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Supplemental Information

CD59 Regulation by SOX2 Is Required for Epithelial Cancer Stem Cells to Evade Complement Surveillance

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Supporting Information

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Supplemental Figures and Legends

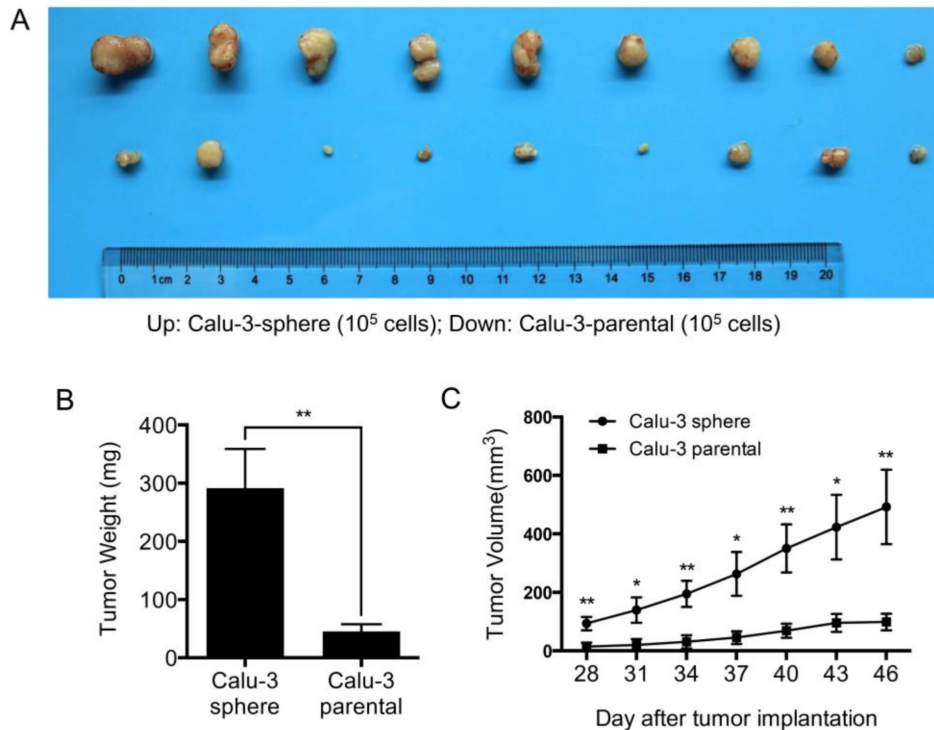

Figure S1. Stem-like Calu-3 sphere forming cells display higher tumor-initiating capability than parental cells, related to results. Same amount of sphere or parental cells were subcutaneously injected into the left or right sides of the nude mouse axilla, respectively. Tumor size was measured twice per week staring from day 28 after implantation, and the experiment was terminated at day 46. (A and B) Tumor image (A) and tumor weight (B) at the end-point of experiments. (C) Tumor growth curve. Data represent mean ± SEM (n=9, independent experiments). *, $P < 0.05$; **, $P < 0.01$.

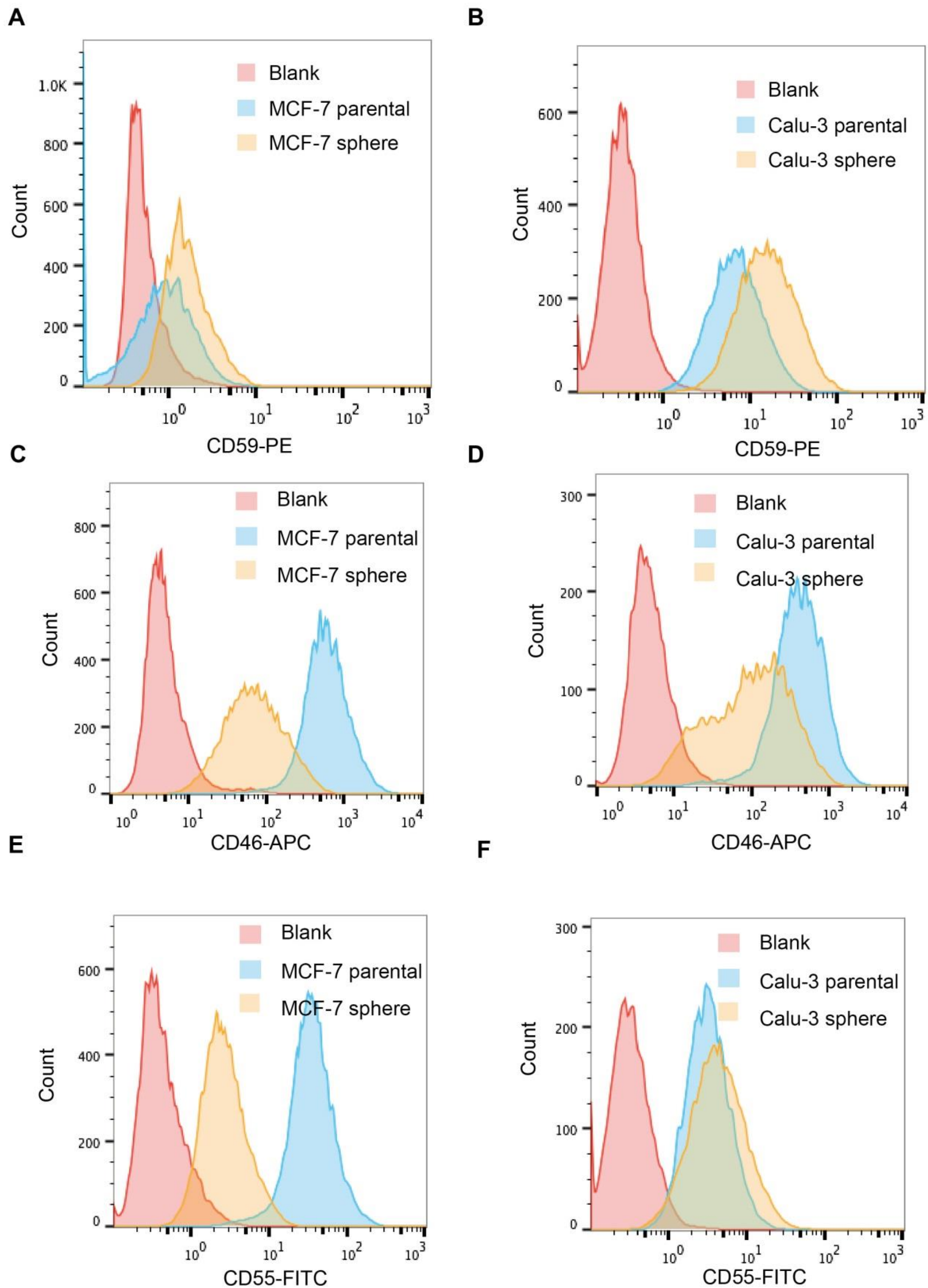

Figure S2. Comparison of the expression level changes in CD59, CD46 and CD55 between the parental and sphere cancer cells, related to Figure 1. (A and B) The CD59 levels were remarkably increased in MCF-7 (A) and

Calu-3 (B) sphere forming cells. (C and D) The CD46 levels were remarkable reduced in MCF-7 (C) and Calu-3 (D) sphere forming cells. (E and F) The CD55 levels were dramatically reduced in MCF-7 sphere forming cells (E) and slightly increased in Calu-3 sphere forming cells (F).

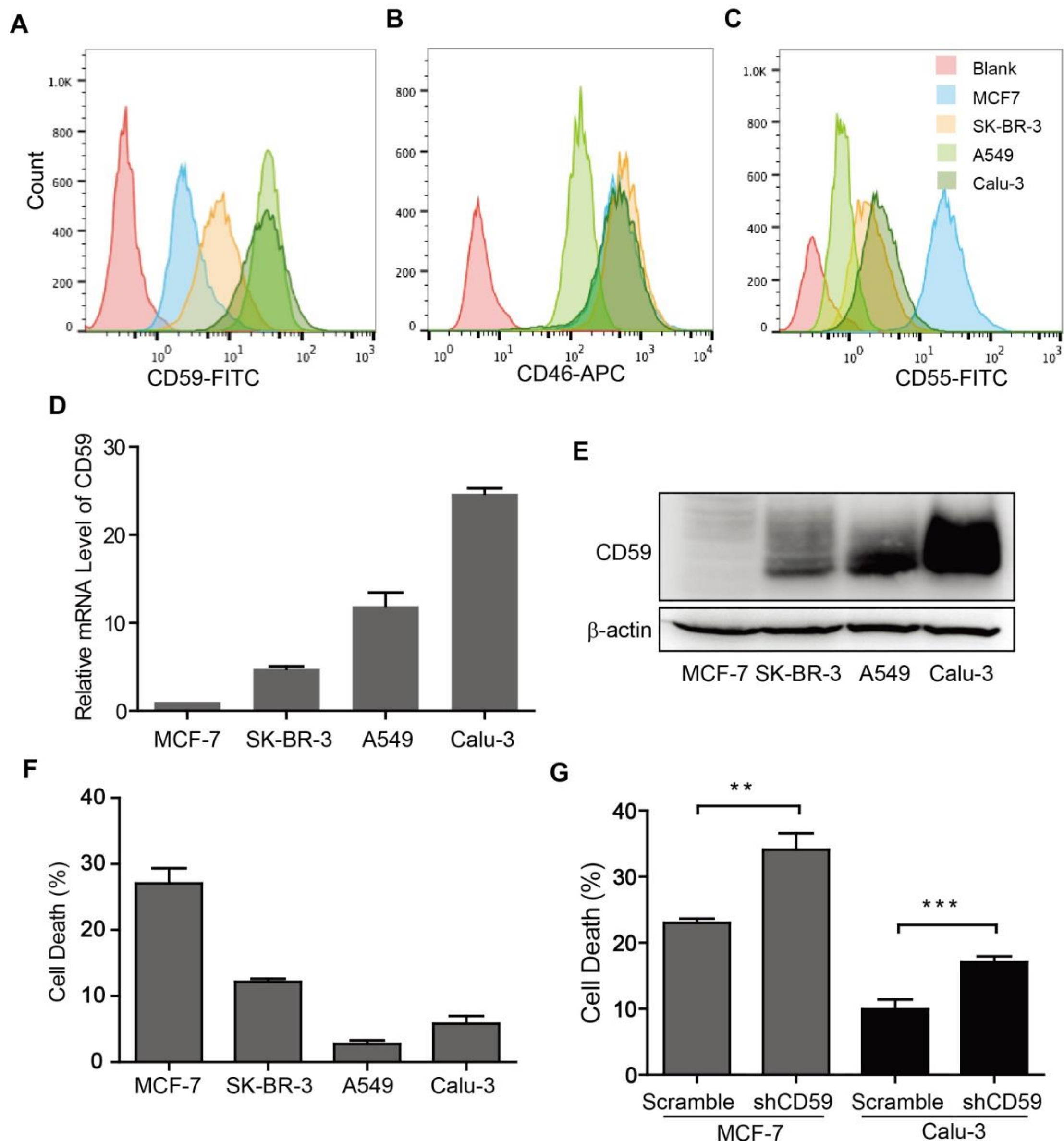

Figure S3. CD59 is the most relevant mCRP that protects parental cancer cells from complement destruction, related to Figure 2. (A-C) The expression levels of CD59 (A), CD46 (B) and CD55 (C) were detected by FACS assays. (D and E) The levels of *CD59* mRNA (D) and protein (E) increased in the order of MCF-7, SK-BR-3, A549 and Calu-3 cells, as detected by qRT-PCR (D) or immunoblotting assays (E), respectively. (F) The order of parental cancer cell sensitivity to cetuximab-induced complement-meditated

destruction measured by CDC assays was highly correlated with CD59 levels (A). (G) CD59-insufficient parental cancer cells were more susceptible than CD59-sufficient parental cells to cetuximab-induced complement-meditated destruction. Data are represented as mean ± SD (n=3, independent experiments); ***P*<0.01; and ****P*<0.001. shSCR: scrambled shRNA; shCD59: specific shRNA against CD59.

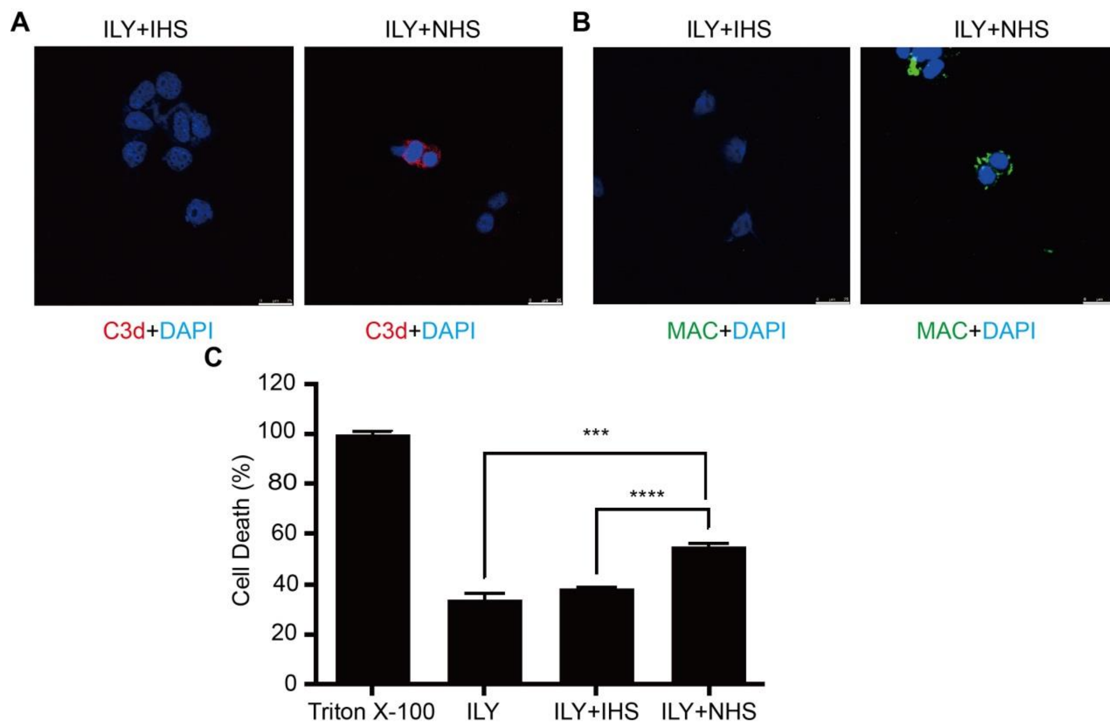

Figure S4. CD59-positive cell death induced by ILY treatment could activate complement in the survived CD59-negative MCF-7 parental cells, related to Figure 3. (A and B) ICC assay: C3d (A) and MAC (B) staining in ILY-treated cells with additional NHS or IHS administration. Scale bar represents 25 μm. (**C**) CDC assay: cell death induced by ILY treatment activated complement by NHS, leading to higher cell death rate than ILY alone and ILY plus HIS groups. Data are represented as mean ± SD (n=3, independent experiments); ***P<0.001; and ****P<0.0001. Triton X-100 induced total cell lysis as a positive control.

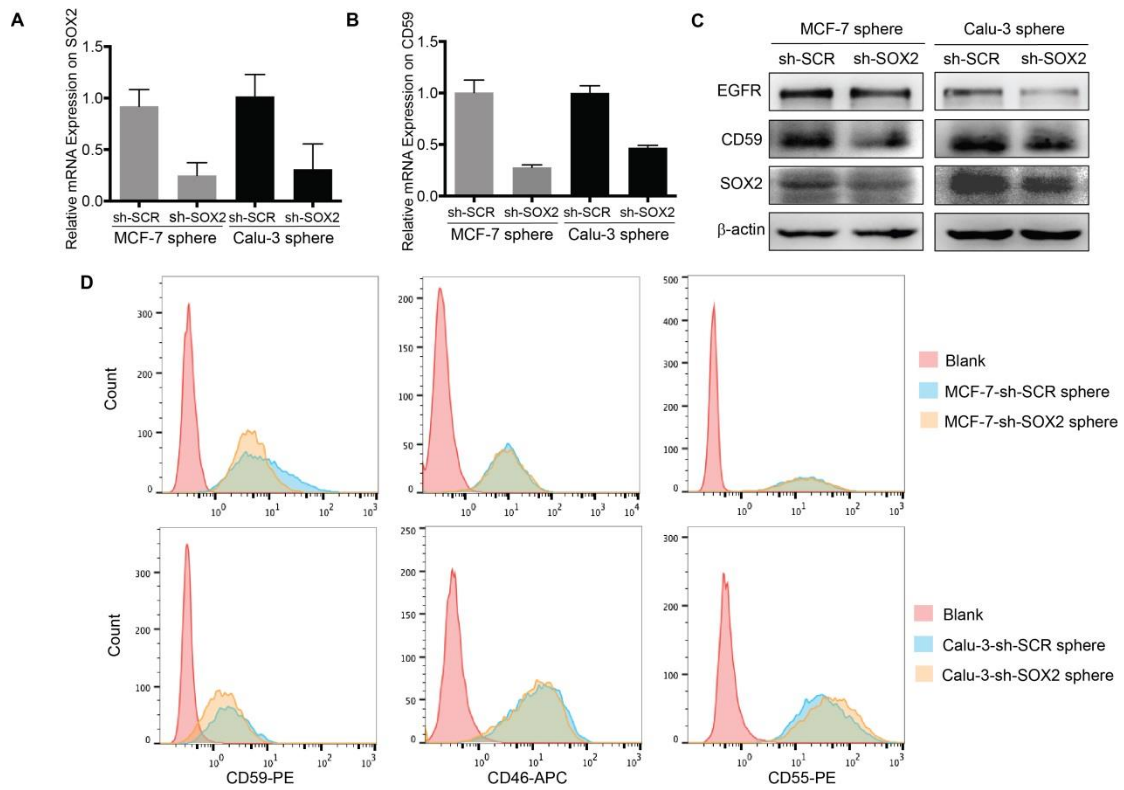

Figure S5. SOX2 insufficiency reduced the expression of CD59 in stem-like sphere forming cells, related to Figure 4. (A and B) SOX2 insufficiency (A) induced by specific shRNA reduced *CD59* transcription (B) in sphere forming cells. The mRNA levels of *SOX2* (A) and *CD59* (B) were measured by quantitative RT-PCR. Data are represented as mean ± SD (n=3, technical repeat). (C) Immunoblotting assay: The protein levels of EGFR, CD59 and SOX2 were remarkably reduced due to *SOX2* knocking-down in sphere forming cells. (D) FACS assay: The membrane level of CD59 but not of CD46 and CD55 was reduced by *SOX2* knocking-down.

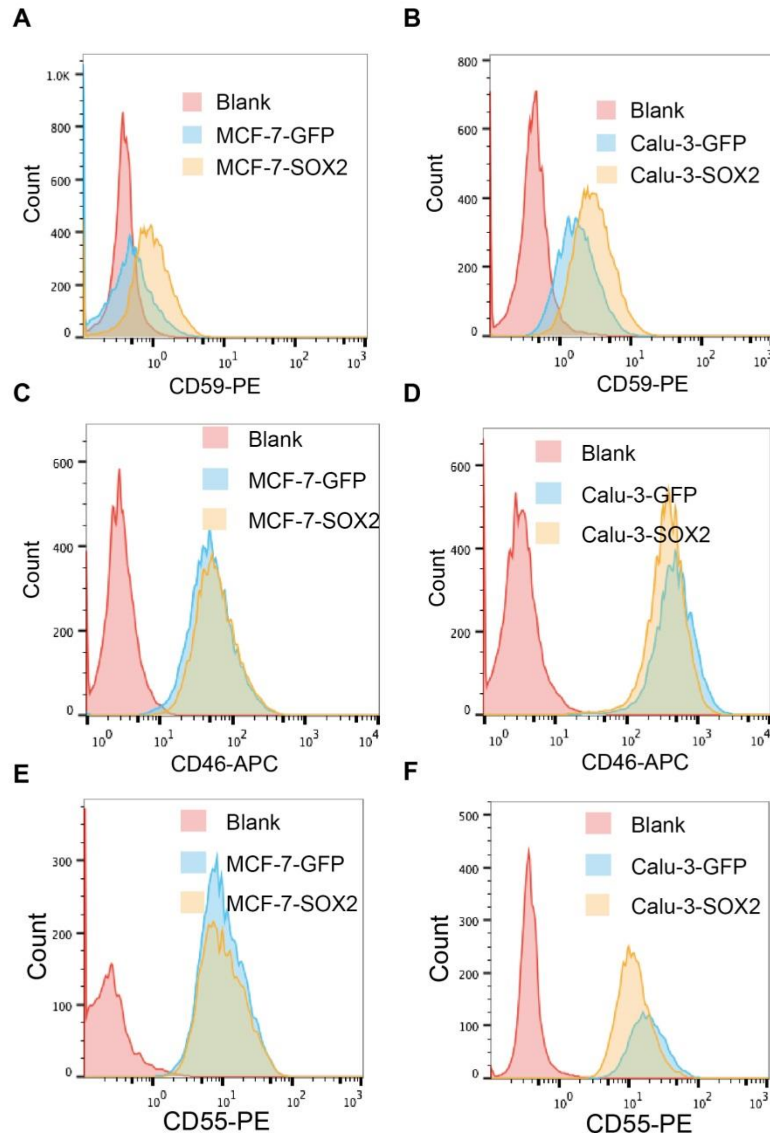

Figure S6. Ectopic SOX2 regulation effects on the expression of CD59, CD46 and CD55 in parental cells detected by FACS assay, related to Figure 5. (A and B) Ectopic SOX2 increased CD59 expression in MCF-7 (A) and Calu-3 (B) cells. (C-F) The CD46 (C and D) or CD55 (E and F) expression level was not increased by ectopic SOX2 in MCF-7 (C and E) or Calu-3 (D and F) cells, and ectopic SOX2 reduced the expression levels of CD46 (D) and CD55 (F) in Calu-3 cells.

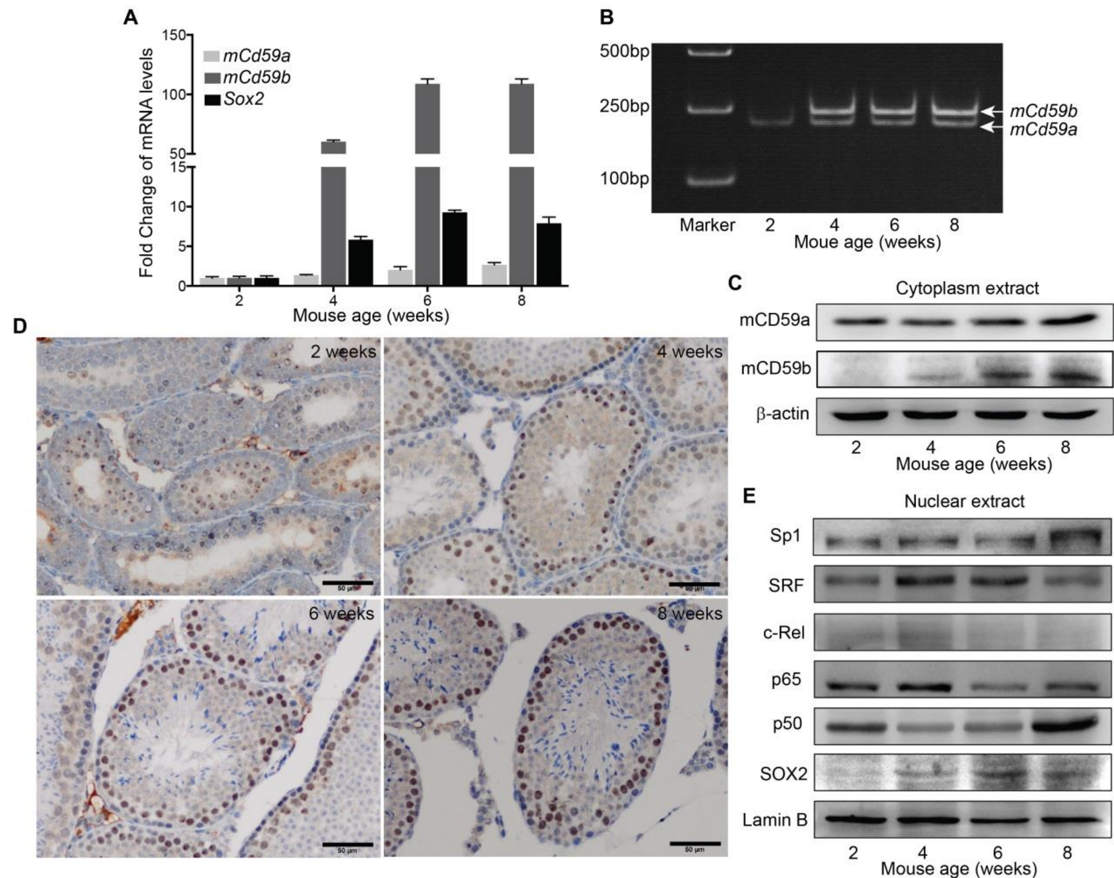

Figure S7. The expression of mCD59b but not of mCD59a is highly correlated with age with the level of SOX2 but not of other recognized transcription factors for *mCd59a* and *mCd59b* in mouse testis, related to Figure 6. Testis samples were collected from mouse with age of week 2, 4, 6 and 8. (A) Quantitative RT-PCR: the expression levels of *Sox2* and especially *mCd59b* but not of *mCd59a* were dramatically increased with age. Data were presented as mean ± SD, and experiments were biologically triplicated, n=3. (B) Reverse transcription PCR: a pair of primers that can amplify both *mCd59a* and *mCd59b* was employed, and PCR products (204 bp for *mCd59a*, and 237 bp for *mCd59b*) were separated by 5% PAGE. (C) Immunoblotting assay: cytoplasm level of mCD59b but not of mCD59a increased with age in mouse

testis samples. (D) SOX2 level increased with age detected by immunohistochemistry assay. Scale bar: 50 μm. (E) Immunoblotting assay: nuclear level of SOX2 but not of other recognized transcription factors for *mCd59a* and *mCd59b* increased with age in mouse testis.

Table S1. The commercial antibodies used in this study, related to methods.

Antibodies	**Manufacturers**	**Applications in this study**	**Catalog Number**
SOX2 (D6D9) XP Rabbit mAb	Cell Signaling Technology	WB (1:1,000), ChIP (5μg/test), IHC (1:100)	3579
Normal Rabbit IgG	Millipore	ChIP (5μg/test)	12-370
CD59 (H-7)	Santa Cruz Biotechnology	WB (1:500)	sc-133170
β-actin (C4)	Santa Cruz Biotechnology	WB (1:1,000)	sc-47778
NFKB p65 (F-6)	Santa Cruz Biotechnology	WB (1:500)	sc-8008
NFKB p50 (E-10)	Santa Cruz Biotechnology	WB (1:500)	sc-8414
c-Rel (B-6)	Santa Cruz Biotechnology	WB (1:500)	sc-6955
Lamin B (M-20)	Santa Cruz Biotechnology	WB (1:500)	sc-6217
Sp1 (E-3)	Santa Cruz Biotechnology	WB (1:500)	sc-17824
goat anti-mouse IgG-HRP	Santa Cruz Biotechnology	WB (1:10,000)	sc-2005
goat anti-rabbit IgG-HRP	Santa Cruz Biotechnology	WB (1:10,000)	sc-2004
EGF Receptor (D38B1) XP™ Rabbit mAb	Cell Signaling Technology	WB (1:1,000)	4267s
CREB-1 (24H4B)	Santa Cruz Biotechnology	WB (1:1,000)	sc-271
Anti-Smad3 (phosphoS423+S425) antibody (EP823Y)	abcam	WB (1:1,000)	ab52903
anti-Smad3 antibody	Arigo Biolaboratories	WB (1:500)	ARG53570
FITC Mouse Anti-Human CD59	BD Pharmingen	FACS (20μL/test)	555763
PE mouse Anti-Human CD59 (H19)	BD Pharmingen	FACS (20μL/test)	560953
PE anti-mouse/human CD44	BioLegend	FACS (5μL/test)	103007
PE anti-human CD46 (TRA-2-10)	BioLegend	FACS (5μL/test)	352401
FITC anti-human CD55 (JS11)	BioLegend	FACS (5μL/test)	311305
Hu CD55 PE (IA10)	BD Pharmingen	FACS (5μL/test)	561901

APC anti-human CD46 (TRA-2-10)	BioLegend	FACS (5μL/test)	352405
CD24-PerCP-Vio700	Miltenyi Biotec	FACS (10μL/test)	130-101-258
CD133/1 (AC133) -PE	Miltenyi Biotec	FACS (10μL/test)	130-098-826
Anti-C5b-9 antibody	abcam	IHC (1:200) ICC (1:100)	ab55811
Mouse Complement Component C3d Antibody	R&D SYSTEMS	IHC (1:200) ICC (1:100)	AF2655
p-CREB-1 (Ser133)	Santa Cruz Biotechnology	WB (1:500)	sc-101663
p300 (N-15)	Santa Cruz Biotechnology	WB (1:500)	sc-584
CBP (451)	Santa Cruz Biotechnology	WB (1:500)	sc-1211
SRF (D71A9) XP Rabbit mAb	Cell Signaling Technology	WB (1:1000)	5147
Alexa Fuor®488 goat anti-rabbit IgG (H+L)	Life Technologies	ICC (1:1000)	A-11034
Alexa Fuor®594 goat anti-mouse IgG (H+L)	Life Techonlogies	ICC (1:1000)	A-11005
peroxidase-conjugated affinipure goat anti-rabbit IgG H&L	Proteintech Group Inc.	IHC (1:200)	SA00001-2

Table S2. The sequences of primers and shRNA, related to methods.

Primers	5' to 3'
mouse *Sox2* CDS Forward primer	ATGTATAACATGATGGAGACGGAG
mouse *Sox2* CDS Reverse primer	TCACATGTGCGACAGGGGCA
human *SOX2* CDS Forward primer	ATGTACAACATGATGGAGACGGAG
human *SOX2* CDS Reverse primer	TCACATGTGTGAGAGGGG
human *SOX2* shRNA Forward primer	AATTAGGAGCACCCGGATTATAAATCTCGAGATTTATAATCCGGG TGCTCCTTTTTTTTAT
human *SOX2* shRNA Reverse primer	AAAAAAAAGGAGCACCCGGATTATAAATCTCGAGATTTATAATCC GGGTGCTCCT
CD59 shRNA Forward primer	CCGGGCTAACGTACTACTGCTGCAACTCGAGTTGCAGCAGTAG TACGTTAGCTTTTTG
CD59 shRNA Reverse primer	AATTCAAAAAGCTAACGTACTACTGCTGCAACTCGAGTTGCAGC AGTAGTACGTTAGC
scramble shRNA Forward primer	AATTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTT AACCTTAGGTTTTTTT
scramble shRNA Reverse primer	AAAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGA CTTAACCTTAGG
-2000 to -1 bp upstream *CD59* exon1 Forward primer	GAACATATAAGTGGAGATGTCCA
-2000 to -1 bp upstream *CD59* exon1 Reverse primer	GCCCCTCAGGATGCCCTT
-1000 to -1 bp upstream *CD59* exon1 Forward primer	TGGCCAGAGATAAACATGCAGT

-1000 to -1 bp upstream *CD59* exon1 Reverse primer	GCCCCTCAGGATGCCCTT
-350 to -1 bp upstream *mCd59b* exon1 Forward primer	GGGTTGAAAGAAGTAGAAGGAA
-350 to -1 bp upstream *mCd59b* exon1 Reverse primer	GGCTTAACATAACCCAGTGTTAG
human *CD59* qRT-PCR Forward primer	GCCAGTCTTTAGCACCAGTTG
human *CD59* qRT-PCR Reverse primer	TACACTTGTAACCCAGCTTTGG
human *CD59* ChIP Forward primer	AACAGTAGCTACCAGCTAAGTTGA
human *CD59* ChIP Reverse primer	AGACCCAAACAAAATGTTATGCGT
mCd59a qRT-PCR Forward primer	CTGACTCTAAGATTGCAGATTTGG
mCd59a qRT-PCR Reverse primer	TGAAGAAACCACCGGTTGGAA
mCd59b qRT-PCR Forward primer	TGTAGCCGGAAGGCAAGTGTATCA
mCd59b qRT-PCR Reverse primer	ACAAGTCCCACTGACAGCATTTGC
mCd59a/b reverse transcription PCR Forward primer	GATTCCTGTCTCTATGCTGTA
mCd59a/b reverse transcription PCR Reverse primer	CAAAATGGCCACCAGAAC

SI Materials and Methods

Cell culture and reagents

All cell lines in this study were purchased from the Type Culture Collection Cell Bank, Chinese Academy of Sciences. Mouse NIH/3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Human lung Calu-3 and cervical HeLa cancer cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and human breast MCF-7 cancer cells were maintained in Eagle's minimum essential medium supplemented with human recombinant insulin (PeproTech, Rocky Hill, NJ) at a final concentration of 0.01 mg/ml, 10% fetal bovine serum and 1% penicillin/streptomycin. The human breast SK-BR-3 cancer cells were maintained in McCoy's 5a medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The human lung A549 cancer cells were maintained in F-12K medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Normal human serum (NHS) as a complement resource was pooled from 10 healthy persons and aliquoted and then stored at −80°C until use. Heat-inactivated human serum (IHS) was prepared in a 65°C water bath for 30 min as a negative control. The anti-mCD59b polyclonal antibody was generated as previously described (Chen et al., 2015). Information regarding

the commercial antibodies used in this study is shown in Table S1.

Plasmid construction and lentiviral transduction

The coding DNA sequences (CDS) of human and mouse SOX2 were obtained by PCR amplification from cDNA pools of human A549 cells and mouse testis, respectively. These sequences were inserted into the pEGFP-N1 via EcoRI and BamHI endonuclease sites for transient SOX2 overexpression in HeLa or NIH/3T3 cells. The human SOX2 CDS was also cloned into the pCDH cDNA cloning and expression lentivector (Cat#CD511B-1, System Biosciences, Palo Alto, CA 94303) for stable SOX2 overexpression in MCF-7 or Calu-3 cells. The pLKO.1-TRC cloning vector (Plasmid #10878, Addgene, Cambridge, MA) was utilized to construct shRNA plasmids of scramble (SCR) and CD59-specific shRNA. The pLKO.3G cloning vector (Plasmid #14748, Addgene, Cambridge, MA) was used to construct shRNA plasmids of scramble (SCR) and SOX2-specific shRNA. The pCDH, pLKO.1 or pLKO.3G plasmid was co-transfected in 293FT cells with pMD.2G and psPAX2 plasmids to generate *SOX2* overexpression, *CD59* knock-down or *SOX2* knock-down lentivirus, respectively. The lentivirus was subsequently added to MCF-7 or Calu-3 culture medium with 8 μg/ml of polybrene (Sigma-Aldrich, USA) for 24 hours of incubation. The cells transfected with the CD59 knock-down lentivirus were selected using 5 μg/ml of puromycin (Sigma-Aldrich, USA), whereas the cells transfected with the *SOX2* overexpression or *SOX2* knock-down lentivirus

were sorted by GFP. Information regarding the primers for the SOX2 CDS cloning and RNAi targets is shown in Table S2.

Quantitative real-time PCR (qRT-PCR)

Total RNA from cells or mouse tissues was extracted with TRIzol reagent (Invitrogen, Grand Island, NY) and transcribed into cDNA using a Reverse Transcription System (Promega, Madison, WI). The input cDNA was standardized and then amplified for 40 cycles with SYBR Green Master Mix (Invitrogen, Grand Island, NY) and gene-specific primers on an ABI Prism 7900HT machine (Applied Biosystems, Waltham, MA). The *ACTB* gene encoding β-actin was used as an endogenous control, and the samples were analyzed in triplicate. The primers for qRT-PCR are listed in Table S2.

Reverse transcription PCR

The reverse transcription PCR was performed as previously described (Donev et al., 2008). In briefly, 200 ng of the cDNA from 2-, 4-, 6- and 8-week mouse testis were used as templates, respectively, and a pair of primers that can amplify both mCd59a and mCd59b was used in PCR for 22 cycles. The PCR products were separated in 5% poly-acrylamide gel, then the gel was scanned by ImageQuant RT ECL 350 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The primers for reverse transcription PCR are listed in Table S2.

Dual-luciferase reporter assay

We used a dual-luciferase reporter assay to identify the regions with promoter

activity in *mCd59b* and human *CD59* according to a previous report (Du et al., 2014). Various size fragments upstream of *mCd59b* and human *CD59* exon 1 were cloned and inserted into the pGL3 Basic Vector (Promega, Madison, WI); the primer sequences are shown in Table S2. Double-stranded DNA fragments with critical site mutations (see Figure 4C and Figure 6A) for SOX2 activity were synthesized by SBS Genetech Co., Ltd (Beijing, China). Then, the pGL3-derived plasmids together with the pRL-TK plasmid were co-transfected into HeLa or NIH/3T3 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Grand Island, NY). After culturing for 48 hours, the dual-luciferase activities were measured using the dual-Luciferase Reporter Assay System (Promega, Madison, WI) on a Bio-Tek synergy HT microplate reader (Winooski, VT).

Chromatin immunoprecipitation (ChIP) assay

We performed ChIP assays as previously described (Du et al., 2014), and the related primers are shown in Table S2 and Figure 6A.

Immunoblotting assay

Immunoblotting assays were performed according to the standard protocol, and the related antibodies were shown in Table S1.

Fluorescence-activated cell sorting (FACS) analysis

Cells were detached using 0.25% trypsin/EDTA. After washing with PBS, the cells were incubated with fluorescein-conjugated antibodies for 30 minutes

and then washed and re-suspended in PBS. Flow cytometric analysis was performed with Cytomics FC 500 MPL (Beckman Coulter, Brea, CA) and analyzed with FlowJo software (Ashland, OR). Cell sorting was performed with a MoFlo XDP (Beckman Coulter, Brea, CA) according to the related fluorescence.

Immunohistochemistry (IHC) assay

For immunohistochemical staining, mouse tissue paraffin sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase for 15 min at 37°C and rinsed in PBS, followed by high-pressure antigen retrieval in citrate buffer. Then, the sections were incubated with rabbit anti-SOX2 monoclonal antibody (1:100; Cell Signaling Technology, Danvers, MA) at 4°C overnight. After rinsing 3 times in PBS, the tissue sections were incubated with peroxidase-conjugated affinipure goat anti-rabbit IgG H&L (1:200; Proteintech, Chicago, IL) at room temperature for 1 hour. Then, the immunoreactivity was measured using a GTVision III immunohistochemical detection kit (GK500705; Gene Tech, Shanghai, China) according to the manufacturer's instructions.

Immunocytochemical (ICC) staining in intermedilysin (ILY)-treated cells

MCF-7 parental cells were treated by ILY (25 nM) for 2 hours, which could induced CD59-positive cell death rapidly via binding to CD59 (Hu et al., 2008). Further, the survived CD59-negative cells were added by NHS for complement activation or IHS as a negative control. CDC assay was used to determine the

cell death rate. ICC staining was used to detect C3d and MAC deposition in cell membrane according to the standard procedure, in which first antibodies against C3d or MAC, second antibody of Alexa Fuor®594 goat anti-mouse IgG (H+L) for C3d detection, and second antibody of Alexa Fuor®488 goat anti-rabbit IgG (H+L) for MAC detection were indicated in Table S1. The images were taken using a Leica TCS-SP5 confocal microscope.

Reference

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