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Supplemental Information

Myc Depletion Induces a Pluripotent Dormant State Mimicking Diapause

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Conditional Deletion of *c-myc* and *N-myc*. Deletion of the floxed alleles in *c-myc*$^{fl/fl}$; *N-myc*$^{fl/fl}$; *RosaEYFP*$^{fl/fl}$ cells was induced by transient transfection of ESCs with the EF1α-CRE plasmid using Lipofectamine 2000 (Invitrogen, 1639722) according to the manufacturer's instructions. Subsequent deletion of the remaining floxed allele was induced by transient transfection with an EF1α-mCherry-CRE plasmid. mCherry-positive and negative cells were sorted by flow cytometry 24h after transfection. To confirm the deletion, genomic DNA was isolated from sorted or cultured cells using the DNeasy Blood and Tissue kit (Qiagen, 69506) according to the manufacturer's instructions.

Synthetic *c-myc* mRNA Production and Transfection. Synthetic modified mRNA was generated as previously described (Warren et al., 2010) with modifications as follows. RNA was synthesized using the AmpliscribeT7-Flash Transcription kit (Epicentre, Illumina company, Madison, WI) and the capping analogon was directly synthesized using the ScriptCap m7G Capping System and ScriptCap 2'-O-Methytransferase Kit (Cellscript, Madison, WI). mRNA transfection was performed using Lipofectamine 2000 (Invitrogen, 1639722) according to manufacturer's instructions and in culture medium not supplemented with PD0325901 (1i) since it negatively regulates c-Myc protein stability by inhibition of Erk signalling (Sears, 2004). ESCs were transfected with 2 µg/ml of synthetic *c-myc* mRNA or Lipofectamine only. A second mRNA transfection was performed 18h after the first as described above. The cells were analysed after additional 8h.

***In vitro* Differentiation.** ESCs were cultured in 2i on gelatine-coated dishes. For embryoid body (EB) formation ESCs were first harvested by digestion with StemPro® Accutase® for 5 min and dissociated until a single-cell suspension was achieved. The cell suspension was pelleted by centrifugation and resuspended in EB-Medium (DMEM, 15% FCS, 50 µM β-mercaptoethanol, NEAA) at a density of 5.5 x 10^5 cells/ml. The forming EBs were cultured in suspension for 5 days and plated on gelatine-coated tissue dishes thereafter. Adherent cells were further differentiated for 5 days in EB-Medium. To address the pluripotent differentiation potential, cultures were fixed in 4% PFA and stained with antibodies against β-3-tubulin (Tuj1) (Covance, Princeton, NJ, MMS-435P, 1:1000), α-smooth muscle actin (Sma) (Sigma-Aldrich, A2547, 1:500) and Gata6 (R&B Systems, AF1700, 1:100).

Flow Cytometry. Cell cycle analysis and cleaved Caspase 3 staining were performed by fixation and permeabilization with Cytofix/Cytoperm (Beckton Dickinson, 51-2090KZ). Intracellular staining with Ki67-Alexa647 antibody (BD biosciences, 558615) or cleaved Caspase 3-PE (BD Pharmigen, 51-68655X) was done over night at 4°C in PermWash solution (Beckton Dickinson, 51-2091KZ). For cell cycle analysis, samples were then incubated with Hoechst33342 (Sigma, H3570) in PermWash solution for 10 min. For FACS analysis of BrdU incorporation combined with 7-AAD staining, ESCs were chased with BrdU (10 µM final concentration) for 15 min prior fixation. BrdU labelling and staining were performed using the APC BrdU Flow Kit (BD Pharmingen™, 552598) according to the manufacturer's instructions. FACS analysis for Sox2 was performed using the BD™ Mouse Pluripotent Stem Cell Transcription Factor Analysis Kit (Becton Dickinson, 560585) according to the

manufacturer's instructions. FACS analysis was performed on LSRII or LSR Fortessa flow cytometers (Becton Dickinson, San Jose, CA). Data were analysed using the FlowJo software (Tree Star, Ashland, OR). Cell sorting experiments were carried out on a BD FACSAria™ III sorter (Becton Dickinson, San Jose, CA).

Alkaline Phosphatase Staining. Alkaline Phosphatase staining was performed using the Stemgent® Alkaline Phosphatase Staining Kit II (Stemgent, 00-0055) according to the manufacturer's instructions.

Immunofluorescence Staining of ESCs. Cells were fixed for 15 min at room temperature in PBS supplemented with 4% paraformaldehyde (Electron Microscopy Sciences, 19208) and permeabilized in PBS containing 0.5% Triton X-100 for 10 min. The cells were then washed three times with 0.1% Tween 20 in PBS for 5 min at RT and blocked for 1h in PBS supplemented with 0.5 % BSA, 10% goat serum, 0.1% Triton X-100 in PBS. Antibody staining was carried out using the following antibodies, diluted in blocking solution: Nanog (Abcam, ab80892, 1:500); Oct4 (Abcam, ab19857, 1:500). After washing twice in PBS, the cells were incubated for 4h at RT with the secondary antibody donkey anti-rabbit-Alexa Fluor 647 (Invitrogen, A-31573) diluted 1:300 in blocking solution. Following DAPI staining (Sigma, D9542), the slides were mounted with Faramount (Dako, S3035) and analysed on a Zeiss Cell Observer fluorescence microscope (Carl Zeiss, Jena, Germany).

Immunofluorescence Staining of Embryos. Mouse embryos were fixed for 15 min at room temperature in PBS supplemented with 4% paraformaldehyde (Electron Microscopy Sciences, 19208) and washed in PBS. Embryos were then incubated with anti-Ki67-eFluor® 660 (eBioscience, 50-5698-80, 1:500), anti-Nanog (Abcam, ab80892, 1:500) or anti-c-Myc (Santa Cruz Biotechnology, sc-764, 1:500) diluted in PBS supplemented with 0.1% Triton X-100 and 1% BSA (PBS-T) overnight at 4°C. After washing with PBS-T, embryos were further incubated with the secondary antibody Alexa Fluor 488 goat anti-rabbit (Jackson ImmunoResearch Laboratories, 111-545-003) or donkey anti-rabbit Alexa Fluor 647 (Invitrogen, A-31573) in PBS-T for 2h at room temperature. Embryos were washed in PBS-T and mounted in DAPI-mounting solution (Life Technologies, Prolong® Gold Antifade reagent with DAPI, P36935). Fluorescence images were acquired using a confocal microscope (LSM 710, Carl Zeiss) equipped with a 63x and a 40x oil immersion 1.4 objective. Image analysis was performed using FIJI or ImageJ. The mean fluorescence intensity per blastocyst was measured as the ratio between the average mean fluorescent intensity of the projected z-stack image of each embryo and the selected area.

Gene Expression Analysis by Quantitative PCR with Reverse Transcription. Total RNA was isolated using the ARCTURUS® PicoPure® RNA Isolation Kit (Life Technologies, Invitrogen) according to the manufacturer's protocol, and including an on-column DNase digestion (Qiagen, 79254). Reverse transcription was performed using the SuperScript VILO cDNA synthesis kit (Invitrogen, 11754-250) according to the manufacturer's instructions. Real-time quantitative PCRs were performed using the ABI Power SYBR Green Master Mix (Applied Biosystems, 4309155). PCR reactions were run on a ViiA7 machine (Applied Biosystems) and results were analysed using the

ViiA™7 – software 1.1. An endogenous control (*Gapdh*, glyceraldehyde-3-phosphate dehydrogenase) was used to normalize the expression. Technical replicates were carried out for all quantitative PCR reactions. The following primers were used: c-myc-FW, 5'-CAC CAG CAG CGA CTC TGA-3'; c-myc-RV, 5'-GGG GTT TGC CTC TTC TCC-3'; N-myc-FW, 5'-CTC CGG AGA GGA TAC CTT GA-3'; N-myc-RV, 5'-TCT CTA CGG TGA CCA CAT CG-3'; Gapdh-FW, 5'-CCC ATT CTC GGC CTT GAC TGT-3'; Gapdh-RV, 5'- GTG GAG ATT GTT GCC ATC AAC GA-3'.

RNA-seq. Total RNA isolation was performed from 12 samples using ARCTURUS® PicoPure® RNA Isolation Kit (Life Technologies, Invitrogen) according to the manufacturer's instructions. DNAse treatment was performed using RNAse-free DNAse Set (Qiagen). Total RNA was used for quality controls and for normalization of starting material. cDNA-libraries were generated with 10 ng of total RNA using the SMARTer™ Ultra Low RNA Kit for Illumina Sequencing (Clontech) according to the manufacturer's indications. Twelve cycles were used for the amplification of cDNA. The sequencing library was generated using the NEB Next ChIP-Seq kit according to manufacturer's instructions (New England Biolabs). Sequencing reads (100 bp) were generated on the HiSeq2000 platform (Illumina) with three samples per lane. Processing of RNA-seq data was performed as previously described (Cabezas-Wallscheid et al., 2014). The sequenced read fragments were aligned to the mouse reference genome GRCm38 (ENSEMBL release 69) (Cunningham et al., 2015) using GSNAP (version 2012-07-20). The number of read fragments overlapping with each gene were counted using HTSeq (Anders et al., 2015). DESeq2 (Love et al., 2014) was used to test for differential expression. Relative expression levels for each gene were calculated from the read counts after applying a variance stabilizing transformation by estimating the ratio between the mean across replicates for a specific cell population to the mean across all the samples. Genes were grouped based on the sign of their relative expression level (Figure S2D). Gene set enrichment analysis was done as previously described (Cabezas-Wallscheid et al., 2014). The raw data are deposited to ArrayExpress (http://www.ebi.ac.uk/arrayexpress) and are available under the accession E-MTAB-3386. In Data S1, we provide the documented R scripts that were used to analyse the high-throughput transcriptome data.

Microarray Analysis. Total RNA isolation was performed using ARCTURUS® PicoPure® RNA Isolation Kit (Life Technologies, Invitrogen) according to the manufacturer's instructions. DNAse treatment was performed using RNAse-free DNAse Set (Qiagen). Microarray analysis raw data are available for download from Gene Expression Omnibus (http://ncbi.nlm.nih.gov/geo; gene accession number: GSE74337). Microarray gene-expression analysis was made using the GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA). Both raw image (.dat) and intensity (.cel) files were generated utilizing the Affymetrix Gene Chip Operating Software (GCOS). Quality control and RMA background subtraction, quantile normalization and summarization of the intensity data have been performed with the oligo package under the R statistical software (http://www.r-project.org). DEG have been calculated using the limma package and genes with a FDR<0.05 and with a log2 fold change of at least 0.5 have been selected for further analysis. GO-term characterization has been performed with the GOstats package.

SUPPLEMENTAL REFERENCES

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