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Supplemental Information

A *miR-590/Acvr2a/Rad51b* Axis Regulates DNA Damage Repair during mESC Proliferation

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Supplementary Information

I. Supplemental Experimental Procedures

Western blot

Cells were lysed with 1×loading lysis buffer that was diluted from 5×loading lysis buffer (2.5 ml of 0.5 mol/L Tris-HCl at pH 6.8 containing 0.39 g of DTT, 0.5 g of SDS, and 0.025 g of bromophenol blue added to 2.5 ml of glycerine). Equal amount of protein was transferred onto PVDF membranes (BioRad, USA). Protein was detected by primary antibodies against CYCLIN B (sc-752, Santa Cruz, California, USA), P21 (sc-6246, Santa Cruz), CYCLIN E (sc-481, Santa Cruz), GAPDH (sc-47724, Santa Cruz), RAD51B (BS2542, Bioworld, Nanjing, China), p-SMAD2 (BS4172, Bioworld), ACVR2A (BS3669, Bioworld), and SMAD2/3 (BS1838, Bioworld).

Immunostaining

mES cells were fixed for 20 min in 4% paraformaldehyde. The cells were then washed 3 times with PBS followed by treatment with 0.2% Triton X-100 for 7 min. The cells were then washed 3 times with PBS followed by treatment with PBS containing 10% FBS (Gibico) for 1 h at room temperature. The primary antibody was diluted in PBS containing 10% FBS and added to the cells for an overnight incubation at 4°C. The cells were then washed 3 times with PBS, and the secondary antibody was added to the cells for incubation at room temperature for 2 h. The primary antibody used for immunostaining was γ-H2AX (#2577, Cell Signaling Technology, Boston,

USA).

Quantification of fluorescence of γ-H2AX immunostaining and DNA tail comet assay

To quantify the fluorescence degree of γ-H2AX staining of each experiment, mES cell clones areas that should contains more than a hundred cells were selected. Fifty clones were selected in each group to detect the mean fluorescence intensity (MFI) that was expressed as arbitrary units per pixel by using LSM Image Examiner software. The comet assay software project (CASP) was used to analyze the DNA tail of comet assay.

Quantitative real-time PCR (qRT-PCR)

For miRNA

The total RNA was isolated using RNAiso (Takara, Japan). miRNA was subsequently reverse-transcribed to cDNA using the miRNA specific stem-loop reverse-transcription primer (Ribobio, China). The amount of target gene expression ($2^{-\Delta\Delta Ct}$) was normalized via the endogenous small nuclear RNA U6 using miRNA-specific primers (Ribobio). The reaction conditions were performed according to the instructions from Ribobio Co., Ltd with SYBR Green qPCR Mix (BioRad).

For mRNA

The total RNA was isolated using RNAiso (Takara). cDNA was subsequently

reverse-transcribed from mRNA by M-MLV Reverse Transcriptase (Takara). The PCR included 40 cycles of amplification using the Stratagene Mx3000P system with SYBR Green qPCR Mix (BioRad). Expression of target genes ($2^{-\Delta\Delta Ct}$) was normalized against *Gapdh*. The primers are shown below:

P21 (forward, 5'-CGAGAACGGTGGAACTTTGAC-3'; reverse, 5'-CAGGGCTCAGGTAGACCTTG-3')

Cyclin E (forward, 5'-GTGGCTCCGACCTTTCAGTC-3'; reverse, 5'-CACAGTCTTGTCAATCTTGGCA-3')

Cyclin B (forward, 5'- AAGGTGCCTGTGTGTGAACC-3'; reverse, 5'-GTCAGCCCCATCATCTGCG-3')

Rb1 (forward, 5'-TCCACCAGGCCTCCTACCT-3'; reverse, 5'-CCAGGAATCCGTAAGGGTGAA-3')

Acvr2a (forward, 5'-GCGTTCGCCGTCTTTCTTATC-3'; reverse, 5'-GTTGGTTCTGTCTCTTTCCCAAT-3')

Rad51b (forward, 5'-TGACGAATCAAATTACGACCCAT-3'; reverse, 5'-CCTAGTGCAGCTACCAAACAG-3')

Rad9 (forward, 5'- GGCTGTCCATTCGCTATCCC-3'; reverse, 5'-GTGGGGCAAAAAGGAAGCAG-3')

Rad51c (forward, 5'- CAACTGCCTGCATTCAGCAC-3'; reverse, 5'-TGCCAGCAGCTCAGTATAATCA-3')

Brca1 (forward, 5’-AGCCCACCTACTTGTGCTGA-3’; reverse, 5’-TCGATGAGTTGAGGCTCTCTAA-3’)

Oct4 (forward, 5’-GGATGCTGTGAGCCAAGG-3’; reverse, 5’-GAACAAAATGATGAGTGACAGACAG-3’)

Nanog (forward, 5’-CAGGTGTTTGAGGGTAGCTC-3’; reverse, 5’-CGGTTCATCATGGTACAGTC-3’)

Sox2 (forward, 5’-GATCAGCATGTACCTCCCC-3’; reverse, 5’- CCCTCCCAATTCCCTTGTATC -3’)

Gapdh (forward, 5’-GTGTTCCTACCCCCAATGTGT-3’; reverse, 5’-ATTGTCATACCAGGAAATGAGCTT-3’)

Trypan blue staining

Prepare 0.5 ml single cell suspension in PBS, and then add 0.5 ml trypan blue solution (0.4%) (Sigma). Allow to react for 3 min, and then calculate the total viable cells (unstained) and total cells (stained and unstained) by microscope to determine the cell viability. Cell viability (%) = viable cells / total cells ×100%.

Cell apoptosis analysis

Cell apoptosis analysis was performed by using AnnexinV-FITC Cell Apoptosis Detection kit (Keygentec, China).

mES cultured without LIF

The cells were cultured in the DMEM medium (Hyclone) supplemented with 15% FBS (Gibco), 1% NEAA (Invitrogen), 1% Gln (Invitrogen), without LIF and β-mercaptoethanol.

Embryonic bodies (EBs) formation

Single mES cell suspension was transferred into the bacteriologic dishes and cultured in the medium which is the same as the culture mentioned above.

Construction of vector

Luciferase reporter vector

3'UTR sequences of *Acvr2a* were amplified by PCR from mES cell genomic DNA and inserted into the pGL3-luciferase reporter gene vector. The mutant miRNA binding sites were obtained by replacing the miRNA binding site sequence with miRNA seed sequences using the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, USA).

Acvr2a overexpression vector

Fragment of mouse *Acvr2a* was cloned from the pCDNA-*Acvr2a* (Sino Biological, China) by the primers with the restriction enzyme cutting site of BamHI and EcoRI (forward, 5'-GGCCGGCCGATGGGAGCTGCTGCAAAGTT-3'; reverse, 5'-GGCGAATTCTCATAGACTAGATTCTTTGGGAGGA-3') and was inserted into Fugw vector.

Acvr2a-flag overexpression vector:

Fragment of mouse *Acvr2a*-flag was cloned from the pCDNA-*Acvr2a* by the primers with the restriction enzyme cutting site of BamHI and EcoRI (forward, 5'-GGCGGATCCATGGACTACAAGGACGACGATGACAAGGGAGCTGCTGCA AAGT-3'; reverse, 5'-GGCGAATTCTCATAGACTAGATTCTTTGGGAGGA-3) and inserted into Fugw vector.

Acvr2a and Rad51b knockdown vector:

The corresponding base pairs for sh*Acvr2a*- or sh*Rad51b*-specific regions (sh*Acvr2a*-1, CCTGTGGCTAATCACAGCATT; sh*Acvr2a*-2: GGTGTTGGA GGGTGCTATAAA; sh*Rad51b*-1: GCTGAGAGACTGGTTGAGATT; sh*Rad51b*-2: TGTTGACTCCATTGCTTCTGTGGTCAGAA) for RNA interference were designed and cloned into the pLKO.1-TRC cloning vector.

Luciferase assay

NIH3T3 cells (1×10^5 cells per well of 24-well plate) were transfected with 250 ng of luciferase reporter, 5 ng of Renilla vector (Promega), and 50 nM chemically synthesized *pre-miR-590* or pre-miRNA control (Biolend, China) using Fugene HD transfection reagent (Roche, Switzerland). Cell lysates were harvested at 24 h after transfection and subjected to the dual luciferase assay (Promega).

Transfection of pre-miRNA, miRNA mimics and inhibitors

The miRNA mimics (Ribobio) were artificially synthesized short double-stranded oligonucleotides, which can be processed by cells to produce mature miRNA. The miRNA inhibitors (Ribobio) were also artificially synthesized

single-stranded RNAs, which are the antisense oligonucleotides of mature miRNAs. miRNA inhibitors compete with the miRNAs for target mRNAs to inhibit the function of miRNAs. The pre-miRNA was an artificially synthesized oligonucleotide that was longer than the miRNA mimics. The synthesized pre-miRNA sequence was the same as the endogenous pre-miRNA sequence. The pre-miRNA, miRNA mimics and inhibitors were all transfected into cells using the Fugene HD transfection reagent (Roche).

Cell proliferation analysis

Cell proliferation analysis was performed by two different kinds of methods. One is MTS assay that was performed by using CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, USA) which needs to detect the absorption value at 490 nm by using microplate reader.

Another is flow cytometric analysis of 5'-bromo-deoxyuridine (BrdU) incorporation. Cells were exposed to BrdU for 1 h before analyzing the proliferative activity. The BrdU incorporation into mES cells was quantified by BrdU Cell Proliferation Detection kit (Keygentec, China).

Statistical analyses

Student's *t*-test was used for all statistical analyses. Statistical significance was defined as follows: * means $p < 0.05$; ** means $p < 0.01$; and ***means $p < 0.001$. Values were presented as the mean ± SD.

II. Supplemental Figures and Figure Legends

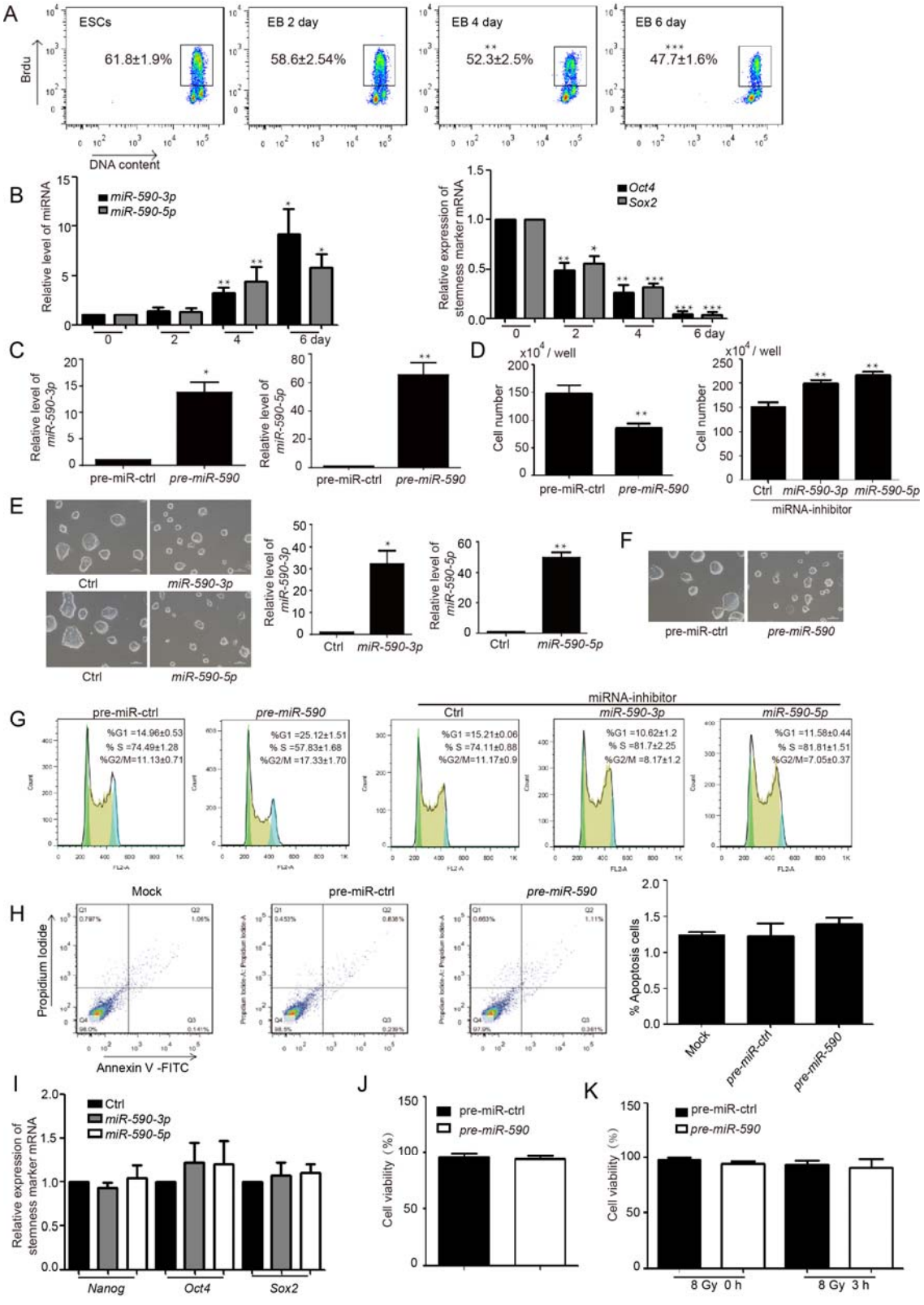

Figure S1

Figure S1, related to Figure 1. *miR-590-3p* and *miR-590-5p* can regulate the proliferation without influencing the stemness maintenance, cell apoptosis and death

(A) Analysis of the proliferation during EBs formation at d2, d4, d6 by FACS analysis of BrdU incorporation. Cells at d0 were mES cells. The figure showed the percentage (%) of population of BrdU positive cells. Data shown are means ± SD of three independent experiments (n=3). **p < 0.01 and ***p < 0.001.

(B) Level of *miR-590-3p/5p* detected by qRT-PCR during EBs formation (left panel). Right panel means detection of level of stemness markers (*Oct4, Sox2*) during EBs formation (right panel). Data shown are means ± SD of three independent experiments (n=3). *p < 0.05, **p < 0.01, and ***p < 0.001.

(C) Detection of *miR-590-3p* and *miR-590-5p* in mES cells transfected with pre-miR-590. Data shown are means ± SD of three independent experiments (n=3). *p < 0.05 and **p < 0.01.

(D) Counting of amount of the total cells of mES cells transfected with *pre-miR-590* or miRNA inhibitors. Data shown are means ± SD of three independent experiments (n=3). **p < 0.01.

(E) Morphology of mES cell clones transfected with *miR-590-3p* or *miR-590-5p* mimics respectively. The scale bar represents 100 μm. Right panel showed the expression level of *miR-590-3p* and *miR-590-5p* in mES cells transfected with *miR-590-3p* or *miR-590-5p* mimics respectively. Data shown are means ± SD of three

independent experiments (n=3). *p < 0.05 and **p < 0.01.

(F) Another kind of mES cells line (46C), transfected with *pre-miR-590* showed smaller clone than control. The scale bar represents 100 μm.

(G) Cell cycle analysis by flow cytometry after propidium iodide (PI) staining. Data shown are means ± SD of three independent experiments (n=3).

(H) Cell apoptosis analysis of mES cells transfected with *pre-miR-590*. Right panel showed the statistical analysis of the percentage of apoptosis cells. Data shown are means ± SD of three independent experiments (n=3).

(I) *miR-590-3p* and *miR-590-5p* cannot influence the stemness by detecting the stemness markers of *Nanog*, *Oct4*, and *Sox2* through qRT-PCR. Data shown are means ± SD of three independent experiments (n=3).

(J) Statistical analysis of the trypan blue staining of mES cells transfected with *pre-miR-590* and control. Data shown are means ± SD of three independent experiments (n=3).

(K) Statistical analysis of the trypan blue staining of *miR-590*-overexpressed mES cells irradiated by the x-ray. 0 h means staining the cell without time to repair the DNA damage after irradiating. 3 h means staining the irradiated cell after 3 h. Data shown are means ± SD of three independent experiments (n=3).

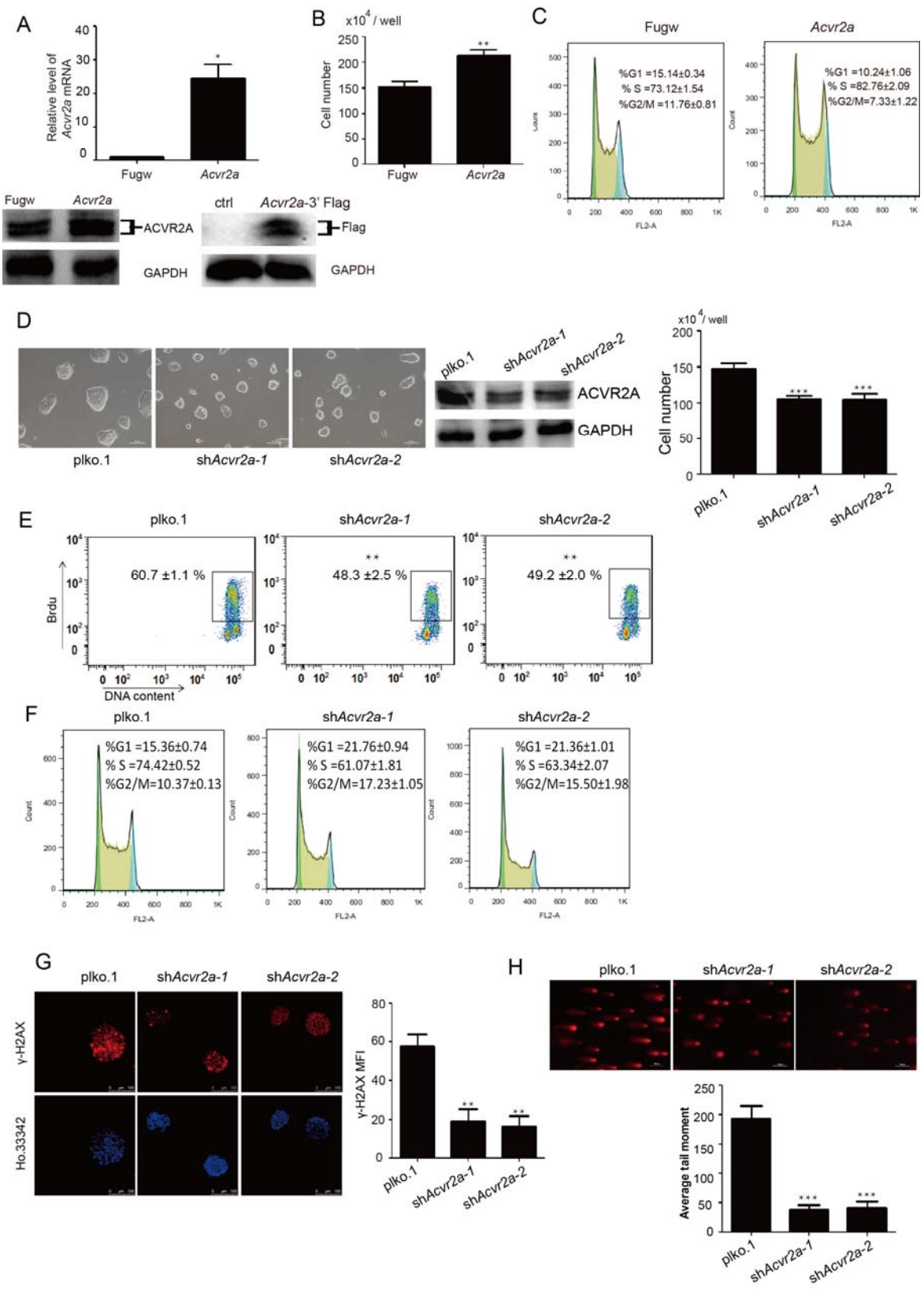

Figure S2

Figure S2, related to Figure 4. Effects of *Acvr2a* on SSB damage repair, DSB

damage repair and proliferation of mES cells

(A) Expression level of *Acvr2a* ectopic expression vector detected by qPCR. Data shown are means ± SD of three independent experiments (n=3). *p < 0.05. Bottom left corner picture showed the effect of overexpression of ACVR2A detected on protein level. Bottom right corner picture showed the bands of *Acvr2a*-flag detected by flag antibody, which determined the two bands of ACVR2A protein.

(B) Counting of amount of the total cells of mES cells overexpressed with *Acvr2a*. Data shown are means ± SD of three independent experiments (n=3). **p < 0.01.

(C) Cell cycle analysis by flow cytometry after propidium iodide (PI) staining. Data shown are means ± SD of three independent experiments (n=3).

(D) Morphology of mES cell clones transfected with sh*Acvr2a-1* or sh*Acvr2a-2* vector. plko.1 is the empty vector. The scale bar represents 100 μm. The effect of the knockdown of *Acvr2a* was detected by western blot. Data shown are means ± SD of three independent experiments (n=3). ***p < 0.001.

(E) Cell proliferation assay by FACS analysis of BrdU labeling. Data shown are means ± SD of three independent experiments (n=3).

(F) Knockdown of *Acvr2a* in mES cells influenced the cell cycle detected by flow cytometry after propidium iodide (PI) staining. Data shown are means ± SD of three independent experiments (n=3).

(G) Immunofluorescence analysis of γ-H2AX (red) to show DSB damage of mES cells. Nuclei were stained with Ho.33342 (Hoechst 33342) (blue). The scale bar

represents 100 μm. Right picture showed the statistical analysis of γ-H2AX mean fluorescence intensity (MFI). Data shown are means ± SD of three independent experiments (n=3). **p < 0.01.

(H) The comet assay shows that knockdown of *Acvr2a* inhibits SSB damage. The scale bar represents 100 μm. The bottom image is the statistical analysis of average tail moment of the mES cells. Data shown are means ± SD of three independent experiments (n=3). ***p < 0.001.

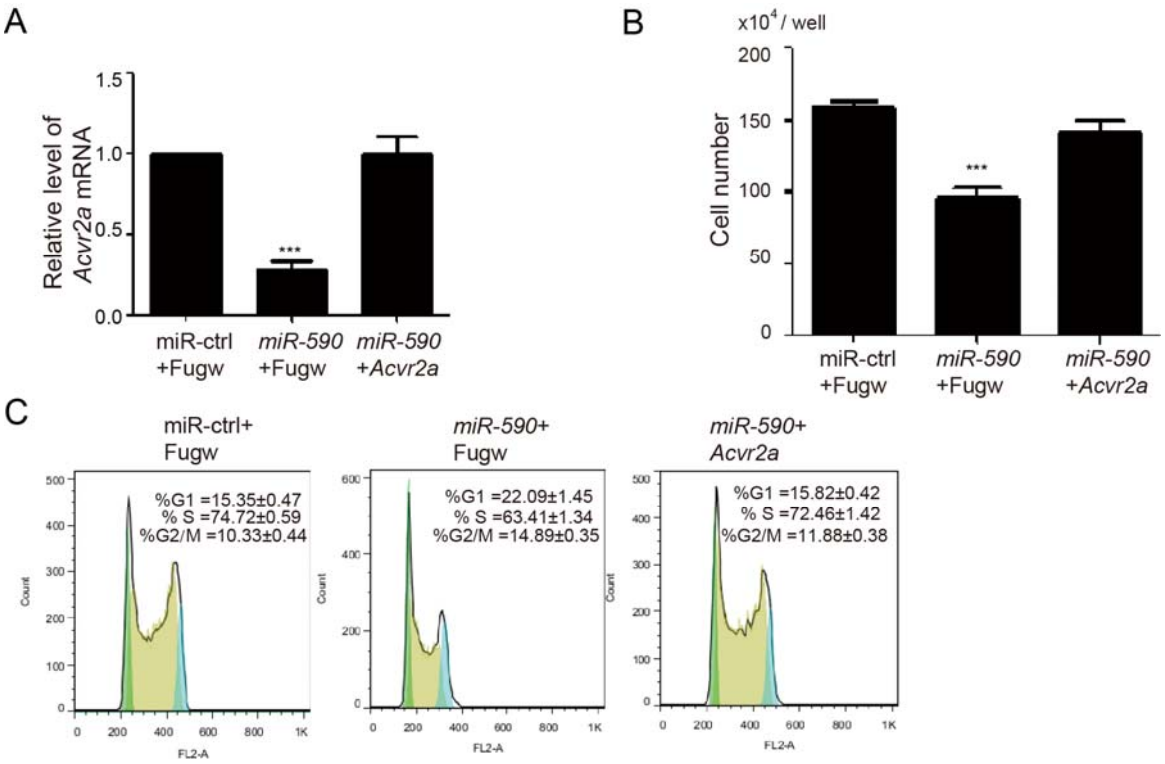

Figure S3

Figure S3, related to Figure 4. *Acvr2a* rescues the function of *miR-590* on cell proliferation and cell cycle

(A) *Acvr2a* expression in the rescue experiment detected by qPCR. Data shown are means ± SD of three independent experiments (n=3). ***p < 0.001.

(B) Counting of total amount of mES cells. Data shown are means ± SD of three independent experiments (n=3). ***p < 0.001 .

(C) Cell cycle analysis by flow cytometry after propidium iodide (PI) staining. Data shown are means ± SD of three independent experiments (n=3).

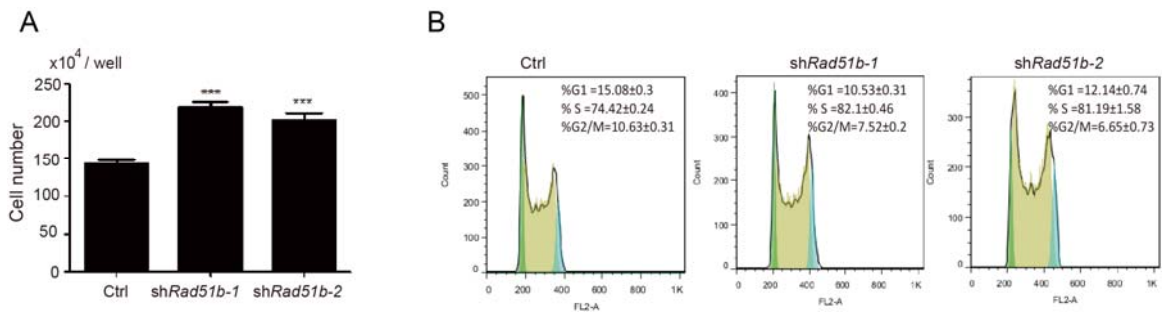

Figure S4

Figure S4, related to Figure 5. Knockdown of *Rad51b* promotes the proliferation

(A) Counting of total amount of mES cells after *Rad51b* knockdown. Data shown are means ± SD of three independent experiments (n=3). ***$p < 0.001$.

(B) Cell cycle analysis by flow cytometry after propidium iodide (PI) staining. Data shown are means ± SD of three independent experiments (n=3).

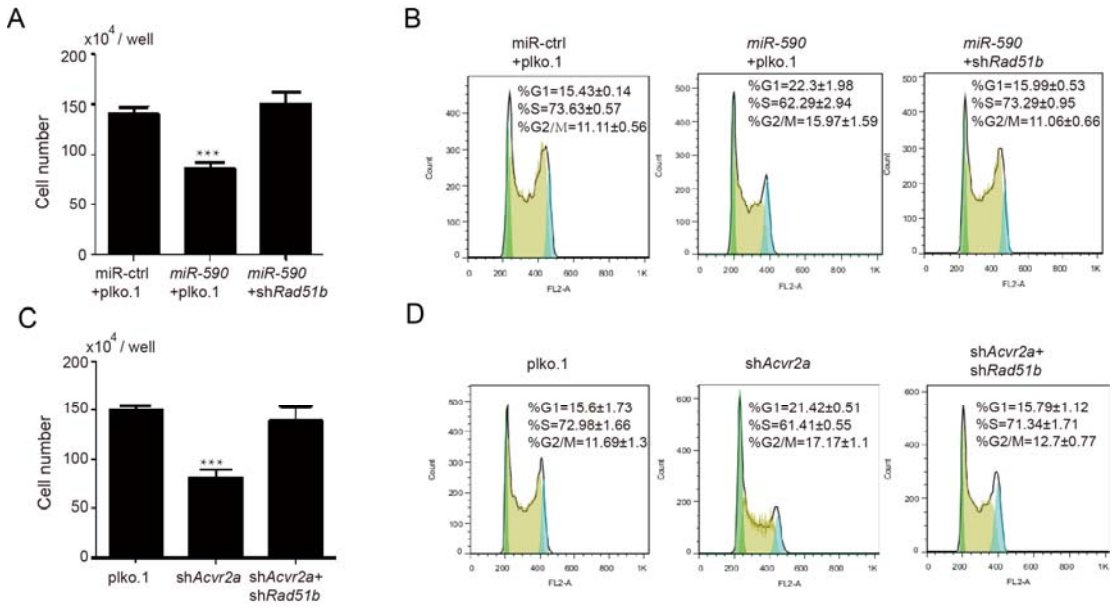

Figure S5

Figure S5, related to Figure 6. *miR-590/Acvr2a/Rad51b* axis regulates the proliferation and cell cycle of mES cells

(A) Counting of total amount of mES cells in the rescue experiment between *miR-590* and *Rad51b*. Data shown are means ± SD of three independent experiments (n=3). ***$p < 0.001$.

(B) Cell cycle analysis of mES cells in the rescue experiment between *miR-590* and *Rad51b* by flow cytometry after propidium iodide (PI) staining. Data shown are means ± SD of three independent experiments (n=3).

(C) Counting of total amount of mES cells in the rescue experiment between *Acvr2a* and *Rad51b*. Data shown are means ± SD of three independent experiments (n=3). ***$p < 0.001$.

(D) Cell cycle analysis of mES cells in the rescue experiment between *Acvr2a* and

Rad51b by flow cytometry after propidium iodide (PI) staining. Data shown are means ± SD of three independent experiments (n=3).