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Supplemental Information

Mthfd2 Modulates Mitochondrial Function and DNA Repair to Maintain the Pluripotency of Mouse Stem Cells

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Supplemental Figures and Tables

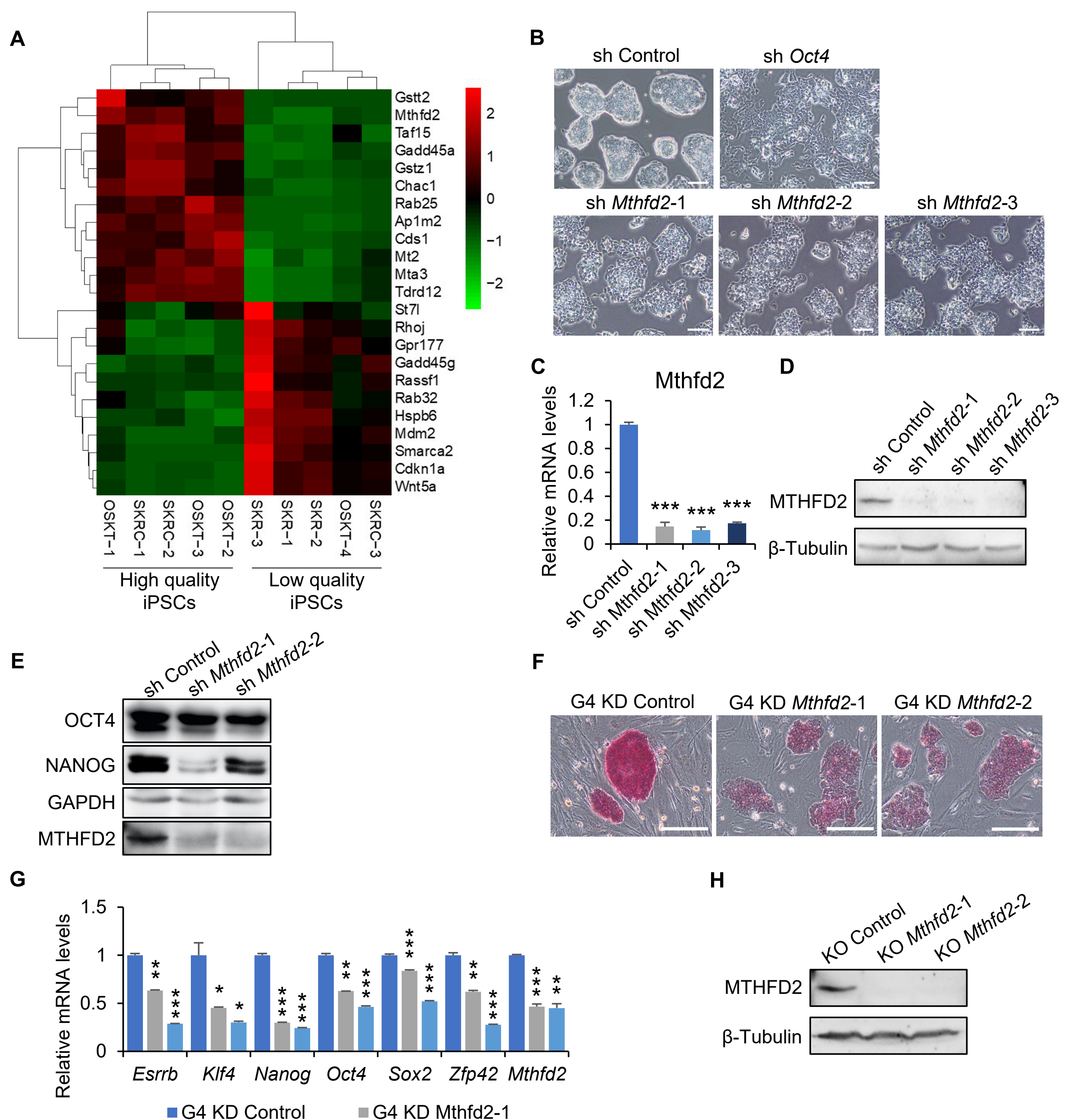


Figure S1. *Mthfd2* Plays Critical Role in Maintaining Pluripotency in mESCs, Related to Figure 1.

(A) Heat-map of differentially expressed genes (DEGs) between iPSCs with different pluripotency. For details, see Table S1.

(B) Representative results from shRNAs screen in mESCs. Scale bar, 100 μm. mESCs treated with sh Control was used as negative control, and mESCs treated with sh *Oct4* was used as positive control.

(C) qRT-PCR analysis of mRNA levels of *Mthfd2* in *Mthfd2* KD mESCs.

(D) Western blot (WB) analysis of the level of MTHFD2 protein in *Mthfd2* KD mESCs. β-Tubulin was used as a loading control.

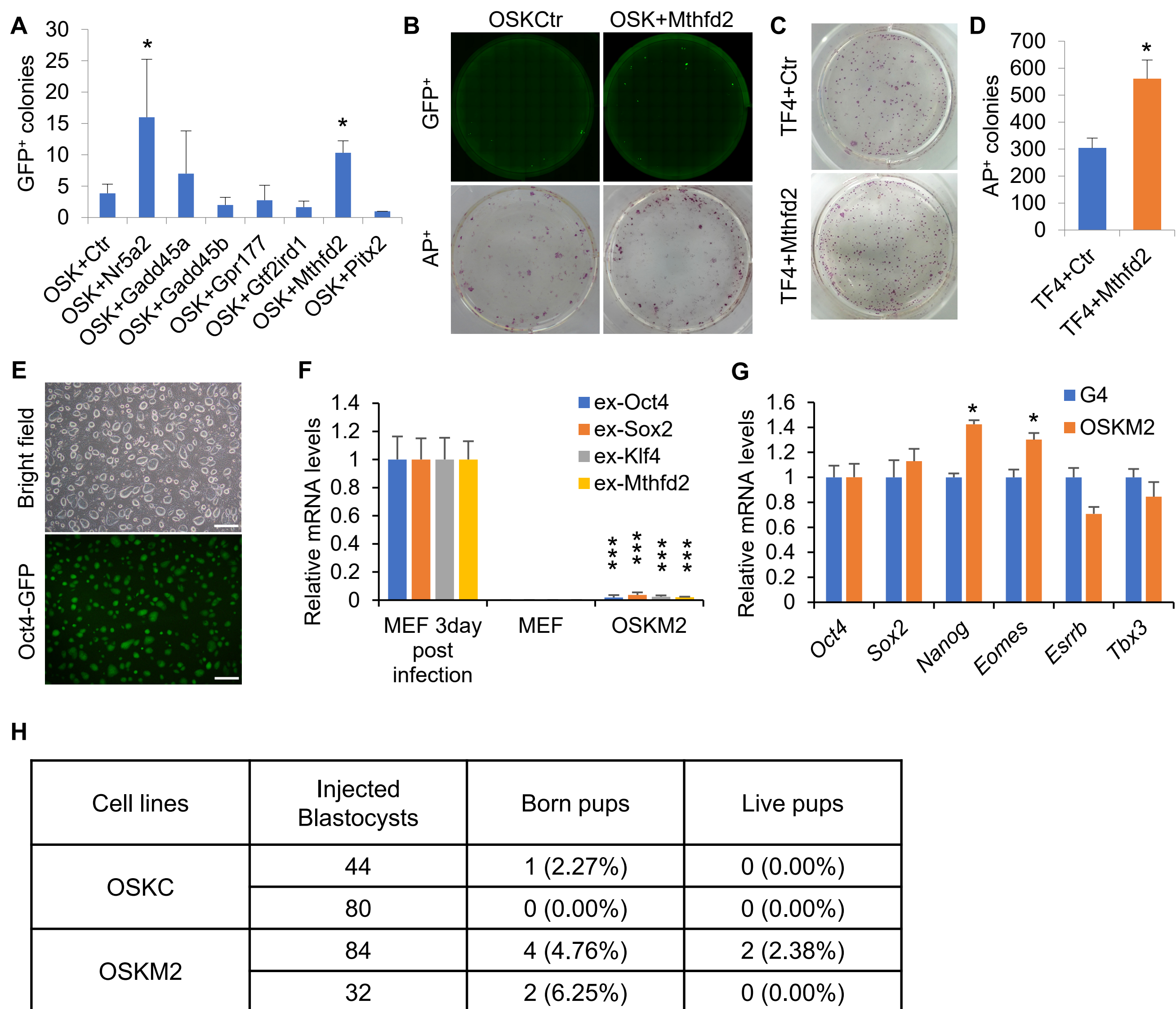
(E) WB analysis of the levels of OCT4 and NANOG protein in *Mthfd2* KD mESCs. GAPDH was used as a loading control.

(F) Representative results of *Mthfd2* KD G4 mESCs with AP staining. Scale bar, 200 μm.

(G) qRT-PCR analysis of mRNA levels of pluripotency marker genes in *Mthfd2* KD G4 mESCs.

(H) WB analysis of the level of MTHFD2 protein in *Mthfd2* KO mESCs. β-Tubulin was used as a loading control.

Data (C and G) are pooled from 3 independent experiments (mean ± SD) relative to EF1-α and the control. *P < 0.05, **P < 0.01, and ***P < 0.001 (Student's t test) compared to the control.



Cell lines	Injected Blastocysts	Born pups	Live pups
OSKC	44	1 (2.27%)	0 (0.00%)
	80	0 (0.00%)	0 (0.00%)
OSKM2	84	4 (4.76%)	2 (2.38%)
	32	2 (6.25%)	0 (0.00%)

Figure S2. *Mthfd2* Facilitates Mouse iPSC Induction, Related to Figure 2.

(A) The reprogramming efficiencies in mESCs medium. OSK + Control (OSKCtr) cells were used as a control. Data are pooled from 3 independent experiments (mean ± SD). *P < 0.05, **P < 0.01, and ***P < 0.001 (Student's t test) compared to the control.

(B) Full-well mosaic images of Oct4-GFP⁺ cells and AP⁺ colonies are shown for OSKCtr and OSKM2 iPSCs in mESCs medium.

(C) The reprogramming efficiency was increased by overexpress (OE) Mthfd2 in TF4 MEFs. Data are pooled from 3 independent experiments (mean ± SD). *P < 0.05, **P < 0.01, and ***P < 0.001 (Student's t test) compared to the control.

(D) *Mthfd2* OE increases the number of AP⁺ colonies following the induction of iPSCs in TF4 MEFs. The results from TF4 + Ctr cells were used as a control.

(E) Phase contrast and Oct4-GFP images of iPSC colonies generated by coinfection of *Mthfd2* and OSK. Scale bar, 500 μm.

(F) qRT-PCR analysis of levels of integrated transgenes in OSKM2 iPSCs. Wild type MEFs and three days after retroviral infection of MEFs were used as controls.

(G) qRT-PCR analysis of mRNA levels of pluripotency marker genes in OSKM2 iPSCs. The G4 mESCs was used as controls.

(H) Analysis of the efficiency of live offspring produced by OSKM2 iPSCs and OSKC iPSCs using tetraploid complementation. Data (F and G) are pooled from 3 independent experiments (mean ± SD) relative to EF1-α and the controls. *P < 0.05, **P < 0.01, and ***P < 0.001 (Student's t test) compared to the control.

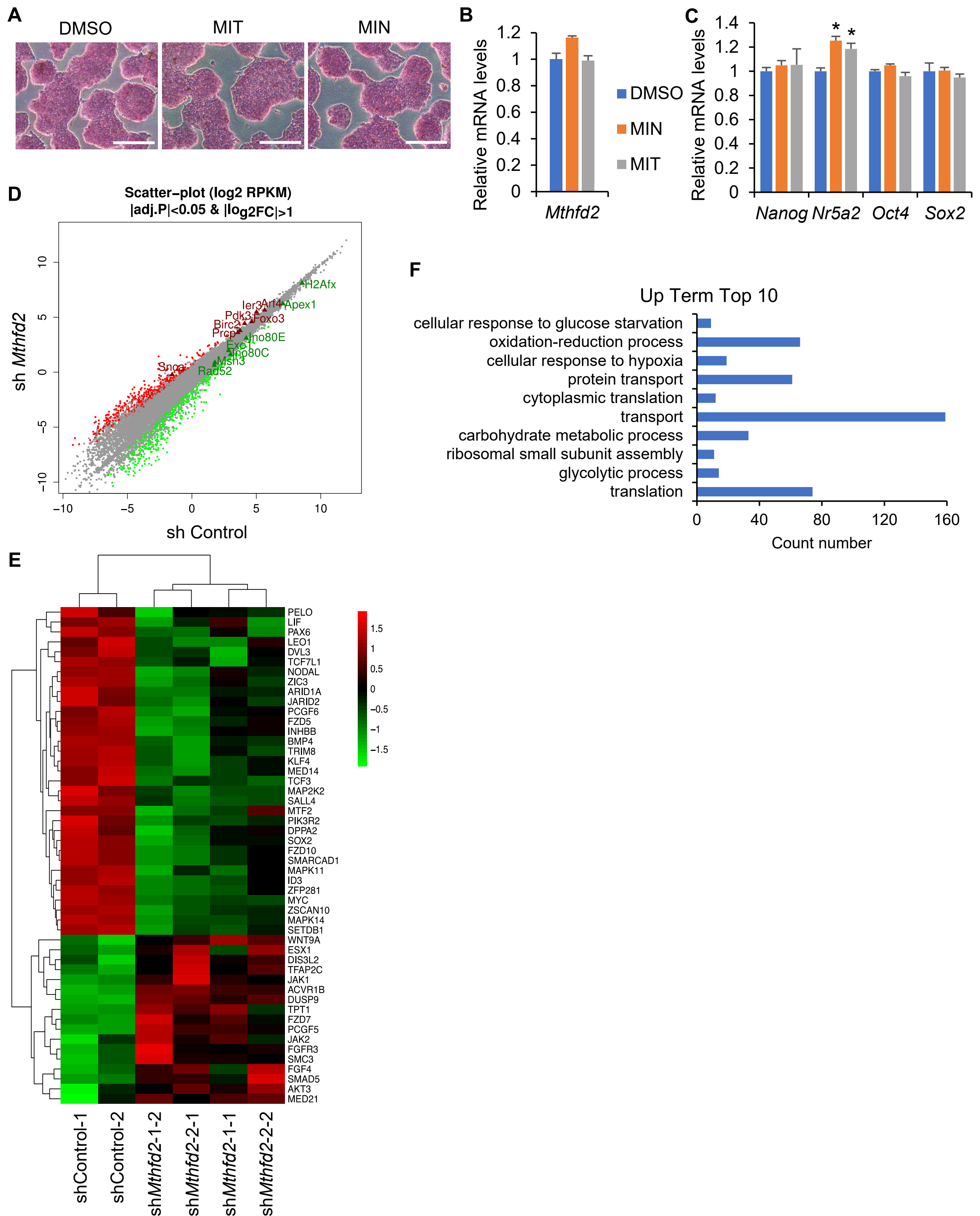


Figure S3. RNA-seq of *Mthfd2* KD mESCs, Related to Figure 3.

(A) Representative results of MI-mESCs with AP staining. Scale bar, 200 μm.

(B and C) qRT-PCR analysis of mRNA levels of *Mthfd2(B)* and pluripotency marker genes (C) in MI-mESCs. Data are pooled from 3 independent experiments (mean ± SD) relative to EF1-α and the control. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (Student's *t* test) compared to the control.

(D) Scatter plot of DEGs analysis based on RNA-seq data. Two biological replicates were used for both Control mESCs and two sets of *Mthfd2* KD mESCs. See Table S2 for details.

(E) Heat-map of DEGs about pluripotency between Control mESCs and *Mthfd2* KD mESCs.

(F) Top 10 of enriched Gene Ontology (GO) terms potentially upregulated by *Mthfd2*.

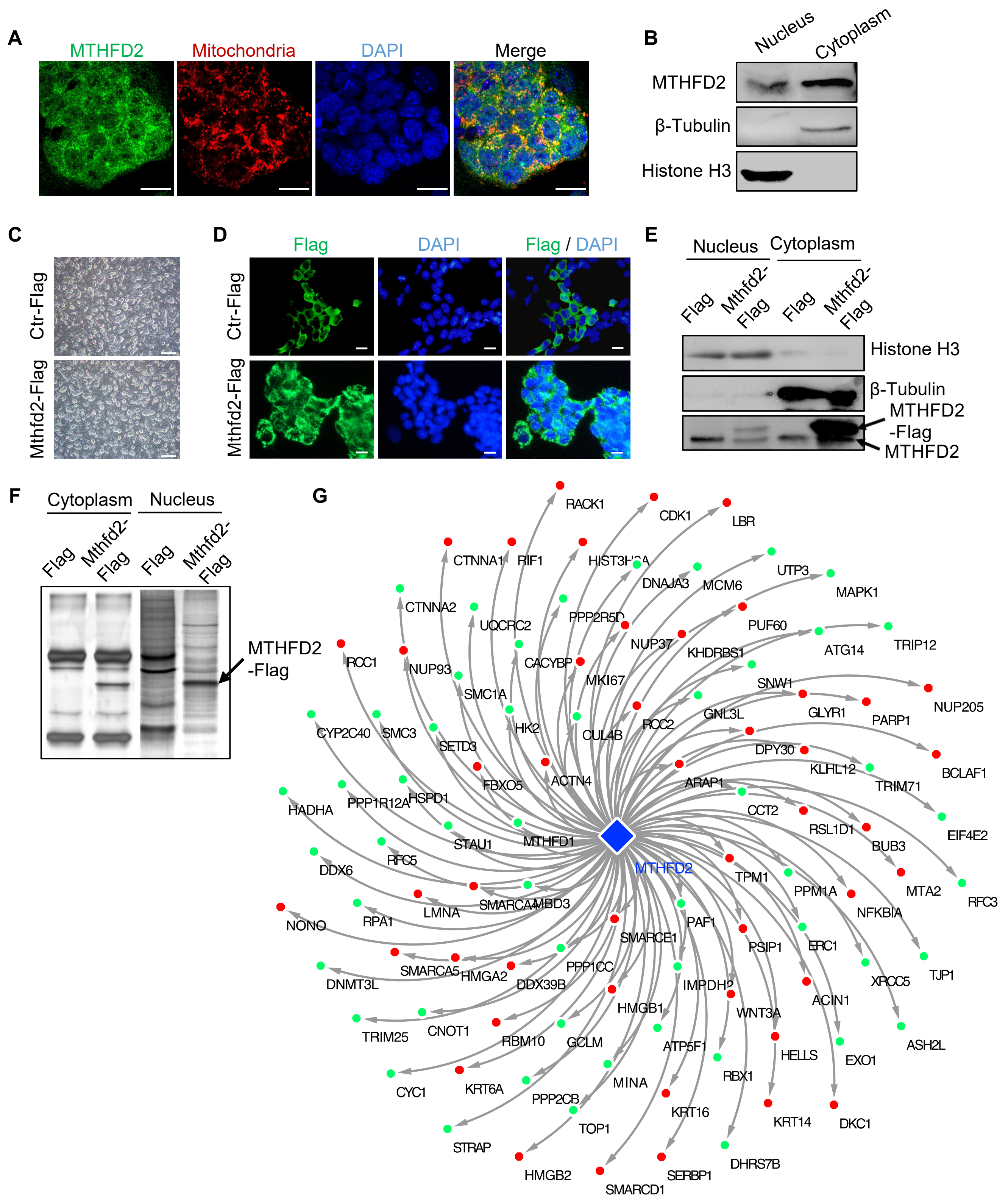


Figure S4. Results of MTHFD2 Interactome Assay, Related to Figure 3.

(A) IF staining for MTHFD2 in the mitochondria and nucleus in mESCs. DAPI was used to indicate the nuclei. Scale bar, 20 μm.

(B) WB analysis of the distribution of the Mthfd2 protein in the cytoplasm and nucleus of mESCs. β-Tubulin and Histone H3 were used as loading controls for the cytoplasmic and nuclear proteins, respectively.

(C) Representative results of Mthfd2-Flag mESCs and control cells (Ctr-Flag). Scale bar, 500 μm.

(D) IF staining for FLAG in Mthfd2-Flag mESCs and Ctr-Flag mESCs. DAPI was used to indicate the nuclei. Scale bar, 20 μm.

(E) WB analysis of the distribution of the MTHFD2 protein in the cytoplasm and nucleus of Mthfd2-Flag mESCs and Ctr-Flag mESCs. β-Tubulin and Histone H3 were used as loading controls for the cytoplasmic and nuclear proteins, respectively.

(F) Cell lysates from Mthfd2-Flag mESCs and Ctr-Flag mESCs were immunoprecipitated with an anti-FLAG antibody. The bound proteins were eluted and resolved by SDS–PAGE and then visualized by silver staining.

(G) Schematic network showing part of the MTHFD2-interacting proteins in mESCs. Red nodes indicate target genes detected in nuclear, green nodes indicate target genes detected in cytoplasm. See Table S3 for details.

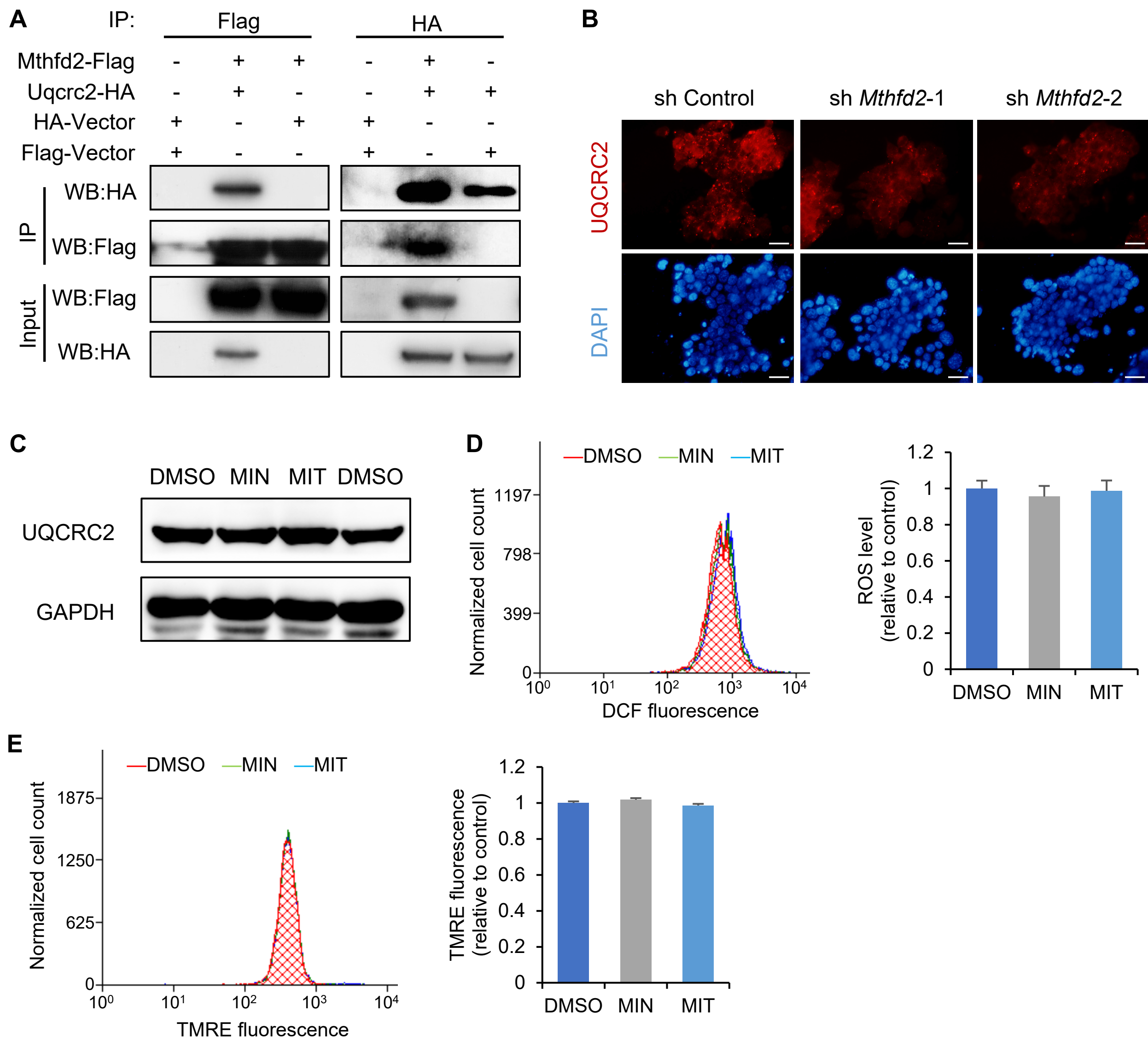


Figure S5. *Mthfd2* function in mESCs is Independent of Its Enzymatic Activity, Related to Figure 3 and 4.

(A) CO-IP results showing the specific interaction between exogenously expressed MTHFD2-Flag and UQCRC2-HA in mESCs. WB was conducted with the indicated antibodies.

(B) Immunofluorescence staining for UQCRC2 in *Mthfd2* KD mESCs. DAPI was used to indicate the nuclei. Scale bar, 50 μm.

(C) WB analysis of UQCRC2 protein level in MI-mESCs. GAPDH was used as a loading control.

(D) Flow cytometry analysis of ROS levels in MI-mESCs. A representative histogram (left) and quantification of the mean fluorescence intensity (MFI) (right) are presented.

(E) Measurements of the mitochondrial membrane potential in MI-mESCs using tetramethylrhodamine methyl ester (TMRE). A representative histogram (left) and quantification of the MFI (right) are presented.

Data are pooled from 3 independent experiments.

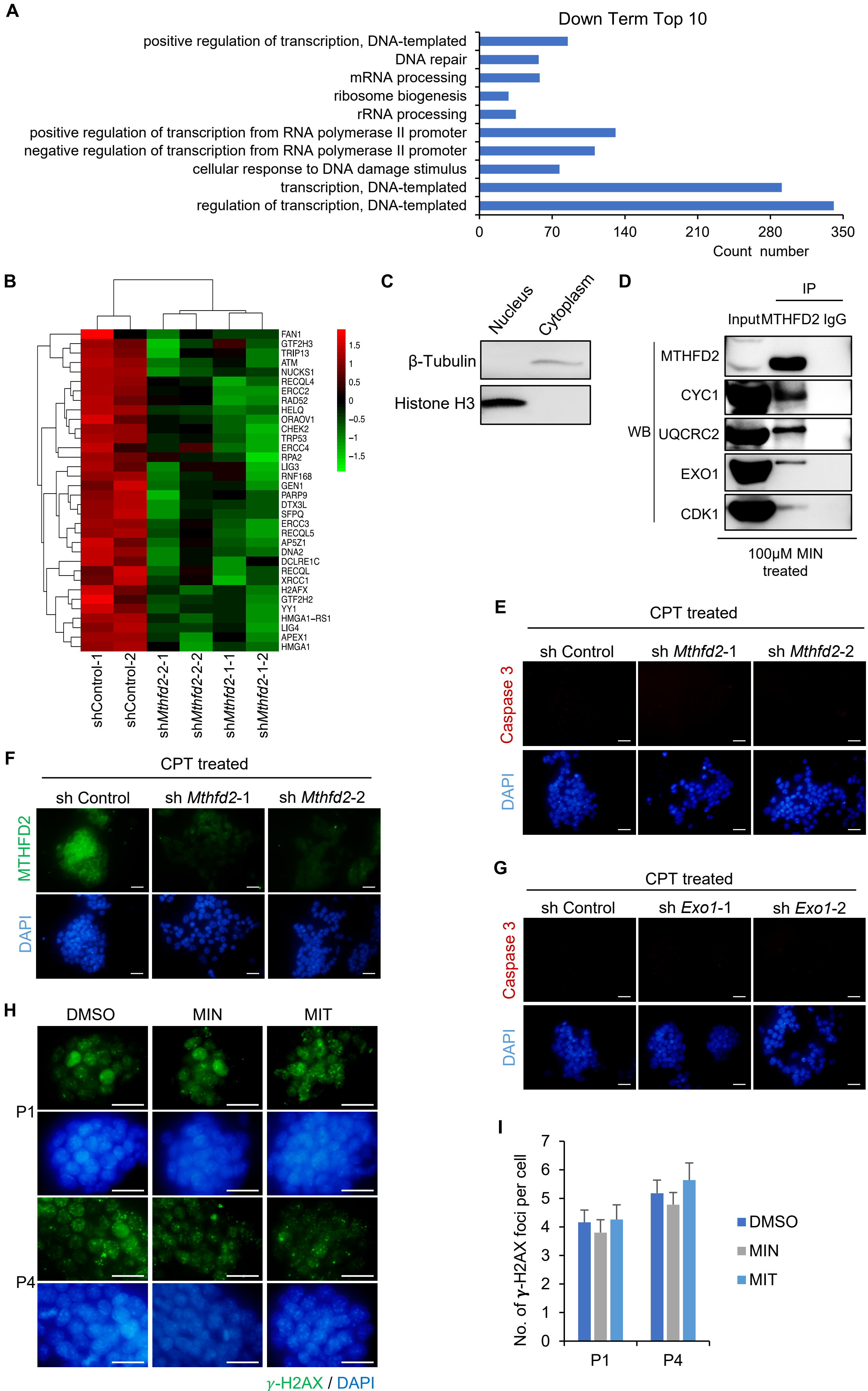


Figure S6

Figure S6. *Mthfd2* Modulates HR Repair Regardless of Its Enzymatic Activity, Related to Figure 5 and 6.

(A) Top 10 of enriched GO terms potently downregulated by *Mthfd2*.

(B) Heat-map of DEGs about DNA repair between Control mESCs and *Mthfd2* KD mESCs.

(C) WB analysis of the distribution of the cytoplasm and nucleus extracts of mESCs. β-Tubulin and Histone H3 were used as loading controls for the cytoplasmic and nuclear proteins, respectively.

(D) CO-IP results showing the interactions between endogenous MTHFD2 and UQCRC2, CYC1, EXO1, CDK1 proteins in MIN-mESCs. WB was conducted with the indicated antibodies.

(E and F) IF staining for Caspase 3 (E) and MTHFD2 (F) in *Mthfd2* KD mESCs post-CPT treatment. DAPI was used to indicate the nuclei. Scale bar, 50 μm.

(G) IF staining for Caspase 3 in *Exo1* KD mESCs post-CPT treatment. DAPI was used to indicate the nuclei. Scale bar, 50 μm.

(H and I) IF staining for γ-H2AX in MI-mESCs. Representative images (H) and quantification of the average number of γ-H2AX foci per cell (I) (n = 50 nuclei) are shown. DAPI was used to indicate the nuclei. Scale bar, 50 μm. Data are pooled from 3 independent experiments (mean $\pm$ SEM). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (Student's *t* test) compared to the control.

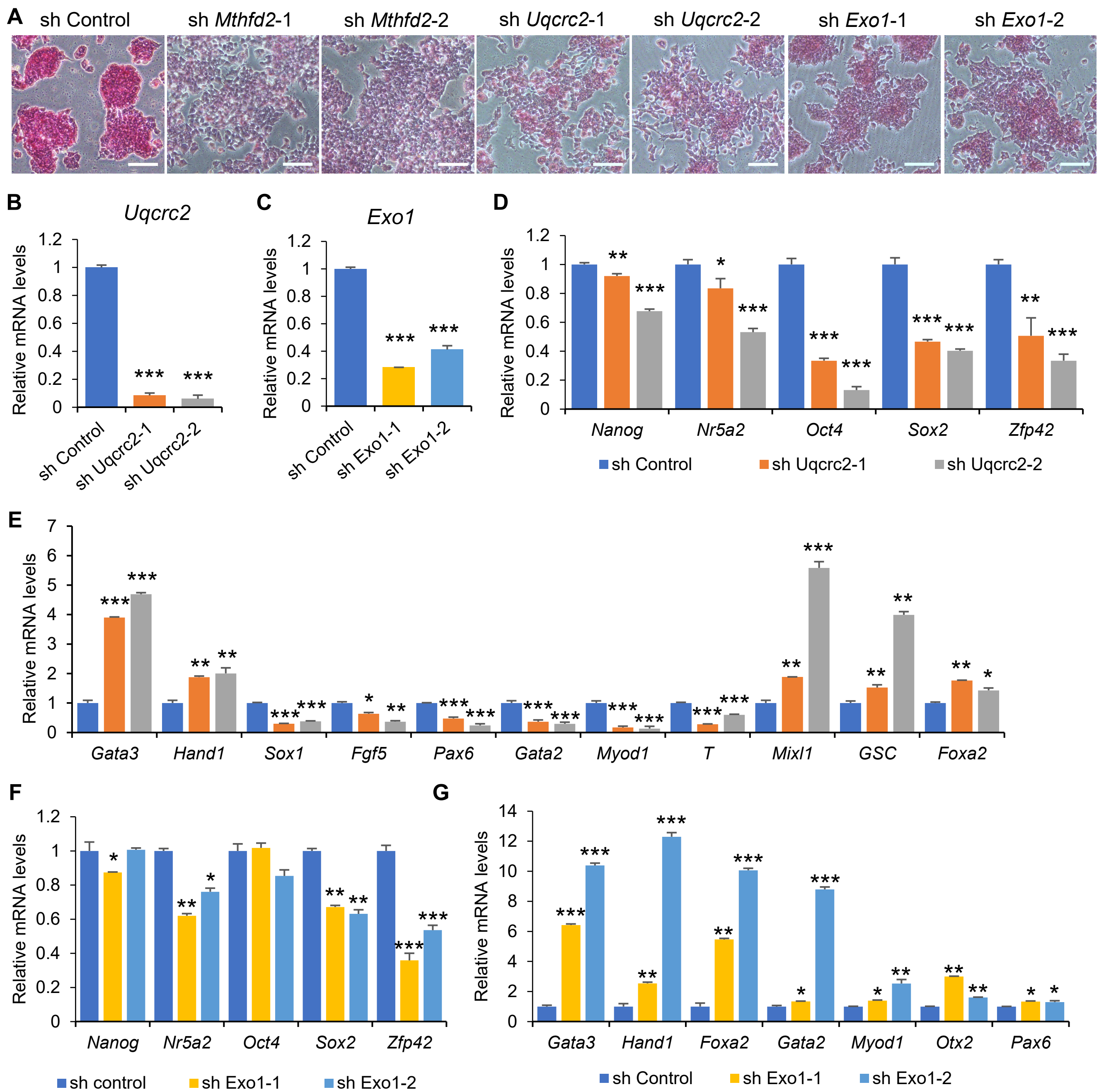


Figure S7. *Uqcrc2* or *Exo1* regulates mESC self-renewal, Related to Figure 7.

(A) Representative results of *Uqcrc2* KD mESCs and *Exo1* KD mESCs with AP staining. Scale bar, 100 μm.

(B and C) qRT-PCR analysis of mRNA levels of *Uqcrc2* and *Exo1* in the samples described in (A).

(D and E) qRT-PCR analysis of mRNA levels of pluripotency marker genes (D) and lineage marker genes (E) in *Uqcrc2* KD mESCs.

(F and G) qRT-PCR analysis of mRNA levels of pluripotency marker genes (F) and lineage marker genes (G) in *Exo1* KD mESCs.

Data (B-G) are pooled from 3 independent experiments (mean ± SD) relative to EF1-α and the control. *P < 0.05, **P < 0.01, and ***P < 0.001 (Student's t test) compared to the control.

Table S4. The List of Overexpression Primer Sequences Used in This Study, Related to Figures 1-7.

The primers used for gene overexpression		
Gene	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')
Mthfd2	ATGGCTTCAGTTTCCTTGTTG	CTAGTTGGTGGCGACTCCG
Uqcrc2	ATGAAGCTCCTCAGCAGGG	TAACTCGTCGAGAAAAGG
Cdk1	ATGGAAGACTATATCAAAATAGAGAAAATTGG	CTACATCTTCTTAATCTGATTGTCC
The primers used for generation of Flag-tagged Mthfd2 plasmid		
Name of plasmid	Primer sequences (5' to 3')	
pCAG-Mthfd2-F	CGACGCGTATGGCTTCAGTTTCCTTGTTG	
pCAG-Mthfd2-Flag-R	CCTTAATTAACTACTTATCGTCATCGTCTTTGTAATCAATATCATGATCCTTGTAGTCTCCGTCGTGGTCCTTATAGTCGTTGGTGGCGACTCCG	
PB-Ubc-Mthfd2-F	CCCTCGAGATGGCTTCAGTTTCCTTGTTG	
PB-Ubc-Mthfd2-Flag-R	CGACGCGTCTACTTATCGTCATCGTCTTTGTAATCAATATCATGATCCTTGTAGTCTCCGTCGTGGTCCTTATAGTCGTTGGTGGCGACTCCG	
The primers used for generation of HA-tagged Uqcrc2 plasmid		
Name of plasmid	Primer sequences (5' to 3')	
pCAG-Uqcrc2-F	CGGGATCCATGAAGCTCCTCAGCAGGG	
pCAG-Uqcrc2-HA-R	CCTTAATTAATTAAGCGTAATCTGGAACATCGTATGGGTATAACTCGTCGAGAAAAGG	
The primers used for GST pull down		
Gene	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')
Exo1	AAGGAAAAAAGCGGCCGCATGGGGATTCAAGGGTTACT	CGGAATTCTTAGTGAAATATTGCTCTCTG
Cdk1	AAGGAAAAAAGCGGCCGCATGGAAGACTATATCAAAATAGAGAAAATTGG	CGGAATTCCTACATCTTCTTAATCTGATTGTCCAAGTCA
Uqcrc2	AAGGAAAAAAGCGGCCGCATGAAGCTCCTCAGCAGGG	CGCGGATCCTTATAACTCGTCGAGAAAAGG
Cyc1	AAGGAAAAAAGCGGCCGCATGGCGGCGGCGGCGGCTT	CGGAATTCTCACTTGGGTGGCCGATAA
Mthfd2	CGCGGATCCATGGCTTCAGTTTCCTTGTTG	AAGGAAAAAAGCGGCCGCCTAGTTGGTGGCGACTCCG

Table S5. The List of shRNA and sgRNA Sequences Used in This Study, Related to Figures 1, 3-7.

The list of shRNA sequences		
Name of shRNA	shRNA sequences (5' to 3')	
sh *Mthfd2*-1F	CGCGTCCCCGTCCAAACAGCGCGGAGTCTTCAAGAGAGACTCCGCGCTGTTTGGACTTTTTGGAAAT	
sh *Mthfd2*-1R	CGATTTCCAAAAAGTCCAAACAGCGCGGAGTCTCTCTTGAAGACTCCGCGCTGTTTGGACGGGGA	
sh *Mthfd2*-2F	CGCGTCCCCGCAGGCATTCCAACCTTAGGTTCAAGAGACCTAAGGTTGGAATGCCTGCTTTTTGGAAAT	
sh *Mthfd2*-2R	CGATTTCCAAAAAGCAGGCATTCCAACCTTAGGTCTCTTGAACCTAAGGTTGGAATGCCTGCGGGGA	
sh *Mthfd2*-3F	CGCGTCCCCGTGCCGATTGCAATGTTGCTTTCAAGAGAAGCAACATTGCAATCGGCACTTTTTGGAAAT	
sh *Mthfd2*-3R	CGATTTCCAAAAAGTGCCGATTGCAATGTTGCTTCTCTTGAAAGCAACATTGCAATCGGCACGGGGA	
sh *Uqcrc2*-1F	GATCCCCGCTGTTGCCTTTCAGAATTCATTCAAGAGATGAATTCTGAAAGGCAACAGCTTTTTA	
sh *Uqcrc2*-1R	AGCTTAAAAAGCTGTTGCCTTTCAGAATTCATCTCTTGAATGAATTCTGAAAGGCAACAGCGGG	
sh *Uqcrc2*-2F	GATCCCCGCAGGTGGCTGAACAATTTCTTTCAAGAGAAGAAATTGTTCAGCCACCTGCTTTTTA	
sh *Uqcrc2*-2R	AGCTTAAAAAGCAGGTGGCTGAACAATTTCTTCTCTTGAAAGAAATTGTTCAGCCACCTGCGGG	
sh *Uqcrc2*-3F	GATCCCCGCTCTAGCTGCAGGTTCTTATTTCAAGAGAATAAGAACCTGCAGCTAGAGCTTTTTA	
sh *Uqcrc2*-3R	AGCTTAAAAAGCTCTAGCTGCAGGTTCTTATTCTCTTGAAATAAGAACCTGCAGCTAGAGCGGG	
sh *Exo1*-1F	GATCCCCGGATACCTACTGTTGGCTTCATTCAAGAGATGAAGCCAACAGTAGGTATCCTTTTTA	
sh *Exo1*-1R	AGCTTAAAAAGGATACCTACTGTTGGCTTCATCTCTTGAATGAAGCCAACAGTAGGTATCCGGG	
sh *Exo1*-2F	GATCCCCGGGTCAAGCCGATTCTCATATTTCAAGAGAATATGAGAATCGGCTTGACCCTTTTTA	
sh *Exo1*-2R	AGCTTAAAAAGGGTCAAGCCGATTCTCATATTCTCTTGAAATATGAGAATCGGCTTGACCCGGG	
Sequences for generation of *Mthfd2* knockout plasmid		
	Forward Primer Sequence (5'to 3')	Reverse Primer Sequence (5'to 3')
Mthfd2-sgRNA-1	CACCGAGTTTCCTTGTTGTCTGCGT	AAACACGCAGACAACAAGGAAACTC
Mthfd2-sgRNA-2	CACCGCGGTTGTTGCGCCCCACGCA	AAACTGCGTGGGGCGCAACAACCGC
Sequences for check *Mthfd2* knockout		
Mthfd2-561	TGCCTTTCAGGGGTTCTC	AAGCGTCCGCATCTCCAC
Mthfd2-623	TTGGCCTAGCTGAGGGAC	GGGAGGAGGGAAGTTGGTA

Table S6. The List of qRT-PCR Primer Sequences Used in This Study, Related to Figures 1, 2 and 7.

Gene	Forward Primer Sequence (5'to 3')	Reverse Primer Sequence (5'to 3')
Mthfd2	CCTTGTTGTCTGCGTTGGCT	ATGACAACGGCTTCATTTCGCA
EF1α	GTGTTGTGAAAACCACCGCT	AGGAGCCCTTTCCCATCTCA
Exo1	TAAACACGTCGAGCCTGTCC	CAGAGCCCAGGAACCTTGTT
Uqcrc2	CCGGGTCCTTCTCGAGATTT	CTGCTTTAACGAACAAGCCGA
Cdk1	TAGACTTCCCAGCAGCCATTC	CACTTCCATCTGGGGGTCAT
Cyc1	ATCGTTCGAGCTAGGCATGG	GCCGGGAAAGTAAGGGTTGA
pMX-1811s	GACGGCATCGCAGCTTGGATACAC	
Oct4 Rv		CCAATACCTCTGAGCCTGGTCCGAT
Sox2 Rv		GCTTCAGCTCCGTCTCCATCATGTT
Klf4-943Rv		GTGGGTTAGCGAGTTGGA
c-Myc Rv		TCGTCGCAGATGAAATAGGGCTG
ex-Mthfd2		CCTGCTGTACTTCTTGCTTGATCTG
en-Oct4	AGTGGGGCGGTTTTGAGTAA	TTCCAAAGAGAACGCCCAGG
en-Sox2	GATCAGCATGTACCTCCCCG	TCCTCTTTTTGCACCCCTCC
Oct4	GGCGTTCTCTTTGGAAAGGTG	AGTTCGCTTTCTCTTCCGGG
Sox2	CCCACCTACAGCATGTCCTAC	AGTGGGAGGAAGAGGTAACCA
Klf4	GACTAACCGTTGGCGTGAGG	GTCTAGGTCCAGGAGGTCGT
Cdx2	AAGACAAATACCGGGTGGTG	CCAGCTCACTTTTCCTCCTG
Eomes	ACCGGCACCAAACTGAGA	AAGCTCAAGAAAGGAAACATGC
Esrrb	TTTCTGGAACCCATGGAGAG	AGCCAGCACCTCCTTCTACA
Fgf5	ATCTACCCGGATGGCAAAGT	TCTCGGCCTGTCTTTTCAGT
Foxa2	GAGCAGCAACATCACCACAG	CGTAGGCCTTGAGGTCCAT
GSC	AGTCAGAAAACGCCGAGAAG	TCGACTGTCTGTGCAAGTCC
Hand1	TCAAAAAGACGGATGGTGGT	GCGCCCTTTAATCCTCTTCT
Klf2	ACCAAGAGCTCGCACCTAAA	GTGGCACTGAAAGGGTCTGT
Nanog	TTCTTGCTTACAAGGGTCTGC	AGAGGAAGGGCGAGGAGA

Nr5a2	TGCTGAGCCCTGAAGCTATT	AGGGTTACTGCCCGTTTTCT
Otx2	AGAGGACGACATTTACTAGGGC	ATTCTTAAACCATACCTGCACC
Pax6	TACCAGTGTCTACCAGCCAAT	TGCACGAGTATGAGGAGGTCT
Zfp42	CAGTTCGTCCATCTAAAAAGGGAGG	TCTTAGCTGCTTCCTTGAACAATGCC
Sox1	ATACCGCAATCCCCTCTCAG	ACAACATCCGACTCCTCTTCC
Sox17	CTCGGGGATGTAAAGGTGAA	CTTTGGCCCACACCATAAAG
T	ATCAGAGTCCTTTGCTAGGTAG	GTTACAATCTTCTGGCTATGC
Tbx3	ATCGCCGTTACTGCCTATCA	TGCAGTGTGAGCTGCTTTCT
Gata3	CTTATCAAGCCCAAGCGAAG	CATTAGCGTTCCTCCTCCAG
Gata2	AGCTGCACAATGTTAACAGGC	AAGGGCGGTGACTTCTCTTG
Myod1	TACAGTGGCGACTCAGATGC	TAGTAGGCGGTGTCGTAGCC
Nodal	ACGTTCACCGTCATTCCTTC	GTAGGGCTGATGCCAACACT
Utf1	CTACGAGGTTCCTTCGACCA	GACTGGGAGTCGTTTCTGGA
KDR	ATGAATTGCCCTTGGATGAG	AGCGTCTGCCTCAATCACTT
Myf5	CCACCAACCCTAACCAGAGA	GTTCTCCACCTGTTCCCTCA
Mixl1	CCATGTACCCAGACATCCACT	CGGTTCTGGAACCACACCT
Acvr1b	GGCTCAGGGTTACCCCTTTT	TTCACGGAACCAAGACCGTT
Spry2	TCCACCGATTGCTTGGAAGT	CACATCTGAACTCCGTGATCG
Tdgf1	CTGCCCAAGAAGTGTTCCCTG	TCGTCACAGACGGCGTTTG
Bmp4	AGGAGGAGGAGGAAGAGCAG	CCTGGGATGTTCTCCAGATG
Nes	GATCGCTCAGATCCTGGAAG	AGGTGTCTGCAAGCGAGAGT

Table S7. Cell Proliferation of *Mthfd2* Knockdown Cells, Related to Figure 1.

First Experiment					
	Day1	**Day2**	**Day3**	**Day4**	
sh Control mESCs	3	29	98	266	x 10^4 cells
sh *Mthfd2*-1 mESCs	3	30.4	110	280	x 10^4 cells
sh *Mthfd2*-2 mESCs	3	28.6	100	270	x 10^4 cells
Second Experiment					
	Day1	**Day2**	**Day3**	**Day4**	
sh Control mESCs	2.4	15.2	68	224	x 10^4 cells
sh *Mthfd2*-1 mESCs	2.4	14	70	218	x 10^4 cells
sh *Mthfd2*-2 mESCs	2.4	13.4	67	210	x 10^4 cells
Third Experiment					
	Day1	**Day2**	**Day3**	**Day4**	
sh Control mESCs	2.6	24	92	210	x 10^4 cells
sh *Mthfd2*-1 mESCs	2.6	20	84.6	206	x 10^4 cells
sh *Mthfd2*-2 mESCs	2.6	22.4	88	220	x 10^4 cells

Supplemental Experimental Procedures

Key Resources Table

Reagent or Resource	**Source**	**Identifier**
Antibodies		
Mthfd2	Santa Cruz	sc-390709
Mthfd2	Abcam	ab151447
Nanog	Abcam	ab70482
Sox2	Abcam	ab97959
SSEA1	Abcam	ab16285
Nestin	Abcam	ab134017
β-Tubulin	Abcam	ab151318
Histone H3	EASYBIO	BE3021
Uqcrc2	Absin	abs116449
Cdk1	Absin	abs115034
Cyc1	Absin	abs104557
Exo1	Absin	abs140694a
Oct 3/4	Santa Cruz	SC-5279
α-Smooth Muscle Actin	Abcam	ab5694
Gata6	Abcam	ab22600
Gapdh	Cell Signaling Technology	2118L
GST	EASYBIO	BE2013
Caspase 3	Cell Signaling Technology	#9664s
HA	EASYBIO	BE2007
HA	EASYBIO	BE2008
Flag	EASYBIO	BE2004
Flag	EASYBIO	BE2005
gamma H2A.X	Abcam	ab11174
Mouse IgG	Beyotime	A7028

Rabbit IgG	Beyotime	A7016
Phospho-CDK Substrate Motif [(K/H) pSP]	Cell Signaling Technology	9477S
Rad51	Abcam	ab133534
Phospho-Threonine-Proline (P-Thr-Pro-101)	Cell Signaling Technology	9391S
Bacterial and Virus Strains		
pSuper-puro	Oligoengine	VEC-PBS-0008
pLVTHM	Addgene	12247
pMXs-Retroviral	Genome Institute of Singapore	N/A
pX330-Green	Massachusetts Institute of Technology	N/A
pCAG-IRES-puro	This paper	N/A
PB-Ubc-Neo	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
Phosphatase Inhibitor Cocktail	Cell Signaling Technology	5870S
Protease Inhibitor Cocktail	Cell Signaling Technology	5871S
MG132	Selleck	S2619
Camptothecin	Selleck	S1288
Doxycycline	Sigma	D9891
Polybrene (Hexadimethrine bromide)	Sigma	107689
Critical Commercial Assays		
Alkaline Phosphatase Detection Kit	Millipore	SCR004
ATP Assay Kit	Beyotime	S0026B
TMRE-Mitochondrial Membrane Potential Assay Kit	Abcam	ab113852
Reactive Oxygen Species Assay Kit	Beyotime	S0033
Cell lysis buffer for Western and IP	Beyotime	P0013
Fast Silver Stain Kit	Beyotime	P0017S
Protein G Agarose	Roche	11243233001
ECL luminescence reagent	Sangon Biotech	C510043
MitoSOX Red Mitochondrial Superoxide Indicator	Yeasen	40778ES50
cycloheximide	MedChemExpress	HY-12320

Antimycin A	Biovision	2247-10
RO-3306	MedChemExpress	HY-12529
MIN	synthesis	N/A
MIT	synthesis	N/A
GelRed	Biotium	41003
Deposited Data		
RNA-sequence data	This paper	SRP149554
Microarray data	(Heng et al., 2010)	GSE19023
Microarray data	(Han et al., 2010)	GSE19164
RNA-sequence data	(Cao et al., 2014)	SRA076823
Experimental Models: Cell Lines		
E14	Genome Institute of Singapore	CRL-1821
G4	Mount Sinai Hospital	N/A
Experimental Models: Organisms/Strains		
Oct4-GFP (OG2) mice	Tongji University; National Institute of Biological Sciences	N/A
Rosa26-M2rtTA Col1a1-tetO-Pou5f1 (TF4) mice	(Gao et al., 2013)	N/A
Recombinant DNA		
pCAG-Mthfd2-puro plasmid	This paper	N/A
pCAG-Mthfd2-Flag-puro plasmid	This paper	N/A
Pb-Ubc-Mthfd2-Flag-Neo plasmid	This paper	N/A
pCAG-Uqcrc2-HA-puro plasmid	This paper	N/A
pSuper-Mthfd2-puro plasmid	This paper	N/A
pLVTHM-Mthfd2-GFP plasmid	This paper	N/A
pSuper-Uqcrc2-puro plasmid	This paper	N/A
pSuper-Cyc1-puro plasmid	This paper	N/A
pSuper-Exo1-puro plasmid	This paper	N/A
pMXs-Oct4 plasmid	(Takahashi and Yamanaka, 2006)	N/A
pMXs-Sox2 plasmid	(Takahashi and Yamanaka, 2006)	N/A
pMXs-Klf4 plasmid	(Takahashi and Yamanaka, 2006)	N/A

pMXs-Mthfd2 plasmid	This paper	N/A
pCAG-Uqcrc2-Neo plasmid	This paper	N/A
pCAG-Cdk1-Neo plasmid	This paper	N/A
pCAG-Mthfd2-Neo plasmid	This paper	N/A
pET-28a (+) (Uqcrc2)	This paper	N/A
pET-28a (+) (Cdk1)	This paper	N/A
pET-28a (+) (Cyc1)	This paper	N/A
pET-28a (+) (Exo1)	This paper	N/A
pGEX-4T-1 (Mthfd2)	This paper	N/A
Software and Algorithms		
Summit 5.2	Beckman Coulter	N/A
Trimmomatic	(Bolger et al., 2014)	N/A
Hisat2 2.1.0	(Kim et al., 2015)	N/A
HT-seq 0.61	(Anders et al., 2015)	N/A
DESeq2 1.18.1	R package (Love et al., 2014)	N/A
GOstats 2.44.0	R package (Falcon and Gentleman, 2007)	N/A
Mascot 2.4	Matrix Science	N/A
CASP comet assay software	Andor Technology	N/A

Contact for Reagent and Resource Sharing

Further information and requests for reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Jianyong Han (hanjy@cau.edu.cn).

Experimental Procedures

Animal Experiments

All animal studies proceeded according to the guidelines of the Institute Animal Care and Use Committee and were approved by the Animal Care and Use Committee of China Agricultural University. We used CD1 (ICR) mice as the embryo donors and recipients, which were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All mice were maintained in specific pathogen-free (SPF) conditions with a 12 h dark/12 h light cycle.

Cell Culture and Transfection.

For G4 and E14 mESCs, Leukemia inhibitory factor (LIF) and serum culture medium were used to maintain mESCs self-renewal. The serum + LIF mESCs medium contained DMEM with 15% fetal bovine serum (FBS), 1% GlutaMAX, 1% sodium pyruvate, 1% nonessential amino acids (NEAA), 0.1 mmol/L β-mercaptoethanol, 1% penicillin/streptomycin (all from Gibco) and 1000 units/mL mouse Leukemia Inhibitory Factor (mLIF, Miltenyi Biotec).

To detect the developmental potential of iPSCs by 8-cell-stage embryo injection and tetraploid complementation, the OSKM2 iPSCs were cultured in 2i+LIF mESCs medium. The 2i+LIF mESCs medium contained DMEM with 15% FBS, 1% GlutaMAX, 1% sodium pyruvate, 1% NEAA, 0.1 mmol/L β-mercaptoethanol, 1% penicillin/streptomycin, 1000 units/mL mLIF, 1 μM PD0325901 (4423, Tocris), and 3 μM CHIR99021 (4192, Tocris).

The MEF cells were maintained on Gelatin in MEF medium, which contained DMEM with 10% FBS, 1% GlutaMAX, 1% NEAA and 1% penicillin/streptomycin.

Plat-E packaging cells (Cell Biolabs), which were used to produce retrovirus, were maintained in MEF medium. All cells were cultured in a humidified incubator at 37°C and 5% CO2.

Transfection of plasmids into mESCs and MEF cells and Plat-E packaging cells was performed using Lipofectamine 3000 (Invitrogen).

Construction of Vectors

In order to overexpress the candidate protein, the coding sequence of full-length mouse candidate genes was cloned into the pMXs retroviral vector, pCAG-IRES-puro vector or PB-Ubc-neo vector. The sequences of primers are showed in Table S4.

The short hairpin RNA (shRNA) targeting mouse *Mthfd2* mRNA or other genes and the control shRNA was cloned into pSuper-puro Vector (Oligoengine). The sequences of shRNA are showed in Table S5.

The sgRNAs for *Mthfd2* were designed by using the website provided by Zhang Feng laboratory (https://zlab.bio/guide-design-resources). The sequences of sgRNA are showed in Table S5. Then the sgRNA sequence was cloned into the pX330-Green vector.

Generation of Gene Knockdown Cells

The pSuper-puro plasmid was transfected into mESCs by Lipofectamine 3000. 24 h later, the mESCs were selected with 2 μg/mL of puromycin for 4 days. All those cells were cultured in serum + LIF mESCs medium.

Generation of Homozygous Knockout Cells

The mESCs were electroporated with pX330-Mthfd2-Green plasmid or pX330-Green control plasmid and cultured in serum + LIF mESCs medium. The plasmid was transiently expressed in cells by electroporation. 3 days later, the GFP-positive cell population were sorted by flow cytometry, and cultured in serum + LIF mESCs medium. Then colonies were picked and dissociated into single cells by TrypLE (Invitrogen). Most cells were cultured to obtain the stable *Mthfd2* KO cell lines, few remaining cells were lysed by Embryonic Cell Lysis Solution (CAT#:130806-1, TIANDZ). The Embryonic Cell Lysis Solution can be used to lyse 2-8 cells, and the lysate can be directly used for PCR. The knockout regions of those cells were amplified by PCR using primers (Table S5) and sequenced. By aligning with the wild type sequence, homozygous knockout cell clones were identified. According the previous reports that Mthfd2 may have an impact on cell proliferation in cancer cell, we prefer to pick the slow-growing colonies. The mESCs electroporated with pX330-Green control plasmid were used as the control cells.

Derivation of Mouse Embryonic Fibroblast Cells (MEFs)

MEFs were isolated from 13.5-day embryos heterozygous for the Oct4-GFP/OSKM transgenic allele. Isolated MEFs in early passages (up to passage 4) were used for further experiments.

Induction of Pluripotent Stem Cells

For iPSCs induction, pMXs retroviral vectors containing coding sequences of mouse *Oct4*, *Sox2*, *Klf4* and candidate genes cDNAs were cotransfected into Plat-E cells using Lipofectamine 2000 (Invitrogen). Culture medium containing virus particles was collected at 24 or 48 h after transfection and filtered through a 0.22 μm filter. MEF cells were infected with equal amounts of OSK and candidate genes viruses mixed with 8 μg/mL polybrene (Sigma-Aldrich). After two rounds of infection, infected cells were changed to serum + LIF mESCs medium or KOSR medium (DMEM supplemented with 10% KnockOut SR XenoFree CTS (KOSR), 1% GlutaMAX, 1% sodium pyruvate, 1% NEAA, 0.1 mmol/L β-

mercaptophenol, 1% penicillin/streptomycin and 1000 units/mL mLIF). The medium was replaced daily. For quantification of reprogramming efficiency using the Oct4-GFP reporter, the transfected MEFs were scanned under a fluorescence microscope in Biostation (Nikon) on day 16 and scored for GFP^+ colonies. On day 18, cells were fixed and stained for alkaline phosphatase activity using an alkaline phosphatase detection kit (Millipore).

Embryoid Body and Teratoma Formation

The iPSCs were grown to approximately 80% confluence and dissociated into single cells in the differentiation medium (DMEM supplemented with 15% FBS, 1% GlutaMAX, 1% NEAA, 0.1 mmol/L β-mercaptophenol). They were seeded at a density of 1×10^5 cells per uncoated 3.5 cm Petri dish. The dishes were taped to a rotary platform in the incubator. Medium was changed every 2 days. Embryoid bodies were collected on day 7 for further differentiation on the uncoated Petri dishes in the differentiation medium.

Approximately 1×10^6 iPSCs were suspended in 150 µL DPBS and injected into NOD-SCID mice to form teratomas. Four weeks after injection, the teratomas were harvested, fixed overnight with 4% paraformaldehyde, embedded in paraffin, sectioned, HE stained, and analyzed.

Production of Chimeric Mice

15-20 iPSCs (with a C57/BL6 background) were injected into an 8-cell stage embryo which was obtained from superovulated female CD1 mice. The reconstructed embryos were cultured *in vitro* and developed into blastocysts. Chimeras were produced by transplantation of injected blastocysts into uterus of pseudopregnant CD1 mice.

Tetraploid Embryo Complementation

The generation of mice by tetraploid embryo complementation was performed using previously described methods (Zhao et al., 2009). Briefly, two-cell embryos were collected from the oviducts of CD-1 females (white coat color) and electrofused by Electro cell manipulator (ECM 2001, BTX Harvard Apparatus) to produce one-cell tetraploid embryos that were then incubated in KSOM until the blastocyst stage. 15-20 iPSCs were injected into each tetraploid blastocyst and approximately 10-14 embryos were transferred to CD-1 pseudopregnant recipient females. All the embryos and live pups

derived from tetraploid blastocyst injection were male, since the injected iPSCs or mESCs were originated from male mice.

Identification of Cell Proliferation

Cells were plated at 2×10^4 cells/well in 12-well plates to examine growth curves. The cells were washed with DPBS, treated with TrypLE, and counted with a LUNA Automated Cell Counter at the indicated times to identify their proliferation.

RNA Purification and cDNA Preparation

Total RNA was extracted from cells using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The RNA was reverse-transcribed using oligo-dT and M-MLV Reverse Transcriptase (Promega).

Quantitative Real-Time PCR

Quantitative Real-Time PCR (qRT-PCR) was run on a Light Cycler 480 II Real-Time PCR System (Roche) using the Light Cycler 480 SYBR Green I Master (Roche). The data was analyzed using the comparative CT ($2^{-\Delta\Delta CT}$) method. The ΔCT was calculated using EF1-α or Gapdh as internal control. All experiments were performed more than three biological replicates. Primer sequences were provided in Table S6.

Immunofluorescence Staining

For immunofluorescence (IF) staining, cells were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature, permeabilized with 0.5% Triton X-100 for 30 min, blocked with PBS containing 5% BSA (Sigma) for 2 h. The cells were then stained with primary antibodies against Oct4 (Santa Cruz SC-5279), Sox2 (Abcam ab97959), Nanog (Abcam ab70482), SSEA1 (Abcam ab16285), Nestin (Abcam ab134017), Gata6 (Abcam ab22600), α-Smooth Muscle Actin (Abcam ab5694), Mthfd2 (Abcam ab151447) at 4°C overnight, followed by staining with the respective secondary antibodies conjugated to Alexa Fluor (Invitrogen). For nuclear staining, the cells were counterstained with DAPI (Sigma) for 5 min.

Western Blot Analysis

Cell extracts were prepared in lysis buffer (20 mM Tris-HCl pH 7.5, 80 mM NaCl, 2 mM EDTA, 10% glycerol, 0.2% NP-40) supplemented with the PIC and phosphatase inhibitor cocktail. Protein concentration was determined with a Bradford Protein Assay Kit (Bio-Rad). Cell lysates were boiled for 5 min and the equal amounts of denatured protein samples were separated by SDS-PAGE gel and transferred to Immobilon PVDF 0.2 mm membranes (Millipore). Membranes were blotted with 5% non-fat milk prepared in Tris-buffer saline-plus 0.1% Tween-20 (TBST) at room temperature for 1 h and then incubated with primary antibodies diluted in 5% non-fat milk overnight at 4°C, followed by the treatment with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h on the next day. After washing with TBST three times, the blotted membranes were exposed with ECL luminescence reagent (Sangon Biotech). For protein degradation assays, 24 h after control and *Mthfd2* shRNA transfection, mESCs were treated with 2ug/mL puromycin for 48h-72h and then treated with 10 mM MG132 (Selleck) for 3 h before harvest(Zhu et al., 2017). The following antibodies were used for western blotting: Mthfd2(Santa Cruz sc-390709; Abcam ab151447), Oct4 (Santa Cruz SC-5279), Gapdh (Cell Signaling Technology 2118L), β-tubulin (Abcam ab151318), Uqcrc2 (Absin abs116449), Cyc1 (Absin abs104557), Cdk1 (Absin abs115034), Exo1 (Absin abs140694a), HA (EASYBIO BE2007-100; EASYBIO BE2008-100), FLAG (EASYBIO BE2004-100; EASYBIO BE2005-100), Phospho-CDK Substrate Motif [(K/H)pSP] (Cell Signaling Technology 9477S), Phospho-Threonine-Proline (P-Thr-Pro-101) (Cell Signaling Technology 9391S). All antibodies were used at the recommended concentration.

Generation of Mthfd2-Flag mESCs and Uqcrc2-HA mESCs

To establish a mESC line stably expressing Mthfd2-Flag, mESCs were electroporated with 4PB (transposase) and the pCAG-Mthfd2-Flag-puro plasmid or PB-Ubc-Mthfd2-Flag-neo plasmid which carried a sequence of Mthfd2 fused with 3xFlag at its C-terminal. Then, the mESCs were selected with 2 μg/mL of puromycin for 4 days or 0.5 mg/mL of G418 for one week. Similarly, to establish a mESC line stably expressing Uqcrc2-HA, mESCs were electroporated with 4PB (transposase) and the pCAG-Uqcrc2-HA-puro plasmid which carried a sequence of Uqcrc2 fused with HA at its C-terminal. Then, the mESCs were selected with 0.5 mg/mL of G418 for one week. All those cells were cultured in serum + LIF mESCs medium.

Subcellular Fraction Isolation

Control and Mthfd2-Flag mESCs cultured in 100 mm petri dishes were washed twice by DPBS, cells were scraped and added 5 mL lysis buffer (10 mM HEPES-NaOH pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM b-mercaptoethanol supplemented with Protease Inhibitor Cocktail (PIC) and phosphatase inhibitor cocktail), vortex shortly, and incubated on ice for 20 min. Then the cell lysate was supplemented with 100 μL of 10% NP-40, vortex shortly, incubated on ice for 1 min, and centrifuged at 16,000g for 10 min. The supernatant was kept as cytoplasmic proteins, and the pellet was used for nuclear protein extraction. Next, the pellet was washed twice by cold DPBS (2 mL/each), then treated by 1 mL of the nuclei lysis buffer (10 mM Tris-HCl, pH 7.6, 420 mM NaCl, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, 2 mM $MgCl_2$ plus PIC and phosphatase inhibitor cocktail), and dispersed by tips and incubated on ice for 40 min, followed by vortex every 5 min. The lysate was centrifuged at 16,000g for 15 min, the supernatant was kept as nuclear proteins (before proceeding to IP assays, 1 mL of lower salt buffer consisting of 10 mM Tris-HCl, pH 7.6, 1 mM DTT, 1 mM PMSF, 2 mM $MgCl_2$ plus PIC and phosphatase inhibitor cocktail was added to adjust the concentration of NaCl to 210 mM). The separated subcellular fractions were used for further assays and analysis.

GST pull down

The bacteria were grown in sterile lysogeny broth (LB) medium with constant swirling (37°C, 180 r/min). After about 3 h, the bacteria were transferred to 500 mL sterile LB medium and incubated at the same condition to the exponential growth phase that measured as the OD600. The expression of interest proteins was induced with IPTG at a final concentration of 1 mM. The bacteria were incubated overnight in a shaker at 16°C. The next morning, the bacteria were centrifuged (3500 r/min, 4°C, 15 min) to pellet the bacteria.

To purify protein, the bacteria pellet was re-suspended in lysis buffer and lysed by sonication on ice for maintaining lower temperature. Then the lysate was centrifuged at 18400 r/min for 45 min at 4°C. Obtained supernatant was purified using a Ni^{2+} affinity chromatography column. Finally, both the precipitation after sonication and the purified proteins were detected by 12% SDS-PAGE.

Briefly, 0.5 mg of His-Uqcrc2/Cyc1/Exo1/Cdk1 fusion proteins in 1000 mL GST binding buffer (250 mM NaCl, 40 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.1 mM PMSF, 0.1 mg/mL BSA) were exposed to GST conjugated Mthfd2 at 4°C for more than 1.5 h,

followed by three times of warm wash using GST binding buffer. Finally, conjugated proteins were eluted by SDS loading buffer boiling at 100°C for 10 min, and examined by western blot analysis.

Intracellular Lactate and Glucose Content Measurement

Intracellular glucose content and lactate accumulation of Control mESCs, *Mthfd2* KD mESCs and *Uqcrc2* KD mESCs were measured after 48 h of culture. Intracellular glucose contents were measured using Liquid chromatography-mass spectrometry (LC-MS). Intracellular lactate accumulations were measured using Gas chromatography-mass spectrometry (GC-MS).

Measurement of ROS and Mitochondrial Superoxide Measurement

For measurement of mitochondrial superoxide, cells were washed with DPBS and dissociated into single cells by TrypLE, and counted with a LUNA Automated Cell Counter. The same number of cells were used for subsequent detection.

For measurement of complete intracellular ROS, 2′,7′-dichloro uorescine diacetat (DCFH-DA, Beyotime) were mixed with dilution buffer HBSS and distributed evenly into each sample tube. Cells were incubated with 50 μM DCFH-DA for 30 min at 37°C (protected from light), mixed upside down every 5 min (protected from light). Cells were washed 3 times with DPBS, resuspended in DPBS and analyzed by flow cytometry on a Moflo-XDP (Beckman). The fluorescence values of 50,000 cells were analyzed for each sample. Fluorescence readings were normalized to cell number. The experiments were repeated three times independently. Data were analyzed as mean fluorescence intensity using summit 5.2 software.

For measurement of mitochondrial superoxide, MitoSOX Red Indicator (Yeasen) were mixed with dilution buffer HBSS and distributed evenly into each sample tube. Cells were incubated with 5 μM MitoSOX Red Indicator for 30 min at 37°C (protected from light), mixed upside down every 5 min (protected from light). Cells were washed 3 times with DPBS, resuspended in DPBS and analyzed by flow cytometry on a Moflo-XDP (Beckman). The fluorescence values of 50,000 cells were analyzed for each sample. Fluorescence readings were normalized to cell number. The experiments were repeated three times independently. Data were analyzed as mean fluorescence intensity using summit 5.2 software.

Mitochondrial Membrane Potential Measurement

For measurement of mitochondrial membrane potential, cells were washed with DPBS and dissociated into single cells by TrypLE, and counted with a LUNA Automated Cell Counter. The same number of cells were used for subsequent detection. Tetramethylrhodamine ethyl ester (TMRE, Abcam) were mixed with dilution buffer HBSS and distributed evenly into each sample tube. Cells were incubated with 50nM TMRE for 30 min at 37°C (protected from light), mixed upside down every 5 min (protected from light). Cells were washed 3 times with DPBS, resuspended in DPBS and analyzed by flow cytometry on a Moflo-XDP (Beckman). The fluorescence values of 50,000 cells were analyzed for each sample. Fluorescence readings were normalized to cell number. The experiments were repeated three times independently. Data were analyzed as mean fluorescence intensity using summit 5.2 software.

Intracellular ATP Measurement

Intracellular ATP levels were measured using the ATP assay kit (Beyotime) according to the manufacturer's instructions. Cells in 6-well plates were harvested and incubated with 200 μL lysis buffer, centrifuged at 4°C, 12,000g for 5 min. The supernatants were taken for subsequent determination. Add 100 μL ATP detection buffer to each hole of 96-well plate, place it at room temperature for 3-5 min, add 20 μL sample and mixed it quickly, then use luminometer to measure RLU value. The concentration of ATP in the sample was calculated according to the standard curve. Relative ATP levels were normalized to protein concentrations.

Drug Treatments

Cells were treated with 1 μM of Camptothecin (CPT, Selleck) for the indicated periods of time to induce DNA damage. Cells were treated with 10 μM MG132 (Selleck) for 4 h to block proteasomal degradation of Uqcrc2. Cells were treated with 100 μg/mL Cycloheximide (CHX, MedChemExpress) to inhibit protein synthesis, 0.5 μM antimycin A (Biovision) to inhibit mitochondrial electron transport chain complex III activity, 9 μM RO-3306 (MedChemExpress) to inhibit Cdk1 activity, 100 μM MIN or 100 μM MIT to inhibit Mthfd2 activity.

DNA Repair Kinetics Assay

For quantifying DSB repair kinetics, cells were treated with 1 μM CPT for 2 h after which the media was replaced with CPT-free media. Cells were immunostained with γ-H2AX antibody (Abcam ab11174) at

different time points post-CPT treatment, as described previously (Mukherjee et al., 2012). To stain for Rad51 foci, cells were treated with 1 μM CPT for 4 h. The cells were fixed with 4% paraformaldehyde/PBS and permeabilized with 0.5% Triton-X/PBS before incubation with antibodies. To obtain clear Rad51 foci, cells were subject to in situ fractionation (Cuadrado et al., 2006). The average number of γ-H2AX foci or Rad51 foci per nucleus was determined after scoring at least 50 nuclei.

Alkaline Comet Assay

Comet assay (alkaline condition) was performed as described (Swain and Subba Rao, 2011; Tice et al., 2000). Briefly, cells were trypsinized and resuspended in ice-colded PBS (Ca^{2+} and Mg^{2+} free) in a concentration of $1x10^5$ cells/mL. An aliquot of 30 μL cells was added to 70 μL of 0.7% low-melting agarose kept at 37°C. The cell-agarose suspension was immediately pipetted and evenly spread onto an area of the comet slides. Slides were kept at 4°C for 10 min followed by immersion in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base, 1% N-lauroylsarcosine, 1% Triton X-100, pH 10.0) for 1 hr at 4 °C in the dark. Slides were then removed, washed, and incubated in cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 30 min. Electrophoresis was carried out at 1 V/cm, 300 mA for 30 min in the dark at 4°C. At the end of the electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris-HCl, pH 7.4) and immersed in ice-colded 100% ethanol at room temperature for 1 h and air dried. Slides were stained with GelRed (Biotium) for 30 min and immediately analyzed. Comets were analyzed by CASP comet assay software (Andor Technology). 50 cells were counted per slide.

Quantification and Statistical Analysis

RNA sequencing analysis

Total RNA was extracted from control mESCs and *Mthfd2* KD mESCs by RNeasy Mini Kit (QIAGEN). To construct and sequence the RNA-seq libraries, we used a polyA selection protocol according to the Illumina TruSeq RNA-Seq library protocol to construct RNA-Seq libraries. RNA-seq library was constructed for each RNA sample, which would make two biological replicates from each sample. Each library was sequenced using an Illumina Hiseq 2500 platform (150 bp pair-end reads). The low-quality reads and adaptor sequences were trimmed with Trimmomatic (Bolger et al., 2014). Clean reads were

aligned to mm10 Hisat2 (Kim et al., 2015). Gene expression levels were calculated by counting the overlap of reads on each gene with HT-seq (Anders et al., 2015) and normalized as RPKM with gene annotation file from Ensembly (release 87) and DESeq2 package in R (Love et al., 2014). Besides, differentially expressed genes were identified by DESeq2 package too. Functional enrichment for Gene Ontology (GO) and KEGG were performing with GOstats package (Falcon and Gentleman, 2007).

Immunoprecipitation and Mass Spectrometry Analysis

3 mg of cytoplasmic and nuclear proteins of control or Mthfd2-Flag mESCs were incubated with 12 μg of Flag antibody at 4°C overnight. Protein G agarose beads (Roche) in 1.5 mL were used to pull down Flag antibody precipitated proteins at 4°C for 1 h, following by washing three times with the lysis buffer and eluting in elution buffer (PH 2.7). The eluted mixture was separated by SDS-PAGE and stained by fast silver stain kit (Beyotime) for identify protein piece. Then the eluted mixtures were sent to analyzed by liquid chromatography-mass spectrometry (LC–MS).

The LC–MS samples were separated on a 2 cm C18 stationary phase column (Phenomenex, USA). While reaching the flow rate of 2 μL/min, the pump flow was split. Then, peptides were separated using a gradient elution that consisted of (a) 0.1% formic acid, (b) 0.1% formic acid in acetonitrile from 1% to 40% surpassing 95 min. Nanospray ESI-MS was performed on a Thermo Q-Exactive high resolution mass spectrometer (Thermo Scientific, USA). The ESI needle voltage was applied to 2 kV, and the isolation width was 4 Da. Raw data from the mass spectrometer were preprocessed with Mascot Distiller 2.4 for peak picking. The resulted peak lists include Mthfd2 and the Mthfd2-Flag samples were searched against Swissprot database using Mascot 2.4 search engine. After filtering the contamination, specific proteins were picked up against the negative control and annotated with UniProt database. Functional enrichment for Gene Ontology (GO) and KEGG were performing with GOstats package (Falcon and Gentleman, 2007). Finally, a potential regulatory network was constructed by Cytoscape.

Statistical analysis

The values reported in the graphs are presented as the mean ± SD or mean ± SEM. Student's *t* test was used to compare treatment groups. For all analyses, a p value less than 0.05 was considered statistically significant. Statistical significance is displayed as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Additional Resources

The SRA website contains detailed data of RNA-seq experiments used in this study: https://www.ncbi.nlm.nih.gov/sra/

Table S1. Microarray Data Analysis and Candidate Gene List, Related to Figures 1 and 2.

Table S2. RNA-Seq data of *Mthfd2* KD mESCs, Related to Figures 3 and 5.

Table S3. List of MTHFD2-Interacting Proteins, Related to Figures 3 and 5.

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