for 3 different cell lines exposed to doxycycline for up to 6 days. A scrambled control cell line is shown on the left and its expression of OCT4 does not change over time. In contrast, both clone 1 and 2 have almost no detectable OCT4 expression after 6 days, confirming the efficacy of shRNA-mediated OCT4 downregulation. (**B**) Stable hESC cell lines that express doxycycline-inducible shRNA against OCT4 were incubated for different times with doxycycline. This led to a decreased expression of the early ectodermal transcription factor OTX2, but increased expression of the early mes-endodermal transcription factor SOX17 and the zinc finger transcription factor GATA2, which is involved in early hemangioblast differentiation from mesodermal precursors. (**C**) Endothelial differentiation of hESCs in response to BMP4 and VEGF leads to endothelial cells with a cobblestone morphology. Cells also formed a vascular network when plated on matrigel. Scale bars, 200μm. (**D**) Flow cytometry demonstrating co-expression of VEGFR2 and VE-cadherin in the majority of endothelial cells generated from CD34 sorted cells and immunofluorescence for endothelial markers CD31 and VE-cadherin shows localization of both proteins at cell borders. Scale bar, 20μm. *P < 0.05, **P < 0.01, ***P < 0.001; ANOVA with post hoc Dunnett's test for comparing gene expression to the control group in panel B. Error bars show s.e.m.

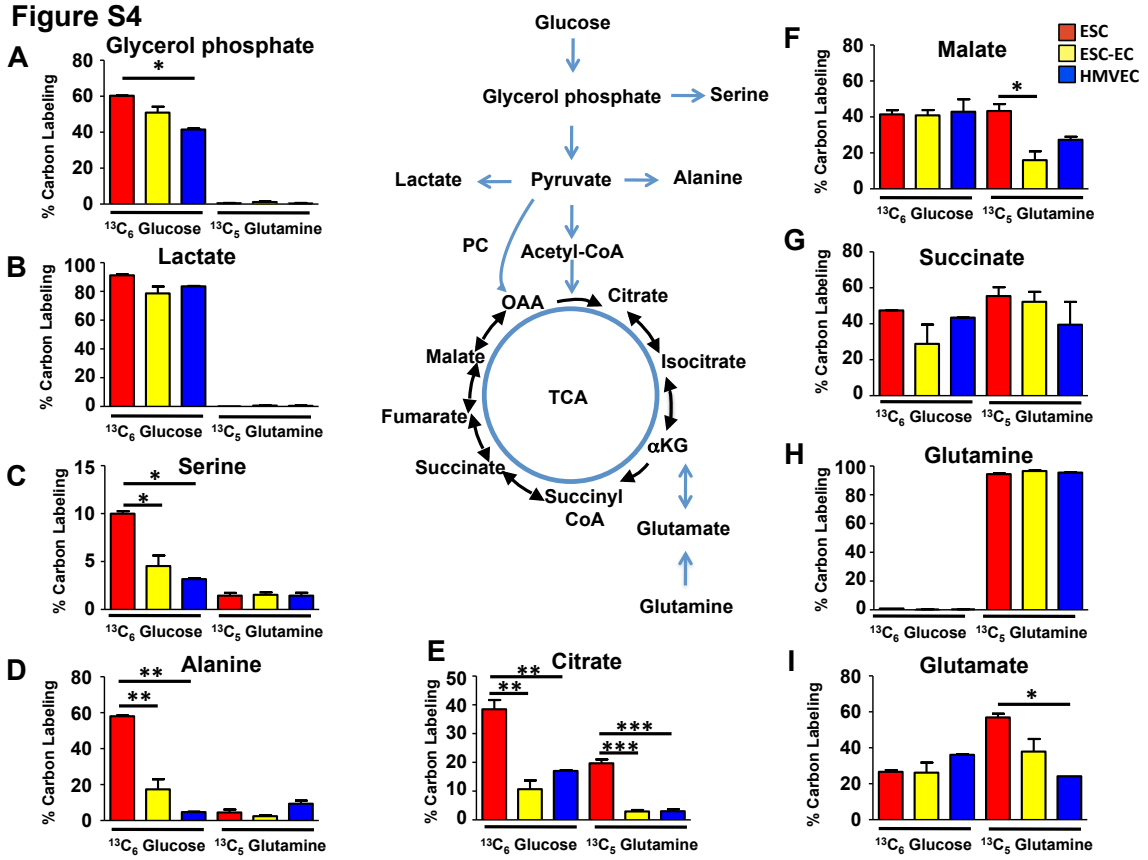

Figure S4. Metabolomic analysis of augmented glutamine metabolism in hESCs. Related to Figure 4. Human ESCs, hESC-derived endothelial cells (ESC-ECs), and human microvascular endothelial cells (HMVECs) as fully differentiated controls were incubated with either [$^{13}C_6$]glucose and unlabeled glutamine or [$^{13}C_5$]glutamine and unlabeled glucose for 24h. Carbon labeling was measured using GC/MS followed by metabolomic analysis. Glucose is converted efficiently into the glycolysis intermediate glycerol phosphate (**A**) and especially into lactate as almost all carbon atoms of intracellular lactate are labeled (**B**), consistent with efficient uptake of glucose into all 3 cell types. Glucose also serves an anabolic role, especially in hESCs, as serine (**C**) and alanine (**D**) are labeled. When cells were exposed to [$^{13}C_5$] glutamine, almost all intracellular glutamine was labeled, indicating that exogenous glutamine is the primary source of intracellular glutamine (**H**); however, the conversion to glutamate was more extensive in hESCs than in fully differentiated endothelial cells (**I**). Glutamine was also taken up in the TCA cycle in both cell types, yet in endothelial cells most labeling was observed in succinate, with lower labeling in malate and almost no labeling in citrate (**E**). In contrast, hESCs demonstrated higher labeling of citrate (**E**). These data suggest that hESCs utilized both glucose and glutamine to generate the TCA cycle intermediate citrate, whereas differentiated endothelial cells primarily relied on glucose-derived citrate. *P < 0.05, **P < 0.01, ***P < 0.001; ANOVA with post hoc Tukey's test for multiple group comparisons. Error bars show s.e.m.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Human ESC culture and differentiation. Human ESCs (H1/WA01 cell line, WiCell, Madison, WI) were cultured on human ESC-qualified matrigel (Corning, Tewksbury, MA) using irradiated mouse embryonic fibroblasts (GlobalStem, Gaithersburg, MD) conditioned medium consisting of DMEM/F12 medium supplemented with 20% knockout serum replacement, L-glutamine, non-essential amino acids, and β-mercaptoethanol (all from Invitrogen, Carlsbad, CA). Prior to use, 10ng/ml basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN) and 100ng/ml heparin (Sigma Aldrich, St Louis, MS) were added. For spontaneous differentiation, medium was not conditioned and bFGF/heparin were left out. For endothelial differentiation, cells were dissociated with accutase (Invitrogen, Carlsbad, CA) for 20' at room temperature and plated at 20,000 cells/cm^2 in conditioned medium containing 10μM of the Rho-associated kinase inhibitor Y27632 (EMD Millipore, Billerica, MA). The next day, medium was changed and after 2 days, endothelial differentiation was started by adding 20ng/ml bFGF, 25ng/ml bone morphogenetic protein 4 (BMP4), and 50ng/ml vascular endothelial growth factor (VEGF) to the medium (all from R&D Systems, Minneapolis, MN). Medium was changed every other day. $CD34^+$ cells were isolated using CD34 microbeads (Miltenyi Biotec, San Diego, CA) and plated on dishes coated with collagen type I (Invitrogen, Carlsbad, CA) in EGM2-MV medium (Lonza, Walkersville, MD). As a control, human lung microvascular endothelial cells (HMVECs) were obtained from Lonza and grown in EGM2-MV medium. To measure the effect of glutamine withdrawal on endothelial differentiation, glutamine was removed from the medium after 2 days of differentiation. Cell permeable dimethyl 2-oxoglutarate was obtained from Sigma Aldrich (St Louis, MS).

OCT4 transcription factor assay and oxidation of OCT4. Nuclear extracts (8μg) were incubated overnight in wells coated with double-stranded DNA containing the OCT4 response element. Next, a primary antibody against OCT4 and a secondary antibody conjugated to horseradish peroxidase are used to detect bound OCT4 according the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). To induce intracellular superoxide formation, cells were treated for 5h with different concentrations of 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ, Sigma Aldrich, St Louis, MS). To determine the oxidation status of OCT4, cells were lysed in RIPA buffer containing freshly dissolved 500μM EZ-Link Maleimide-PEG2-Biotin (Thermo Fisher Scientific, Hampton, NH) and incubated on ice for 4h to label reduced thiols. Afterwards, 50μg of protein was incubated with 150μl NeutrAvidin agarose (Thermo Fisher Scientific, Hampton, NH) and incubated overnight at 4ºC. Sample was eluted by boiling the resin-bound complex in 1x SDS-PAGE sample buffer. Input (50μg of protein) and pulled down protein (containing reduced thiol groups) were loaded next to each other and % reduced protein was quantified using Image J software (NIH, Bethesda, MD).

ATP, GSH/GSSG, and NADPH levels. ATP levels were measured using a commercially available bioluminescent kit (Sigma Aldrich, St Louis, MS) and normalized to protein concentration. Oxidized and reduced glutathione levels were measured using the GSH/GSSG-Glo Assay kit (Promega, Madison, WI). Briefly, cells were cultured in the presence or absence of glutamine for 2 days, trypsinized and 50,000 cells were lysed in either total glutathione lysis or oxidized glutathione lysis reagent. After adding luciferin generation and detection reagents, luminescence was measured. For NADPH measurements, we used a $NADP^+$/NADPH quantification kit (Sigma Aldrich, St Louis, MS). Cells were first cultured in the presence or absence of glutamine for 2 days, then cells were incubated with extraction buffer followed by 2 freeze-thaw cycles. To measure NADPH, $NADP^+$ was degraded at 60ºC for 3h.

Endothelial sprouting assay. Invasion into 3 dimensional collagen gels was quantified using previously published methods (Bayless et al., 2009; Kang et al., 2015). Briefly, rat tail collagen type I was polymerized with 200ng/ml VEGF (R&D Systems, Minneapolis, MN). Human ESCs were differentiated toward endothelial cells for a total of 4 days and the glutamine withdrawal group did not receive glutamine during the final 2 days of differentiation. 50,000 cells were plated on top of the collagen gels in EBM2 medium containing 40ng/ml bFGF, 50μg/ml ascorbate, and 1/250 reduced serum II (Sigma Aldrich, St Louis, MS) as described previously (Kang et al., 2015). After 72h, cells were fixed for 2h with 3% glutaraldehyde in PBS and stained for 20' with 0.1% toluidine blue in 30% methanol. Endothelial sprouting was quantified as the number of structures invading beneath the monolayer per high power field.

Animal experiments. Animal experiments were performed according to the NIH guidelines for the care and use of live animals and were approved by our IACUC committee. Ten week-old male NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice (The Jackson laboratory, strain 005557) lacking mature T, B, and NK cells were used for *in vivo* neovascularization experiments. Human ESCs were differentiated using the endothelial differentiation protocol for 4 days. The first group of cells were maintained in medium containing 4.5mM glutamine for the full 4 days, while the second group of cells received glutamine only for the first 2 days, after which glutamine was withdrawn for 2 days. After a total of 4 days of endothelial differentiation, 4 million cells were injected with 250µl growth factor reduced Matrigel (Corning, Tewksbury, MA). After 7 days, plugs were collected and paraffin embedded. Pictures of 10 random fields of hematoxylin and eosin stained sections were obtained using an Axioplan 2 microscope (Carl Zeiss, Goettingen, Germany) and the number of red blood cells per high power field determined.

Gene expression. Total RNA was extracted using the PureLink Micro-to-midi total RNA isolation kit (Invitrogen, Carlsbad, CA). Reverse transcription was performed on 500ng RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Quantification was performed on a 7900HT fast real-time PCR system (Applied Biosystems, Carlsbad, CA) using FastStart Universal Sybr Green master mix (Roche Applied Science, Indianapolis, IN). The following primers were used for quantification of GATA2 (Fw 5'-AGACGAAGGCAACCATTTTTAGAA-3', Rv 5'-GCCAATCCCGAGGAAGAAC-3'), GLS1 (Fw 5'- TGTGATTCCTGACTTTATGTCTTTTACCT-3', Rv 5'-GACACACCCCACAAATCGG-3'), GLS2 (Fw 5'-TTCAATTTCCACAACTATGACAACCT-3', Rv 5'-ACAGTCTTGTTCCGAATTTCTGC-3'), OCT4 (Fw 5'-AGAAGGATGTGGTCCGAGTGTG-3', Rv 5'-CATAGTCGCTGCTTGATCGC-3'), OTX2 (Fw 5'-CGAGGGTGCAGGTATGGTTT-3', Rv 5'-TTTTGGCAGGTCTCACTTTGTTT-3'), SLC1A5 (Fw 5'-GCCATCATCCTCGAAGCAGT-3', Rv 5'-TCAACTCAGGCTCTGTGCTTCTC-3'), and SOX17 (Fw 5'-AGGGCGAGTCCCGTATCC-3', Rv 5'-ACGACTTGCCCAGCATCTTG-3'). All results were normalized to beta-2-microglobulin (B2M, Fw 5'-ATAAGTGGAGGCGTCGCGCTG-3', Rv 5'-ACAGCTAAGGCCACGGAGCG-3'), and compared to control samples using the $2^{(\Delta\Delta C_t)}$ method.

Flow cytometry. For differentiation quantification, cells (200,000) were incubated with anti-CD34 conjugated to Alexa700 (BD Biosciences, San Jose, CA) for 30' at 4ºC and analyzed on an LSRFortessa cell analyzer (BD Biosciences, San Jose, CA). Mitochondrial ROS levels were quantified after incubation with 5µm MitoSOX for 20' at 37ºC and for normalization, cells were stained with 50nM MitoTracker Green for 30' at 37ºC. Samples were analyzed on a Cyan ADP analyzer (Beckman Coulter, Indianapolis, IN). Anti-OCT4 conjugated to PE and anti-cleaved PARP conjugated to Alexa 700 together with matched isotypes were obtained from BD Biosciences (San Jose, CA). Cells were trypsinized and fixed overnight with methanol at -20ºC. After permeabilization in 0.25% Triton X-100, cells were incubated with antibodies for 1h and analyzed on a BD LSRFortessa flow cytometer (BD Biosciences, San Jose, CA). As a positive control, cells were incubated for 1.5h with Staurosporine (Sigma Aldrich, St Louis, MI).

Immunofluorescence, image acquisition, and fluorescence quantification. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton X-100 in PBS. After blocking with 2% BSA in PBS + 0.05% Tween 20, primary antibodies were applied overnight at 4ºC. Matching secondary antibodies conjugated to Alexa 488 were applied for 1h and cover slips were mounted using ProLong Gold antifade mountant containing DAPI (Invitrogen, Carlsbad, CA). The primary antibodies used were against OCT4 (sc101534, Santa Cruz Biotechnology, Dallas, TX) and NANOG (ab21624, Abcam, Cambridge, MA). Images were obtained on a LSM710 confocal microscope (Carl Zeiss, Goettingen, Germany) using a 63x/1.4 Oil Plan-Apochromat objective and a 25mW Argon laser. Mean immunofluorescence intensity in Figures 1A, S1B, and S2A was analyzed by using the nuclear DAPI stain as a mask. Individual nuclei were then manually selected and the mean intensity measured using Image J software (NIH, Bethesda, MD).

Site-directed mutagenesis. Human OCT4A cDNA was cloned into the pEGFP-N1 vector creating a fusion OCT4-eGFP construct. Site-directed mutagenesis was performed using a QuikChange Site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The following primers were used for site-directed mutagenesis: C63G (Fw 5'-GGGATTCCCCCAGGCCCCCCGCCGTAT-3', Rv 5'-ATACGGCGGGGGGCCTGGGGGAATCCC-3'), C70G (Fw 5'-GCCATCCCCCCACCGAACT CATACGGC-3', Rv 5'-GCCGTATGAGTTCGGTGGGGGGATGGC-3'), C76G (Fw 5'-CCTGGGGC

CCACCGTACGCCATCCC-3', Rv 5'-GGGATGGCGTACGGTGGGCCCCAGG-3'), C115G (Fw 5'-GGTGACGGTGCCGGGCTCCGGGG-3', Rv 5'-CCCCGGAGCCCGGCACCGTCACC-3'), C185G (Fw 5'-AGCCTCAAAGCGGCCGATGGTCGTTTGGC-3', Rv 5'- GCCAAACGACCATCGGCCGCTTTGAGGCT-3'), C198G (Fw 5'-CAAGGGCCGCAGCTTACCCATGTTCTTGAAGCTAA-3', Rv 5'-TTAGCTTCAAGAACATGGGTAAGCTGCGGCCCTTG-3'), C221G (Fw 5'- GAGGGTTTCTGCTTTGCCTATCTCCTGAAGATTTTCATTGTTGT-3', Rv 5'- ACAACAATGAAAATCTTCAGGAGATAGGCAAAGCAGAAACCCTC-3'), C252G (Fw 5'-AGTGTGGGTTTCGGGCCCTGCAGGAAC AAATTC-3', Rv 5'-GAATTTGTTCCTGCAGGGCCCGAAACCCACACT-3'), C279G (Fw 5'-TGGCGCCGGTTACCGAACCACACTCGG-3', Rv 5'- CCGAGTGTGGTTCGGTAACCGGCGCCA -3'). Mutations were confirmed with sequencing (3730xl Analyzer, Life Technologies, Carlsbad, CA). Transfection was done using Lipofectamine 3000 (Life Technologies, Carlsbad, CA) using 1μg of DNA per 500,000 HEK 293T cells. After 24h, nuclear proteins were isolated and an equal amount of protein was used for the OCT4 activity assay. OCT4 sequence alignment was done using CLC Sequence viewer 7.5 (Qiagen, Valencia, CA)

Stable isotope-based metabolomics. Stable isotopes were obtained from Cambridge Isotope Laboratories (Andover, MA) and dissolved in basal medium consisting of 2.125g Dulbecco's minimal essential medium powder and 2.170g Ham's F12 medium powder both lacking glucose and glutamine (D9800-02 and N8542-12, US Biological, Salem, MA) supplemented with 1.425g $NaHCO_3$ (Sigma Aldrich, St Louis, MI) before adjusting the pH to 7.2. ESC medium was made as described above, with the addition of either [$^{13}C_6$]D-glucose and unlabeled glutamine, or [$^{13}C_5$]L-Glutamine and unlabeled glucose. Final concentrations were 17.5mM glucose and 4.5mM glutamine. Medium was added to cells for 24h, after which medium was removed, cells were quickly washed with distilled water and snap frozen in liquid nitrogen. Dishes were stored at -80ºC until GC/MS analysis. Samples were derivatized with trimethylsilyltrifluoroacetamide and metabolomic analysis was performed using an Agilent 7890A GC/5975C MS (GC/MS) at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Isotope labeling of each metabolite was corrected by subtracting its natural isotope distribution as described previously (Fernandez et al., 1996). To calculate the % carbon labeling of metabolites, a weighed average was calculated based on the prevalence of each individual isotopomer.

Generation of stable shOCT4 cell lines. The following shRNAs targeting OCT4 were cloned into the pLKO-Tet-On lentiviral vector after annealing of forward and reverse oligos: Clone 1 Fw 5'-CCGGAGCGATCAAGCAGCGACTATGCTCGAGCATAGTCGCTGCTTGATCGCTTTTTT-3', Rv 5'-AATTAAAAAAGCGATCAAGCAGCGACTATGCTCGAGCATAGTCGCTGCTTGATCGCT-3' and Clone 2 Fw 5'-CCGGAGCGAACCAGTATCGAGAACCCTCGAGGGTTCTCGATACTGGTTCGCTTTTTT-3', Rv 5'-AATTAAAAAAGCGAACCAGTATCGAGAACCCTCGAGGGTTCTCGATACTGGTTCGCT-3'. Plasmids were transfected together with pMD2.G and psPAX2 plasmids (Addgene) into HEK 293T cells to allow for lentiviral production. Two days later, supernatant was collected, filtered through a 0.45μm filter, and added to H1 hESCs together with 4μg/ml polybrene. After 3 more days, selection was started with 0.5μg/ml puromycin. This dose of puromycin was used because it killed all non-transduced hESCs in 2 days. Surviving clones were picked individually and maintained in puromycin for an additional 2 weeks to establish stable cell lines. Several clones were screened for OCT4 downregulation in response to 200ng/ml doxycycline (Sigma Aldrich) by RT-PCR and confirmed by immunoblotting. Three clones (2 containing the first construct, 1 containing the second construct) were subsequently selected and used for experiments.

SUPPLEMENTAL REFERENCES

Fernandez, C.A., Des Rosiers, C., Previs, S.F., David, F., and Brunengraber, H. (1996). Correction of 13C mass isotopomer distributions for natural stable isotope abundance. Journal of mass spectrometry : JMS 31, 255-262.