Supplementary material

Incorporation of a histone mutant with H3K56 site substitution perturbs the replication machinery in mouse embryonic stem cells

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**Supplementary Materials and methods**

**Cell culture, primary cells, and generation of stable cell lines**

Mouse embryonic stem cells (mESCs, J1 strain) seeded on inactivated mouse embryonic fibroblast (MEF) feeder cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Thermo Fisher Scientific, Waltham, Massachusetts, USA, #11995) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, #10099141), 1× nonessential amino acids (NEAAs, Thermo Fisher Scientific, #11140), 1×GlutaMAX (Thermo Fisher Scientific, #35050061), 7 μM β-mercaptoethanol (Ameresco, Framingham, Massachusetts, USA, M8210), 10 ng/ml mouse leukemia inhibitory factor (Merck, Darmstadt, Germany, LIF2050) and 1% antibiotic (Pen-Strep, Thermo Fisher Scientific, #15140122). All mESCs were subpassaged every 2-3 days by digesting the cell clone into a single-cell suspension using 0.25% trypsin-EDTA (Thermo Fisher Scientific, #25200072), and the resulting cells were divided on 3-5 fresh feeder plates.

HeLa cells and primary MEFs were cultured in DMEM plus 10% FBS. Primary MEFs were isolated from the skin of 13-day-old pregnant mice (CF1 strain) by trypsin digestion for 1 hour at 37°C, and the resulting single-cell suspension was collected and cultured in DMEM plus 10% FBS and 1% antibiotic. After 3 passages, the cells were inactivated by X-ray irradiation (8 Gy, 5 min) and used as mESC feeders.

To generate the stable mESC lines, concentrated lentiviruses (~10⁷ IFU) were added to 0.2 ml of mESC single-cell suspension (10⁵ cells) for 30 min under growth conditions. Then, the cell-virus mixture was diluted in 2 ml of growth medium and transferred to a fresh feeder. After 3 days, the transduction-positive cells were sorted by flow cytometry using a fluorescence reporter as a selection marker (FACS, BD Bioscences, San Jose, CA, USA).

**Cell staining and EdU cell proliferation detection**

DNA replication was tested using a Click-iT EdU Staining Kit (Thermo Fisher Scientific, C10634, C10640). Cells in the exponential phase were labeled by adding EdU to the growth medium at a final concentration of 10 μM, and all mESCs were pulsed for 15 min under growth conditions. After labeling, EdU incorporation was immediately ceased by replacing the cells into growth medium without EdU, and the cells were collected for the following Click-iT reaction according to the manufacturer’s instructions for the kit. The cells were finally detected by FACS, and fluorescence was quantified with arbitrary units. The ratio of EdU-positive cells and the EdU mean value were analyzed using FlowJo software.

mESC pluripotency was roughly evaluated by quantifying the para-nitrophenol product formation catalyzed by alkaline phosphatase (SIDANSAI, Shanghai, China, #1101-050). Other
molecular-grade reagents were all obtained from Merck.

Replication refiring activity was assayed by detecting the remaining EdU incorporation after Hydroxyurea (HU, Merck, H8627) pretreatment for blocking. Before EdU addition, 4 mM HU was added to the cell medium, and the cells were cultured for 4 hours. Then, EdU was added to the culture, and the cells were incubated for another 15 min. EdU staining was performed according to the method described above.

Antibodies and other reagents

Antibodies against histone H3 (Abcam, Cambridge, UK, ab1791), H3K4me3 (Abcam, ab8580), H3K9me3 (Abcam, ab8898), H3K36me3 (Abcam, ab9050, or Abclonal, Wuhan, China, A2366), H3K56me1 (Abcam, ab66857), H3K56me3 (Rockland, Philadelphia, Pennsylvania, USA, #600-401-194), H3K56ac (Abcam, ab76307), GFP (Abcam, ab290), PCNA (Abcam, ab29), Chk1 (Proteintech, Wuhan, China, 10362-1-AP), MCM2 (Abclonal, A0502), MCM3 (Abcam, ab4460), Phospho-Tyrosine (p-Tyr-102) (CST, Massachusetts, USA, #9416), Phospho-Threonine (CST, #9381), ORC1 (Abcam, ab85830), ORC6 (Abclonal, A5426) and HA (Abcam, ab9110) were used for western blot detection; GAPDH (Novus, St. Louis, Missouri, USA, NB300-221) and β-actin (Santa Cruz Biotechnology, Dallas, Texas, USA, SC47778) were used as internal loading controls for total protein western blotting. Other chemical reagents of biotechnology grade were purchased from Merck or Amresco.

DNA fiber assay

DNA combing analysis was performed as described previously (Bianco et al., 2012). Briefly, cells were incubated consecutively with 25 μM CldU and 250 μM IdU for the indicated times. Next, cells were harvested and were embedded into agarose plugs to perform the DNA extraction procedure. Genomic DNA was stained with YOYO-1 and was resuspended in MES buffer. DNA fibers were stretched on silanized coverslips using the Dip Coater instrument (HTLAB, Shanghai, China, HTDC-300) and were crosslinked to the glass surface by baking at 60°C. Slides were incubated with primary antibodies (Abcam ab6326 for detection of CldU; Merck B2531 for detection of IdU) after denaturation of DNA with NaOH, washed with PBS and incubated with Cy3- or 488-conjugated secondary antibodies (Jackson ImmunoResearch, Pennsylvania, USA, 112-165-003; 115-545-062). Fibers were examined using a 63× confocal microscope (Carl Zeiss, Oberkochen, Germany, LSM800). At least 100 replication tracts were quantified for each samples.

Duolink proximity ligation assay
The Duolink® PLA was performed using Duolink In Situ Orange Starter Kit (Merck, DUO92101). Briefly, cells were seeded and cultivated on glass coverslips to 70% confluency, fixed in 4% formaldehyde for 15 min and permeabilized in 0.2% Triton X-100 for 10 min. After blocking for 1 h, cells were incubated with primary antibodies against HA (Abcam, ab9110; Santa Cruz Biotechnology, SC7392) and Chk1 (Santa Cruz Biotechnology, SC8408) or MCM3 (CST, #4012), Chk1 (Santa Cruz Biotechnology, SC8408) and MCM3 (CST, #4012) at 4°C overnight and then processed the following reaction according to the manufacturer’s instructions. Slides were examined using a 63× confocal microscope (Carl Zeiss, LSM800). PLA signals correspond protein-protein interaction couples.

**Coimmunoprecipitation for histone mutants**

Vectors of histone H3 mutants fused with EGFP were transiently transfected into mESCs using Xfect™ mESC Transfection Reagent (Takara, #631320). After 48 hours of transfection, the cells were collected, and nuclear extracts were prepared with salt extraction methods. Briefly, the cell pellets were resuspended in 5 packed cell volumes of hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and protease inhibitors (EDTA-free, Roche)), and the suspension was transferred to a Dounce homogenizer (type A) and lysed with ~10 strokes after incubation for 10 min on ice. The lysate was then centrifuged at 1000 ×g for 15 min to pellet the nuclei, which were resuspended in low-salt buffer (20 mM HEPES (pH 7.9), 25% glycerol, 20 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and protease inhibitors (EDTA-free, Roche)). The histone mutants and their binding proteins were extracted by adding a 1/4 volume of high-salt buffer (20 mM HEPES (pH 7.9), 25% glycerol, 1.2 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and protease inhibitors (EDTA-free, Roche)) and incubated on a rocker for 30 min. The extract was then centrifuged at 25000 ×g for 30 min at 4°C, and the supernatant was diluted with HEPES-glycerol buffer to lower the KCl concentration to 150 mM. Finally, the histone mutants were captured by incubating the extracts with GFP-trap beads (Chromotek, Munich, Germany, GTA-20) for 2 hours at 4°C. Unbound proteins were removed by extensively rising with washing buffer (20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and protease inhibitors (EDTA-free, Roche)).
**Supplementary Figures**

**Supplementary Figure S1.** Construction and identification of mESC strains expressing ectopic histone H3K56 mutants. (A) Lentivirus-mediated ectopic expression of histones H3K56A, H3K56M, H3K56Q, H3K56R and H3WT in mESCs. (B) Representative photographs (upper and middle panels) and alkaline phosphatase staining (bottom panel) of the positive clones carrying histones H3WT, H3K56A, H3K56M, H3K56Q and H3K56R. Scar bar, 200 µm. (C) Western blot of chromatin-deposited ectopic histone H3WT and H3K56 mutants. An HA tag on ectopic histones was used for the assay, endogenous H3 was used for chromatin control, and β-actin was used as the whole-lysate control. (D) Nuclear extracts from H3K56 mutants were subjected for GFP-trap pull-down followed by immunoblotting. The expression levels of H3K56 mutants compared to endogenous H3 were detected and the ratios were shown. The signal intensities were calculated by Image J.
Supplementary Figure S2. Detection of EdU and PI double staining of H3K56 mutants and H3WT by flow cytometry. Representative FACS images of EdU staining of stable mESC lines carrying H3.1WT, H3K56A, H3K56M, H3K56Q, H3K56R. The boxed region indicates the cells in S phase. The red dashed line indicates the mean value of EdU intensity in H3WT or H3K56 mutants.
Supplementary Figure S3. Cell cycle assay of the H3K56A and H3K56M strains by flow cytometry. The percentages of G1-, S- and G2/M-phase cells were determined by analyzing the intensity of PI staining. Data from three independent experiments are presented as the mean±SEM. *P<0.05, **P<0.01 (two-tailed t-test, n=3).

Supplementary Figure S4. DNA fiber combing assays of H3K56 mutants. Confocal images of DNA fibers in H3K56 mutant cell lines. Cells were labeled for 10 min with 25 μM CldU and were further incubated for 20 min with 250 μM IdU. DNA fibers were analyzed by DNA combing as described in the text. Red: CldU. green: IdU. Scar bar, 10 μm.
**Supplementary Figure S5.** Effect of HU treatment on EdU incorporation. (A) Flow cytometry analysis of the cell cycle by PI staining after HU treatment for 4 h. (B) Representative FACS images of EdU-labeled mESCs after HU blockade treatment. The square region indicates the cells in S phase, and the red dashed region indicates the cells sensitive to HU blockade.
**Supplementary Figure S6.** Immunoprecipitation analysis of chromatin regulators that interact with histone mutants. (A) Schematic procedure for purification and immunoprecipitation of HA-tagged histone mutants. (B) Co-IP results obtained using the bait protein HA in mESC nuclear extracts carrying histone mutants. Chk1 was detected in IP products by western blotting.
Supplementary Figure S7. MCM3 interacts with Chk1 in H3K56A and H3K56M cell lines. (A-B) Duolink proximity ligation assay using antibodies against Chk1 (mouse) and MCM3 (rabbit) were performed in H3K56A and H3K56M cell lines. Red spots represent protein-protein interaction couples. The control shown indicates the omission of the primary antibody against Chk1. Scar bar, 10 µm, scar bar of the enlarged images, 5 µm.
Supplementary Figure S8. RNA-seq analysis of transcripts in mESCs expressing H3K56A. (A) RPKM value statistics of the detected genes *MycL1* and *Tritl* in the Chr. 4 qD2.2 region in H3WT and H3K56A strains. (B) Volcano map of the differentially expressed genes of H3K56A vs H3WT. The dot plot indicates the genes that were differentially expressed in H3K56A compared with H3WT.

Supplementary Figure S9. H3K56ac distribution in non-amplified regions. The H3K56ac ChIP DNA from H3WT and H3K56A/M cells were used for qPCR detection. *Eif3j1*, *Ccr3*, *Matn3* and *Angpt4* in non-amplified genome regions were used for test.
Supplementary Figure S10. Binding strength of deposited histone H3 with the replisome candidates. 

(A) Diagram of the experimental procedure. H3WT-GFP coding vectors were transfected into the HeLa cell line to construct a stable cell strain. The cells were treated with C646 and with DMSO as the vehicle, and the resulting nuclear extract products were used for GFP-trap. The captured products of the H3-GFP complex were used for western blotting to detect the replisome candidates. 

(B) Immunoblotting of replisome candidates of MCM2, MCM3 and Chk1. GFP was used as an ectopic H3-captured loading control, and internal H3K56ac was used to detect the inhibitory effect of p300.
Supplementary Figure S11. Immunoblotting assay of histone modifications in mESCs expressing H3K56A and H3K56M. Whole-cell lysates of mESCs carrying H3K56A/M were used for western blotting. H3 was used as the loading control.

Supplementary Figure S12. Knockdown of Chk1 decreases the phosphorylation levels of MCM3. MCM3 were immunoprecipitated from nuclear extracts and total phosphorylation level on the isolated MCM3 were tested. p-Thr and p-Tyr were detected in IP products by western blot using specific antibodies.
Supplementary Figure S13. Effect of Chk1 on tumor formation of H3K56A or H3K56M strains. (A) Teratoma formation by H3K56A cells transfected with shNC and shChk1 in nude mice. The image shows a carcinoma 4 weeks after injection of the mESCs. (B) Statistical analysis of the teratoma weight in mESCs, as shown in Figure A. Error bars indicate SD (n=10 mice in each group), and p values were obtained by t-tests. (C) Teratoma formation by H3K56M cells transfected with shNC and shChk1 in nude mice. The image shows a carcinoma 4 weeks after injection of the mESCs. (D) Statistical analysis of the teratoma weight in mESCs as shown in Figure C. Error bars indicate SD (n=10 mice in each group), and p values were obtained by t-tests.
Supplementary Figure S14. Effects of Ras signal firing on H3K56ac modification in mESCs. Wild-type Ras (RasWT) and activated Ras (RasY40C) were expressed in mESCs by gene transfection. The resulting whole-cell lysate was used for western blotting to detect histone H3K56ac. Total H3 was used as the loading control. Ectopic tagged GFP was used as a transfection control, and empty GFP was used as a negative control for Ras.
### Supplementary Tables

**Supplementary Table S1:** The list of proteins identified by Mass Spectrometry detection that exclusively interacted with H3K56A. High-score binding partners (no less than 50%) are listed.

| Description                                                                                                                                                                                                 | Score  |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
| WD repeat-containing protein 18 OS=Mus musculus GN=Wdr18 PE=1 SV=1 - [WDR18_MOUSE]                                                                                                                          | 79.71  |
| **Serine/threonine-protein kinase Chk1** OS=Mus musculus GN=Chek1 PE=1 SV=2 - [CHK1_MOUSE]                                                                                                                  | 78.04  |
| **Leucine-rich repeat protein SHOC-2** OS=Mus musculus GN=Shoc2 PE=2 SV=2 - [SHOC2_MOUSE]                                                                                                                   | 63.07  |
| **V-type proton ATPase subunit B, brain isoform** OS=Mus musculus GN=Atp6v1b2 PE=1 SV=1 - [VATB2_MOUSE]                                                                                                     | 56.69  |
| **Y-box-binding protein 3** OS=Mus musculus GN=Ybx3 PE=1 SV=2 - [YBOX3_MOUSE]                                                                                                                             | 55.48  |
| **40S ribosomal protein S9** OS=Mus musculus GN=Rps9 PE=2 SV=3 - [RS9_MOUSE]                                                                                                                             | 53.94  |
| **Importin subunit alpha-4** OS=Mus musculus GN=Kpna3 PE=1 SV=1 - [IMA4_MOUSE]                                                                                                                           | 53.81  |
**Supplementary Table S2: Sequences of RT-qPCR primers.**

| Gene   | Strand | Sequence                    |
|--------|--------|-----------------------------|
| Skint3 | F      | CCAGTCCTTGCCCCCATTAGG       |
|        | R      | GAGTTCTGTCCGCCCTCCACAT      |
| Skint9 | F      | AGTTTGTGATCCACCTGCTG       |
|        | R      | TTCTCCGATTTGAAACAGGGCA      |
| Mycl1  | F      | ACTCCTAGTCTGGAAGCCAGT       |
|        | R      | ACATCAATCTCTCTCACCCTGAG    |
| Bmp8b  | F      | GTCCGGGAACTCCTATGCTA       |
|        | R      | GACCCGGTGCTCGGGATCG        |
| Matn3  | F      | TCCGGGACACAAGTGCTGCTCT     |
|        | R      | TCGGGCTTCTCTGATGTCGTA       |
| Angpt4 | F      | TGTTGGAGGCACAGGTCTCTAA     |
|        | R      | ATGTGTTGCTTCCAGTGCTCT      |
| Ccr3   | F      | AAAAACTTGCAAAAACCTGAGAAGCTA|
|        | R      | GCCATTCTACTTGTCCTGGTGA       |
| Eif3j1 | F      | TCGGGATGTCTGTATTTCATTGGGA  |
|        | R      | GTGGCCTTTATCCCCCTCC        |
| β-globin| F     | GGCCTTGCTAAGCGGTTGG       |
|        | R      | AGTCTGTCTTTCTGGAAGTCAAT    |
| Map3k1 | F      | GGAGGATCTGTGGCTCACCT     |
|        | R      | GACGGTTGGGACACCTGTA       |
| Wnt6b  | F      | CGGGCAGAAAGGTTCTGGAG       |
|        | R      | CACCCGCTGCCCTGTACTCT       |
| Bbc3   | F      | GCGGGAGGAGCAAGAGAGAG          |
|        | R      | CACGGATCCCTGGGTGAAGG          |
| Pou4f2 | F      | CAGTGGCGAGAGCTTTGCTT         |
|        | R      | GGCACAGCAGACTCTCATCCA       |
| Snail1 | F      | GCACCCACACTGGTGAGAAG        |
|        | R      | GTGCTGTGGAGCAAGGACA       |
| Lef1   | F      | ATCCTGGGCAGAAGATGCGCA       |
|        | R      | AGGCAGCTGTCTAGTGGA       |
| Wt1    | F      | GTGCCAATCCAGGCTGCAA        |
|        | R      | TGGAGCTGTCTGAGCAGAGA       |
| GAPDH  | F      | ATGACATCAAGAAAGGTGTTG       |
|        | R      | CATAACCAGGAAATGAGCCTTG       |
**Supplementary Table S3:** The list of differential genes up- or down-regulated by more than 1.5-fold in H3K56A cell lines (the expression profile of the control cells was subtracted).

| H3K56A_up   | H3K56A_down   |
|-------------|---------------|
| Rn4.5s      | 1700037H04Rik |
| Tmem38b     | 2310036O22Rik |
| Gm20594     | 4930502E18Rik |
| Ifi2711     | 4930539J05Rik |
| Sfmbt2      | A2m           |
| Hist2h2bb   | Asb6          |
| C030037D09Rik | Atf3         |
| Fat2        | Atp4  |
| Rnu12       | Atp5g3 |
| Nkx2-1      | Atp5l |
| Tnc         | Bbc3 |
| 2310014L17Rik | Ccno   |
| Naaa        | Cd34 |
| Rab6b       | Cdkn1c |
| Dpysl2      | Chac1 |
| Camk2n2     | Crebzf |
| Map3k1      | Crtap |
| Med12I      | Dbr1 |
| Mir5109     | Ddr2 |
| Gm16880     | Dis3l |
| Evi2a-evi2b | Dll1 |
| Rims1       | Dmrt1 |
| Synpo       | Dpm3 |
| Slc13a4     | Dpp4 |
| Malat1      | Dpp7 |
| Negr1       | Dusp9 |
| Hif3a       | Dyrk1b |
| Lars2       | E130012A19Rik |
| Nova2       | Efnb2 |
| Satb1       | Eif1 |
| Tshz3       | En2 |
| Dsg3        | Epas1 |
| C230081A13Rik | Etv5   |
| Pcnxl2      | Flywch2 |
| Smim6       | Foxd3 |
| D8Ertd82e   | Foxn4 |
| Rmrp        | Ftl1 |
| 4932413F04Rik | Gas8  |
| Gene  | Gene  |
|-------|-------|
| Pira6 | Gpatch3 |
| Raph1 | H19 |
| Kenq1ot1 | H1fx |
| Xist | Herpud1 |
| Lrc19 | Hist1h2af |
| Cldn4 | Hmgn2 |
| Auh | Hpcal4 |
| C430002E04Rik | Hspb1 |
| Sos2 | Id3 |
| Elmod2 | Ier5l |
| Slco4c1 | Ino80b |
| Ddx60 | Irak3 |
| Zfp318 | Itgb7 |
| Gabrb2 | Kdm5d |
| Igf1r | Klk10 |
| Map1b | Lef1 |
| Pigr | Lpar1 |
| Rhox8 | Mdk |
| Ceacam20 | Mep1b |
| Cplx2 | Mfap4 |
| Ednrb | Mrpl52 |
| Klk14 | Mrps11 |
| Mylk4 | Mt2 |
| 1700010I02Rik | Mthfr |
| Pcdha12 | Myo1g |
| Pcdha6 | Ndufc1 |
| Adh6-ps1 | Notum |
| Pcdha10 | Ormd13 |
| Gm20753 | P3h4 |
| Fam124a | Phactr1 |
| Dcc | Pkp2 |
| Emp2 | Pou4f1 |
| Pcdha1 | Pou4f2 |
| Pcdha3 | Psmd9 |
| Pcdha8 | Pttg1 |
| Pcdhac1 | Rarg |
| Ffar2 | Rbm3 |
| Grm1 | Rgs16 |
| Usp17la | Ripply1 |
| Pcdha9 | Rnasek |
| Cabp7 | Robo4 |
| Rgs7bp | Rplp1   |
|--------|---------|
| Hmr    | Rps28   |
| Il1b   | Rusc2   |
| Rab3c  | Sall3   |
| D830030K20Rik | Sec61b |
| Tfap2a | Sesn2   |
| BC021785 | Shf   |
| Pcdha11 | Siah1b  |
| Tctex1d4 | Slc1a1 |
| Hs3st3b1 | Snai1   |
| Clec4a2 | Sox21   |
| Ch1I   | Sox3    |
| Spsb4  | Spic    |
| Slc13a3 | Surf6   |
| Chrna4 | Syce1   |
| Gm16576 | Tappbl  |
| Dner   | Tarbp2  |
| Rimkla | Tbx3    |
| Fam134b | Tchp    |
| Col12a1 | Tex19.1 |
| Eif4e3 | Trib3   |
| Ccl3   | Tspy12  |
| CK137956 | Ttc28  |
| Gm3002 | Uba1y   |
| Fgfr4  | Ube2s   |
| Trabd2b | Urm1    |
| Mycl1  | Wbp1    |
| Pcdha2 | Wdr46   |
| Pcdha5 | Wnk3    |
| Cabyr  | Wt1     |
|        | Zfand2a |
|        | Zfp428  |