PNAS
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Supplementary Information for

Cops5 safeguards genomic stability of embryonic stem cells through regulating cellular metabolism and DNA repair

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Supplementary Materials and Methods
Figures S1 to S6
Table S1

Other supplementary materials for this manuscript include the following:

Dataset S1

Supplementary Materials and Methods

Vector construction

pX330-U6-Chimeric_BB-CBh-hSpCas9 (pX330) vector was used to construct gene knockout plasmids targeting *Cops5*, *Cops8* and *Xpa*. sgRNAs were designed based on a website (http://cripsr.mit.edu/). The primers of sgRNA targeting *Cops5* were: Forward: CACCGGGCCGCCTTGAGAATGCAAT, Reverse: AAACATTGCATTCTCAAGGCGGCCC. The primers of sgRNA targeting *Cops8* were: Forward: CACCGGTCAGTTGGACAGCGAATC, Reverse: AAACGATTCGCTGTCCAACTGACC. The primers of sgRNA targeting *Xpa* were: Forward: CACCGCACACGTTGGCAGATCAAAG, Reverse: AAACCTTTGATCTGCCAACGTGTGC. *Cops5* gene was first mutated in the sgRNA recognition site by quick mutagenesis and then cloned into the pBS31 vector, resulting in pBS31-*Cops5m*. pX330 *p53* KO plasmid was a gift from Dr. Jing Huang. *Mtch2* gene was amplified from cDNA and cloned into pCAGIPuro.

Western blot

Cells were harvested and resuspended in RIPA lysis buffer (Beyotime, P0013), followed by sonication for 1 min and centrifugation at 12,000 rpm for 15 min. The protein concentration was measured by BCA protein concentration determination kit (Beyotime, P0012). The samples were resolved by SDS-PAGE, followed by transferring onto a PVDF membrane (GE Healthcare, 10600021). Membranes were probed with primary antibodies, anti-Cops2 (Bethyl, IHC-00179), Cops5 (ABclonal, A1766), Cops8 (Biomol, PW8290), Nanog (Bethyl, A300-397A), Oct4 (Santa Cruz, sc5279), β-Tubulin (Huada, AbM59005-37B-PU), Flag (Sigma, F1804), p53 (CST, #2524), cleaved caspase 3 (CST, #9661), HA (Santa Cruz, Sc7392), Mtch2 (Abcam, ab113707), γH2AX (Millipore, 05-636). Bound primary antibodies were recognized by HRP-linked secondary antibodies, goat anti-mouse IgG (GE Healthcare, NA931) and goat anti-rabbit IgG (GE Healthcare, NA934V), at room temperature for two hours. Immunoreactivity was detected by ECL Plus (Beyotime). The pictures were taken by automatic chemiluminescence imaging analysis system (Tanon).

Co-immunoprecipitation (co-IP)

Cells were lysed in lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, and 2 mM EDTA) with protease inhibitor (Roche) and PMSF at 4°C on rotator for 30 min. Cell lysate was then centrifuged at 17,000 g for 30 min, and supernatant was collected. For FLAG co-IP, cell lysates were incubated with anti-Flag M2 magnetic beads (Sigma, M8823) at 4°C overnight. For HA co-

IP, cell lysates were incubated with HA antibody (sc-7392, Santa Cruz) or IgG (A9044, Sigma) at 4°C overnight, and then with protein G beads (17-0618-01, GE Healthcare) at 4°C for 4 hours. The beads were washed three times, and then boiled in 2×SDS loading buffer for 5 min. The samples were subjected to Western blot for further detection.

Quantitative reverse transcription PCR

Total RNA was extracted from cells by Trizol (Invitrogen, 15596018). 1 μg RNA were used for cDNA synthesis by Transcriptor cDNA Synth. Kit 2 (Roche, 4897030001) with random primers according to the manufacturer's instruction. PCR reactions were performed with SYBR Green Master (Roche, 04913914001) in a Bio-RAD MyiQ2 System. PCR cycling conditions were 95°C for 2 min, 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s, and then a melting curve of the amplified DNA was acquired. Quantification of target genes was normalized with β-Actin. Primer information was listed in SI Appendix, Table S1.

Colony forming assay

500 cells were plated into a well of 6-well plate, and cultured for 6 days in ESC medium. Phase-contrast images were captured, and colonies numbers were counted under microscope.

Embryoid body (EB) differentiation

To form EBs, ESCs were cultured in 25 μl hanging drops (1000 cells/drop, ESC medium without LIF, with or without Dox), and EBs were collected on day 4.

Cell cycle analysis

Cells were harvested, fixed by 70% ethanol, and stained with staining buffer (PBS supplied with 5 μg/ml propidium, 1 mg/ml RNase A, 1% Triton X) for 30 min at 37°C. Cells were then analyzed by FACSCalibur flow cytometer (BD Biosciences). The results were analyzed by Modfit software.

Cell apoptosis analysis

Annexin V-PI apoptosis detection kit (Beyotime, C1062) was used for cell apoptosis assay. Cells were harvested, washed by PBS once, resuspended in Annexin V-PI binding buffer supplied with PI (5 μg/ml) and Annexin V (10 μg/ml), incubated on ice for 15 min, and analyzed by FACSCalibur flow cytometer (BD Biosciences). The results were analyzed by FlowJo software.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 30 min, and permeablilized with 0.2% Triton X-100 for 30 min, followed by blocking in 5% goat serum at room temperature for 2 hours. Cells were then

incubated with the primary antibody, anti-γH2AX (Millipore, 05-636), overnight at 4°C. Then cells were washed and incubated with Goat Anti-Mouse IgG FITC (Jackson, 115-095-003) secondary antibody. Hoechst 33342 (Sigma, B2261) or DAPI (Invitrogen, P36935) was used for nucleus staining. Images were captured with Zeiss Axio-Imager Z1 fluorescence microscope.

ROS detection

Cells were harvested and incubated with 5 μM H2DCFDA (MCE, HY-D0940) in PBS at 37 ℃ for 30 min in the dark. One sample without staining served as a negative control. Cells were then analyzed by flow cytometer.

Measurement of extracellular acidification rate (ECAR)

ECAR assay was performed using Glycolysis Assay kit (Abcam, ab197244) according to the manufacturer's instructions. 1×10^6 cells were harvested and washed in respiration buffer once. Cells were then resuspended in 150 μl respiration buffer, and mixed with 10 μl reconstituted Glycolysis Assay Reagent. All samples were transferred to 96-well plate, and the fluorescence signals (Ex/Em = 380/615 nm) were measured every 1.5 min for 2 hours by BioTek microplate reader at, at 37°C.

For ECAR assay using the Seahorse XF^e24 Analyzer, on day 1, cells were plated at a density of 1×10^5 cells/well on gelatin coated XF24 seahorse plates. On day 2, the culture medium was removed, and cells were washed with XF assay medium for three times. Then cells were cultured with 500 μl XF assay medium for 1 hours at 37 °C in a non-CO_2 incubator. Glucose (10 mM), oligomycin (1 μM), 2-deoxy-glucose (2-DG, 50 mM) were added at 20 min, 45 min, and 70 min, respectively. Data were collected and analyzed by the Seahorse XF^e24 Analyzer and Wave software.

Measurement of oxygen consumption rate (OCR)

OCR assay was performed using Extracellular O_2 Consumption Assay kit (Abcam, ab197243) according to the manufacturer's instructions. 1×10^6 cells were resuspended in 150 μl ESC medium, mixed with 10 μl reconstituted Extracellular O_2 Consumption Reagent, and transferred to 96-well plate. One drop of pre-warmed high sensitivity mineral oil was added to seal the sample. Then the fluorescence signals (Ex/Em = 380/650 nm) were measured every 1.5 min for 2 hours by BioTek microplate reader, at 37°C.

Comet assay

Comet assay was carried out using Comet Assay Kit (abcam, ab238544) according to the manufacturer's instructions. Briefly, comet agarose (75 μl/well) was added onto the comet slide to

create a base layer, and the slide was incubated at 4°C for 15 min. Cells were harvested and resuspended at 1×106 cells/ml in cold PBS. Cells and comet agarose were mixed at 1/10 ratio (V/V) thoroughly, and was immediately transferred onto the base layer (75 µl/well). The slide was incubated at 4°C for 15 min in the dark. The slide was immersed in lysis buffer (14.6 g NaCl, 20 ml 500 mM EDTA, 10 ml 10×Lysis Buffer solution, DMSO 10 ml, adjust volume to 100 ml with ddH2O) for an hour at 4°C in the dark, and then in alkaline solution (12 g NaOH, 2 ml 500 mM EDTA, adjust volume to 1L with ddH2O) for 30 min at 4°C in the dark. Gel electrophoresis was performed at 20 V, 30 min. The slide was immersed in pre-chilled ddH2O for 2 min and washed twice more. After incubating with cold 70% Ethanol for 5 min, the slide was taken out to air dry. 100 µl/well of diluted Vista Green DNA Dye was added and incubated at room temperature for 15 min. Images were captured with Zeiss Axio-Imager Z1 fluorescence microscope. Comets were analyzed using CASP comet assay analysis software (Andor Technology), and 100 cells were calculated in each sample.

siRNA transfection

For one well on 6-well plate, 125 µl OPTI-MEM (31985070, Invitrogen) and 3.75 µl lipofectamine 3000 Reagent (L3000015, Invitrogen) are mixed together, and 5 µl 20 µM siRNA is diluted in 125 µl OPTI-MEM. The diluted lipofectamine 3000 reagent was added into siRNA mixture, mixed well, and incubated at room temperature for 10 min. 5×10^5 ESCs were plated on a well of 6-well plate. The siRNA and lipofectamine mixture was added to cells. Control siRNA sequences: UUCUCCGAACGUGUCACGUTT, ACGUGACACGUUCGGAGAATT. siMtch2-1: GCGACAAGUAUGUCAGCUUTT, AAGCUGACAUACUUGUCGCTT; siMtch2-2: GGUCCAUGGUACAGUUUAUTT, AUAAACUGUACCAUGGACCTT; siMtch2-3: GGAAAUAUGAGCCGAGGAATT, UUCCUCGGCUCAUAUUUCCTT.

Supplementary Figures

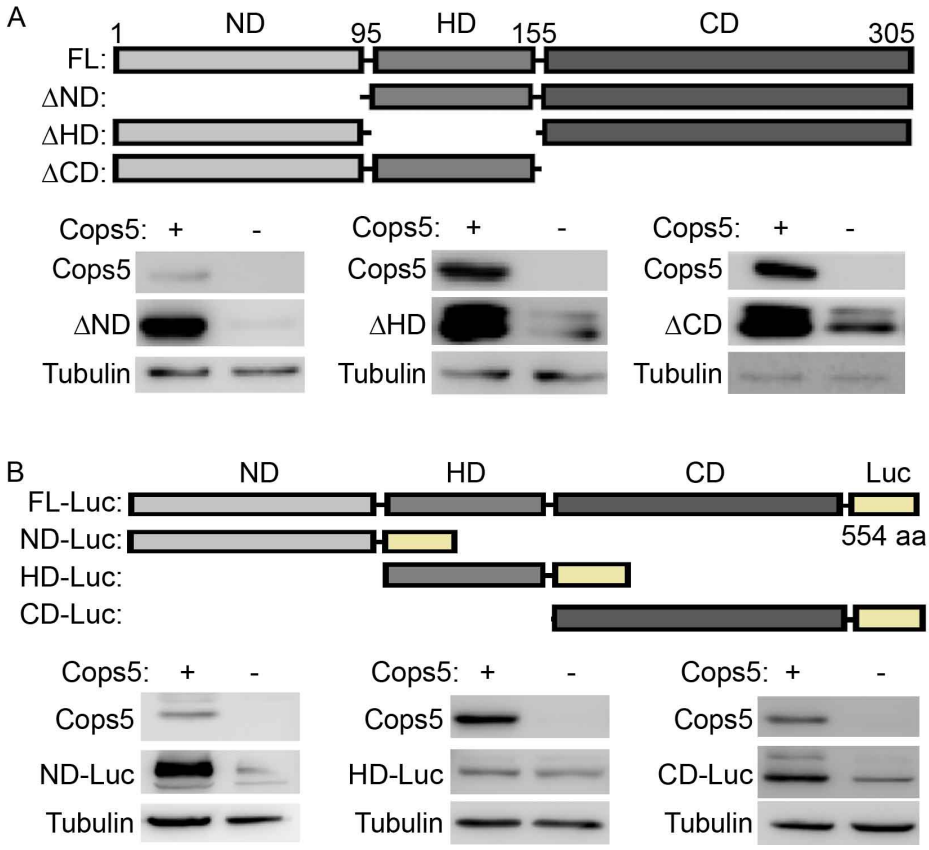

Figure S1. The N-terminal and C-terminal domains mediates *Cops5* KO induced Nanog degradation. (related to Figure 2)

(A) Plasmids expressing Flag-tagged ΔND, ΔHD, or ΔCD Nanog mutants were transfected into *iC5; C5 KO* ESCs with or without Dox. 48 hours after transfection, cells were harvested for Western Blot.

(B) Plasmids expressing ND, HD, and CD fused with luciferase were transfected *iC5; C5 KO* ESCs with or without Dox. 48 hours after transfection, cells were harvested for Western Blot.

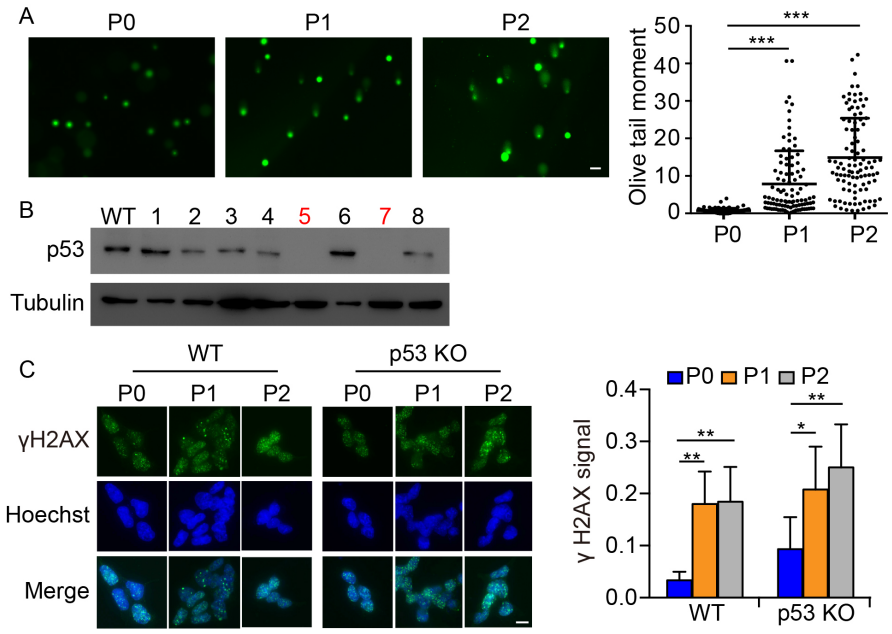

Figure S2. ***p53* KO does not rescue DNA damage in *Cops5 KO* ESCs. (related to Figure 3)**

(A) Comet assay of *iC5; C5 KO* ESCs upon *Cops5* KO. Left panel shows the representative images. Scale bar, 50 μm. Comet tail lengths of 100 cells per sample are quantified and shown in the right panel. (B) Western blot to screen for *p53 KO* clones in *iC5*; *C5 KO* ESCs. Clone 1-8 were ESC lines derived from single colonies after transfection of p53 sgRNA into *iC5*; *C5 KO* ESCs. Clone 5 and 7 were validated as *iC5*; *C5 KO*; *p53 KO* ESCs, and were named as p53 KO-1 and p53 KO-2 in Figure 3, respectively. (C) Immunofluorescence images of γH2AX in *iC5; C5 KO* ESCs and *iC5; C5 KO; p53 KO* ESCs, cultured with (P0) or without Dox for 48 (P1) and 96 hours (P2). Scale bar, 10 μm. Relative fluorescence intensities of γH2AX were quantified in around 100 nuclei for each condition and plotted in the right panel.

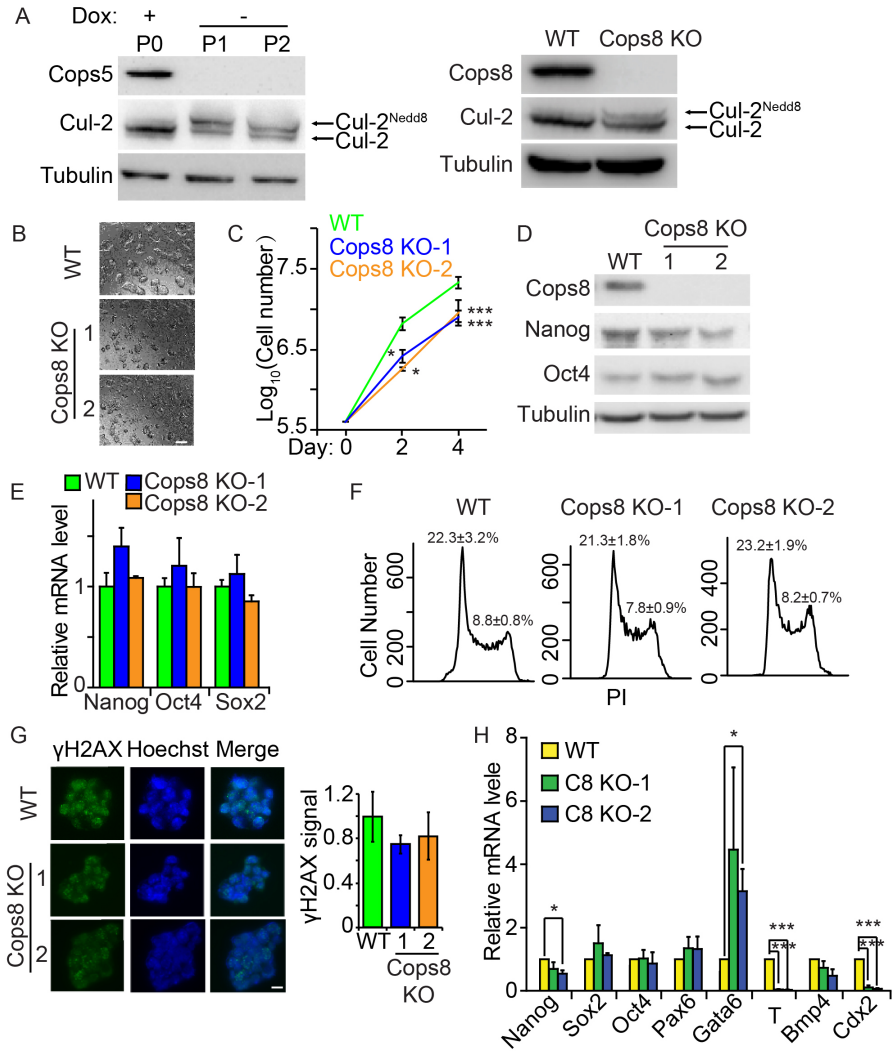

Figure S3. *Cops8 KO* ESCs display no obvious phenotype, except for slightly reduce growth rate. (related to Figure 2 and 3)

(A) Western blot for Cul-2 in *iC5; C5 KO* ESCs upon Dox withdrawal, as wells as WT and *Cops8 KO* ESCs. The upper and lower bands in the Cul-2 blot are neddylated and unneddylated Cul-2, respectively. (B) Phase contrast images of WT and *Cops8 KO* ESCs. Scale bar, 100 μm. (C) Growth curves of WT and *Cops8 KO* ESCs. (D) The protein levels of pluripotency factors, Nanog and Oct4, in WT and *Cops8 KO* ESCs, measured by Western blot. (E) The mRNA levels of pluripotency genes, *Nanog*, *Oct4*, and *Sox2*, in WT and *Cops8 KO* ESCs, measured by quantitative RT-PCR. (F) Cell cycle analysis of WT and *Cops8 KO* ESCs. (G) Immunofluorescence images of γH2AX in WT and *Cops8 KO* ESCs. Scale bar, 10 μm. Relative fluorescence intensities of γH2AX were quantified in around 100 nuclei for each condition. Quantification results are shown in the right panel. (H) Quantitative RT-PCR analysis of pluripotency and differentiation genes in day 4 EBs formed by WT and *Cops8 KO* ESCs.

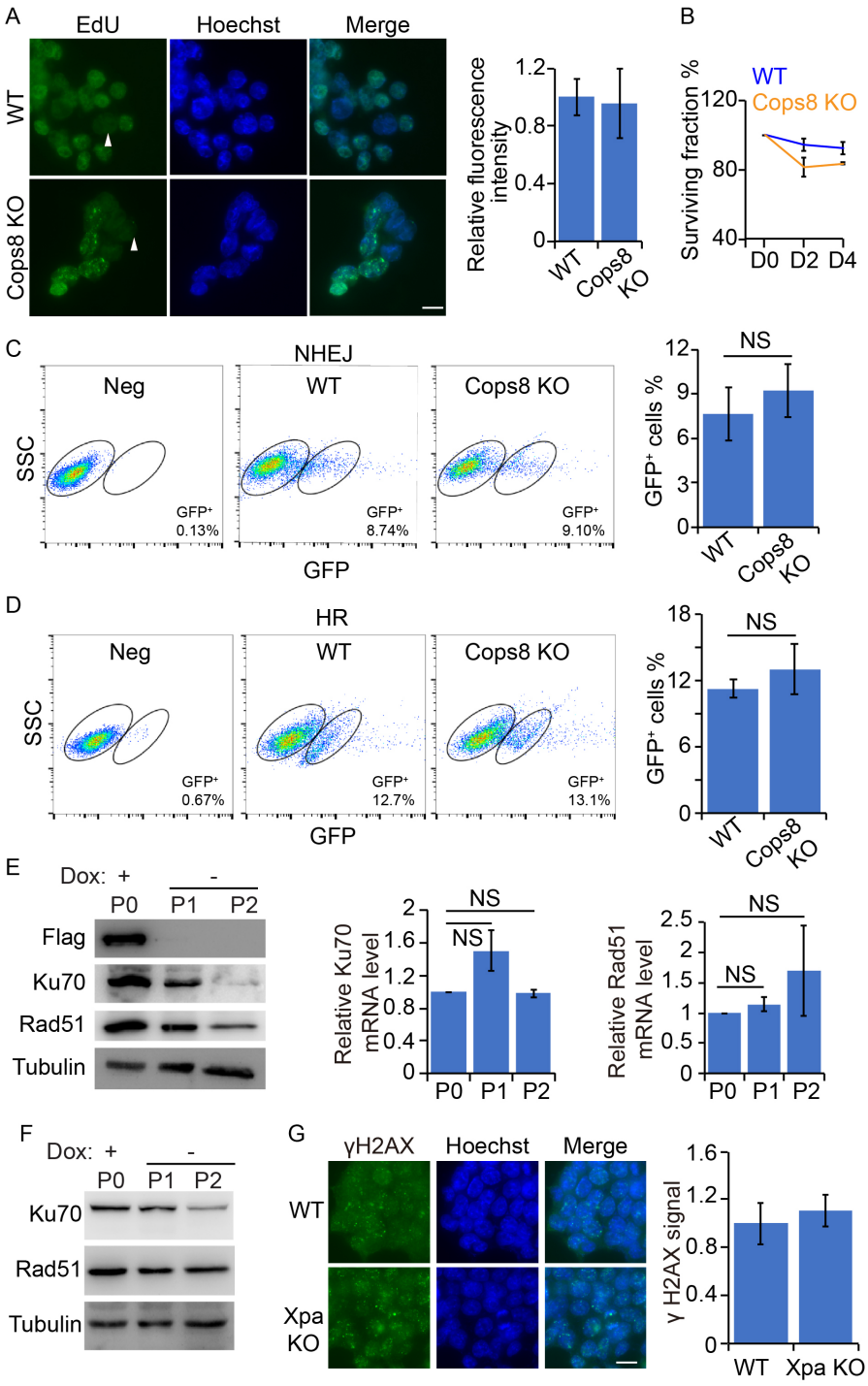

Figure S4. No DDR defects in *Cops8 KO* ESCs. (related to Figure 4)

(A) GGR assay for *WT* and *Cops8 KO* ESCs. Representative images are shown in the left panel. Triangles indicate non-S phase cells. Scale bar, 10 μm. Relative fluorescence intensities of 50 non-S phase cells were quantified and plotted in the right panel. (B) TCR assay for WT and *Cops8 KO* ESCs. WT and *Cops8 KO* ESCs were cultured in medium with or without 3 μg/ml illudin S for two passages. Surviving fraction was defined as the ratio of live cells with illudin S treatment to live cells

without illudin S treatment. (C and D) Measurement of NHEJ (C) or HR (D) in WT and *Cops8 KO* ESCs. WT and *Cops8 KO* ESCs with integrated NHEJ or HR reporter were transfected with *I-Sce*I expressing plasmid. 48 hours after transfection, the percentage of GFP^+ cell was analyzed by flow cytometry. The left three panels show the result of a representative experiment. The right panel shows the quantification result of three independent experiments. (E) Protein and mRNA levels of Ku70 and Rad51, key factors for NHEJ and HR, respectively, in *iC5; C5 KO* ESCs at various time points after Dox withdrawal. (F) Ku70 and Rad51 protein expression in *iC5; C5 KO; p53 KO* ESCs at various time points after Dox withdrawal. (G) Immunofluorescence images of γH2AX in WT and *Xpa KO* ESCs. Scale bar, 10 μm. Relative fluorescence intensities of γH2AX were quantified in around 100 nuclei for each condition and plotted in the right panel.

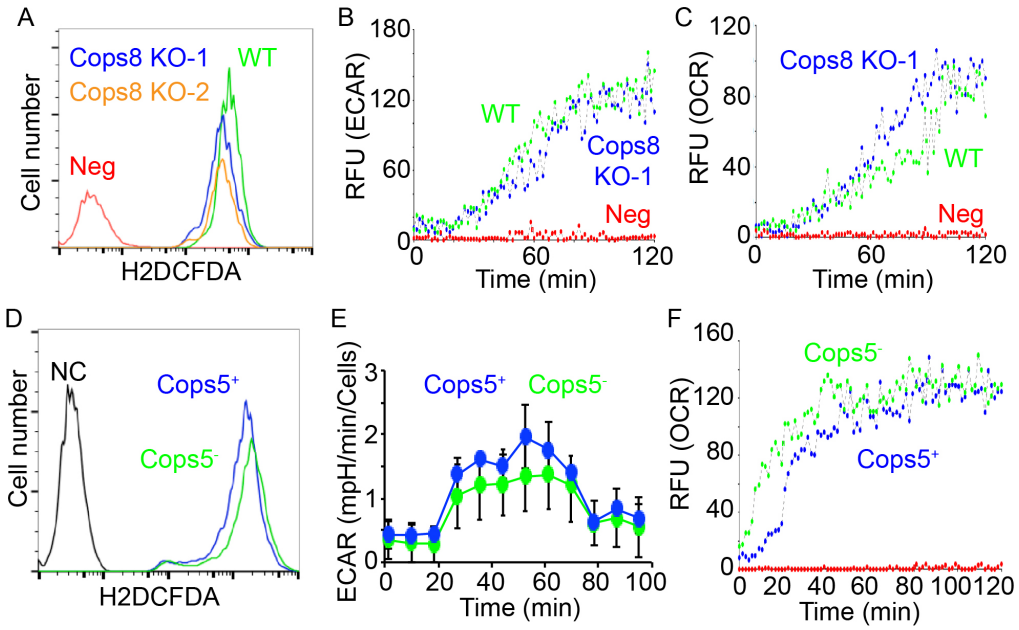

Figure S5. *Cops8* KO does not affect cellular metabolism. (related to Figure 5)

(A-C) Measurement of ROS (A), ECAR (B), and OCR (C) in WT and *Cops8 KO* ESCs. (D-F) Measurement of ROS (D), ECAR (E), and OCR (F) in differentiated *iC5; C5 KO* cells cultured with or without Dox for 48 hours. *iC5; C5 KO* ESCs were differentiated for 4 days in medium with Dox, but without LIF. On day 4, differentiated cells were cultured with or without Dox for 48 hours. (E) ECAR assay was performed with the Seahorse XFe/XF Analyzer.

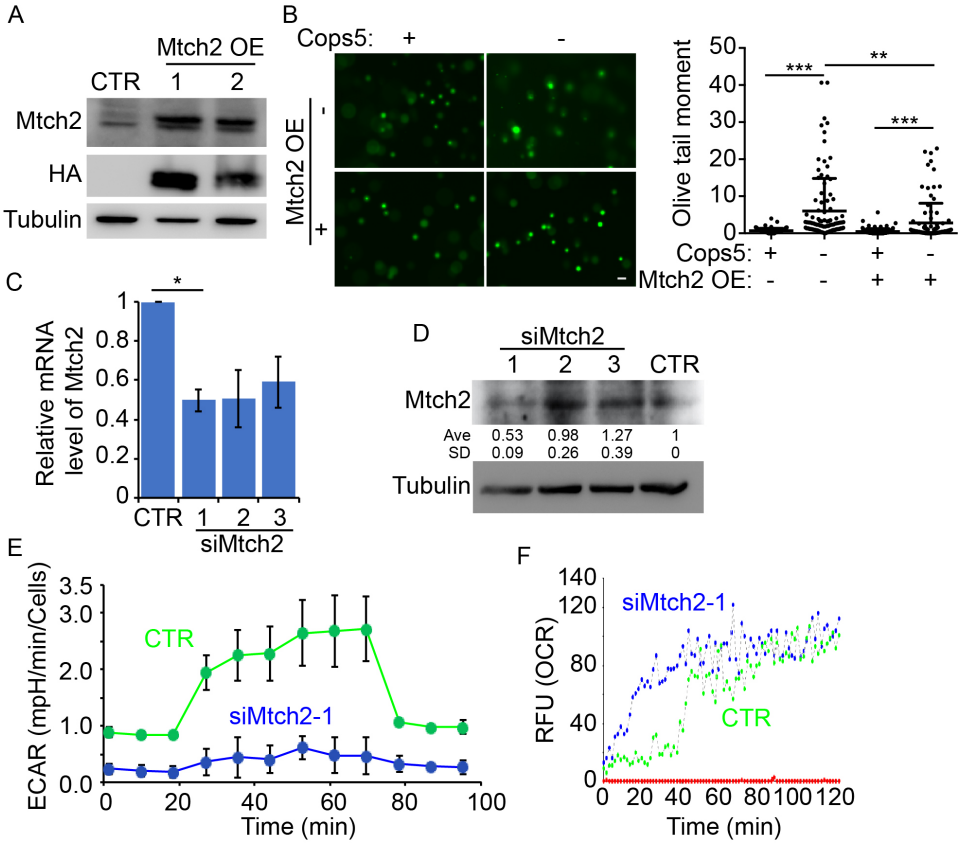

Figure S6. Knockdown of Mtch2 reduces glycolysis in ESCs. (related to Figure 6)

(A) Western blot to validate the overexpression of Mtch2 in iC5; C5 KO; Mtch2 OE ESCs. The upper band in the Mtch2 blot is the exogenous HA tagged Mtch2, while the lower band is the endogenous Mtch2. (B) Comet assay of in iC5; C5 KO and iC5; C5 KO; Mtch2 OE ESCs with or without Dox for 48 hours. Left panel shows the representative images. Scale bar, 50 μm. Comet tail lengths of 100 cells per sample are quantified and shown in the right panel. (C and D) The efficiency of Mtch2 knockdown was analyzed by quantitative RT-PCR (C) and Western blot (D). ESCs were transfected with siRNAs targeting Mtch2 or non-targeting control siRNA (CTR). Forty-eight hours after transfection, cells were harvested for quantitative RT-PCR (C) and Western blot (D). Quantification results of the Western blot analysis are shown below the corresponding Mtch2 bands (n=3). Only siMtch2-1 siRNA downregulates Mtch2 at both RNA and protein levels. Thus, only siMtch2-1 siRNA was used for subsequent experiments. (E-F) Measurement of ECAR (E), and OCR (F) in Mtch2 knockdown and control ESCs. ESCs were transfected with siRNAs targeting Mtch2 or negative control siRNA (CTR). ESCs were transfected with siMtch2-1 or control siRNA. Forty-eight hours after transfection, cells were harvested for ECAR assay with the Seahorse XFe/XF Analyzer (E) and OCR assay (F).

Supplementary Table

Table S1. Primers for quantitative RT-PCR.

Gene	Forward primer	Reverse primer
Nanog	TACAAGGGTCTGCTACTGAGATGC	TTGGGACTGGTAGAAGAATCAGGG
Oct4	ATCAGCTTGGGCTAGAGAAGGATG	AAAGGTGTCCCTGTAGCCTCATAC
Sox2	GCGGAGTGGAAACTTTTGTCC	CGGGAAGCGTGTACTTATCCTT
Fgf5	GAAACTCGGATACAGCATCCCTCT	GGATCGCTACAGAGAATCCCACTT
Nestin	CTGGATCTGGAAGTCAACAGAGGT	ATCCTCAGTTTCCACTCCTGTAGC
Gata4	GCTATGCATCTCCTGTCACTCAGA	CCAAGTCCGAGCAGGAATTTGAAG
Gata6	CTTCTCCTTCTACACAAGCGACCA	ATACTTGAGGTCACTGTTCTCGGG
Hand1	AAGGATGCACAAGCAGGTGAC	TTTAATCCTCTTCTCGCCGGG
T	CATCGGAACAGCTCTCCAACCTAT	TACCATTGCTCACAGACCAGAGAC
Bmp4	ACAGCGGTCCAGGAAGAAGAAT	TGCACAATGGCATGGTTGGT
Cdx2	CAGTCCCTAGGAAGCCAAGTGAAA	AAGTGAAACTCCTTCTCCAGCTCC
Mtch2	CATCCTCCTTTGACCGAGTTATC	ACGTGGAAGGGATGTGTAATG
Ku70	CAGCTTGTCTTCCTCCCTTATG	CTTGTCTATCTGCTCCTGGTTG
Rad51	GGCAGCGATGTCCTAGATAATG	CAGTGCATACCTGGATTCTACC
Actin	CAGAAGGAGATTACTGCTCTGGCT	TACTCCTGCTTGCTGATCCACATC