miR-144/451 represses the LKB1/AMPK/mTOR pathway to promote red cell precursor survival during recovery from acute anemia

Xiao Fang,[1,2,*] Feiyang Shen,[2,*] Christophe Lechauve,[3] Peng Xu,[3] Guowei Zhao,[3] Jacobi Itkow,[2] Fan Wu,[2] Yaying Hou,[2] Xiaohui Wu,[2,4] Lingling Yu,[2,4] Huiqing Xiu,[2] Mengli Wang,[2] Ruiling Zhang,[2] Fangfang Wang,[2] Yanqing Zhang,[2] Daxin Wang,[1] Mitchell J. Weiss[3] and Duonan Yu[2,5,6,7]

[1]Clinical Medical College of Yangzhou University, Yangzhou, China; [2]Jiangsu Key Laboratory of Experimental & Translational Non-Coding RNA Research, University School of Medicine, China; [3]Department of Hematology, St. Jude Children's Research Hospital, Memphis, TN, USA; [4]Department of Pediatrics, Jingjiang People's Hospital, Yangzhou University, Jingjiang, China; [5]Institute of Comparative Medicine, Yangzhou University, China; [6]Institute of Translational Medicine, Yangzhou University School of Medicine, Yangzhou, China and [7]Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Disease and Zoonosis, Yangzhou, China

**XF and FS contributed equally to this work.*

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Correspondence: dnyu@yzu.edu.cn

Supplementary Data

Supplementary Materials

Animals and genotyping

Our miR-144/451 KO mice lacking a 388-bp segment of genomic DNA containing the bicistronic miR-144 and miR-451 locus were described previously. [1] p53ER knock-in (KI) mice were kindly provided by Gerard Evan (University of Cambridge, UK) and have been maintained in our laboratory since 2006. [2] These mice were backcrossed onto a C57BL/6J background for 10 generations. All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of the Yangzhou University School of Medicine and the Children's Hospital of Philadelphia. For genotyping miR-144/451 mice, genomic DNA isolated from mouse tails was amplified by polymerase chain reaction (PCR) with 3 primers: Forward (F), 5′-TTC TGC CTG TAA CTC TGG ATC CCT AAG AGA-3′; Reverse 1 (R1), 5′-GGG TAC CCA GAC TAG TAC ATC ATC TAT A-3′; and Reverse 2 (R2), 5′-ATC CCC TCG AGG GAC CTA ATA ACT TC-3′. For genotyping p53ER mice, the following primers were used for PCR with tail genomic DNA as the template: Forward (F), 5′-CCT CCA GCC TAG AGC CTT CCA AGC-3′; Reverse 1 (R1), 5′-GGT GAG ATT TCA TTG TAG GTG CC-3′; and Reverse 2 (R2), 5′-GCA CAC AAA CTC TTC ACC CTG C-3′.

Cell culture

G1E and G1E-ER4 erythroid cells were grown in culture as previously described. [3] Where indicated, G1E-ER4 cells were treated with 25 nM estradiol for varying lengths of time to induce differentiation. The isolation of erythroid progenitors from embryonic day 14.5 (E14.5) FLs and the growth of erythroid progenitors in maturation medium were described previously. [4] In short, E14.5 embryos were genotyped and erythroid precursors were isolated from individual embryos by using the EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit (StemCell Technologies). Purified lineage-negative cells were grown in erythroid maturation medium consisting of IMDM with 10% FCS, 2 mM L-glutamine, 10 μM 1-thioglycerol, 5% Gibco® Protein-Free Hybridoma Medium II (PFHM II), and 5 U/mL of erythropoietin (Amgen). For retroviral infections, cells were grown in expansion

medium consisting of StemPro34 medium (Invitrogen) supplemented with 2 mM L-glutamine, 10 μM 1-thioglycerol, 1 μM dexamethasone, 0.5 U/mL of erythropoietin, and 1% murine stem cell factor (SCF)-conditioned medium. After 72 h of expansion, cells were washed and resuspended in maturation medium.

Cell treatment

Erythroid cells isolated from FLs were subjected to different concentrations (0, 0.5, and 1 μM) of 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrrazolo[1,5-a]-pyrimidine (AMPK inhibitor, compound C, CC; EMD Biosciences, Inc.) and 5′-phosphoribosyl-5-aminoimidazole-4-carboxamide (AICAR; Calbiochem). At 24 to 48 h after treatment, the cells were analyzed for apoptosis by flow cytometry.

Antibodies

The following flow cytometry antibodies were purchased from BD Biosciences (San Jose, CA): APC rat anti-mouse Ter119 (cat. # 557909) and PE or FITC rat anti-mouse CD71 (cat. # 553267 or 553266, respectively). The following antibodies were used for Western blot analysis: anti-GAPDH (Santa Cruz Biotechnology, cat. # sc-25778), anti–β-actin (clone AC-15, A3854, Sigma-Aldrich), anti-p53 (Santa Cruz Biotechnology, cat. # sc-6243), anti–14-3-3ζ (Santa Cruz Biotechnology, cat. # sc-1019), anti-VDAC1 (Abcam, cat. # ab14734), and anti-p-ATG13 (Ser318, Rockland, cat. # 600-401-C49). All other antibodies were purchased from Cell Signaling Biotechnology (Danvers, MA); they included antibodies against the following: MO25α/Cab39 (cat. # 2716), AMPKα (cat. # 2603), p-AMPKα (Thr172, cat. # 2531), TSC2 (cat. # 3990), p-TSC2 (Ser1387, cat. # 5584), Raptor (cat. # 2280), p-Raptor (Ser792, cat. # 2083), p70S6K (cat. # 9202), p-p70S6K (T389, cat. # 8209), S6 (cat. # 2317), p-S6 (cat. # 2211), eIF4B (cat. # 3592), p-eIF4B (cat. # 5399), p-p53 (Ser15, cat. #9284), LKB1 (cat. # 3047), p62 (cat. # 5114), cMyc (cat. # 13987), ULK1 (cat. # 8054, cat. # 5869, cat. # 14202), and ATG13 (cat. # 13273).

Western blot analysis

Protein lysates from purified erythroblasts were prepared using cell lysis buffer with 1 mM DTT and 1:500 protease inhibitor mixture (Sigma-Aldrich). Protein concentrations were determined using a Pierce bicinchoninic acid (BCA) assay kit (Thermo Scientific, Rockford, IL), and 10 μg of protein was resolved by 4.5%–15%

gradient SDS-PAGE. After being transferred to 0.2-μm polyvinylidene fluoride membranes (Whatman), proteins were detected with primary antibodies, as described above, and HRP-conjugated secondary antibodies. Horseradish peroxidase–conjugated anti–β-actin antibody was used as a loading control. Western blots were developed using SuperSignal West Pico chemiluminescent substrate. The secondary antibodies, markers, and reagents for Western blot analysis were obtained from Thermo Scientific.

Fluorescence-activated cell sorting (FACS)

The expression of RBC surface markers and cell death were analyzed with an LSRII or LSRFortessa Cell Analyzer System (BD Biosciences). Cells grown in culture or primary cells isolated from tissues were washed and resuspended in PBS containing 0.1% BSA (FACS buffer) before being stained with fluorescence-labeled antibodies. Data analyses were performed with FlowJo software (TreeStar). The Annexin V Early Apoptosis Detection Kit (Cat. # 553786) was obtained from BD Biosciences, and assays were performed according to the manufacturer's instructions. The nucleation of erythroid cells was quantitated by staining with 5 mM Hoechst 33342 (Sigma) for 1 h at 37 °C; cell viability was quantitated by flow cytometry after staining cells with Live/Dead® Near-IR Fixable Dead Cell Stain (Invitrogen) for 30 min or 7AAD for 10 min at 4 °C. Erythroid subpopulations (nucleated erythroblasts and reticulocytes) were sorted on the basis of CD71/Ter119 expression by using a FACSAria cytometer (BD Biosciences). $CD71^{+}/Ter119^{+}/FSC^{high}$ cells were defined as nucleated cells, whereas $CD71^{+}/Ter119^{+}/FSC^{low}$ cells were considered reticulocytes. [1]

Real-time PCR

Total RNAs from sorted primary cells or cells grown in culture were extracted with TRIzol reagent (Invitrogen), treated with deoxyribonuclease (DNase), and converted to cDNAs by using the PrimeScript™ First Strand cDNA Synthesis Kit (Takara Bio, Inc., Dalian, China) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed to detect cDNA levels, using an ABI 7900 Sequence Detection System with the SYBR Green RT-PCR Reagents Kit (Applied Biosystems, Foster City, CA). An All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD) was used according to the manufacturer's

instructions to detect miRNA expression. A 2-step PCR was performed, with an incubation at 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s and 60.5 °C for 30 s. The RNA U6 was used as an internal control for miRNA detection, and β-actin mRNA was used as an internal control for mRNA expression. All the amplifications were performed in triplicate. The forward primers for *miR-451* and U6 were 5′-AAACCGTTACCATTACTGAGTT-3′ and 5′-CGCTTCGGCAGCACATATAC-3′, respectively, and the Universal Adaptor PCR Primer (GeneCopoeia, Rockville, MD) was used as the reverse primer.

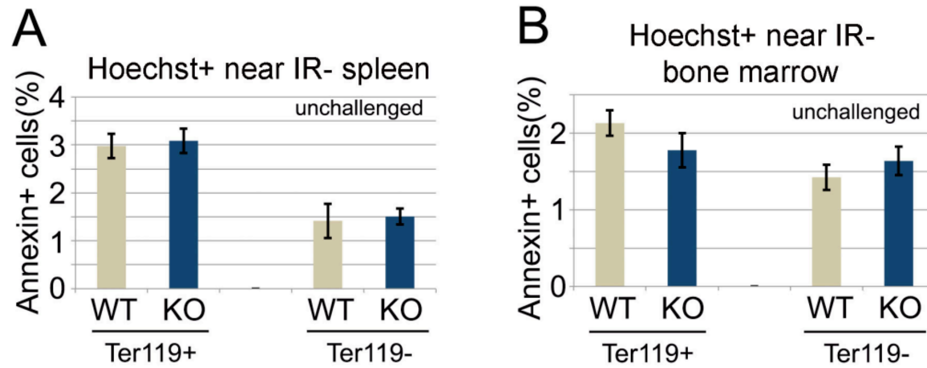
Dual-luciferase reporter assay

A 409-bp Cab39 3′-UTR fragment was amplified by PCR from Cab39 cDNA and cloned into the *Eco*RI and *Xba*I sites of the modified pGL3 luciferase reporter pGL3-BS (Promega Corporation, Madison, WI). [1] The primer sequences used were as follows: forward, 5′-GCAGTTCAACGACGAGAAGA-3′ and reverse, 5′-TGTCAGCGATGTATCACCGT-3′. The mutant constructs of the Cab39 3′-UTR were generated using the oligonucleotide-directed PCR mutagenesis assay described elsewhere. [1] Co-transfection of *Renilla* vectors with the Cab39 3′-UTR luciferase reporter plasmid or pGL3-BS empty control vector was performed using Lipofectamine 2000 (Invitrogen Life Technologies). After 24 h, the dual-luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega) and a Veritas™ Microplate Luminometer (Promega) according to the manufacturer's instructions.

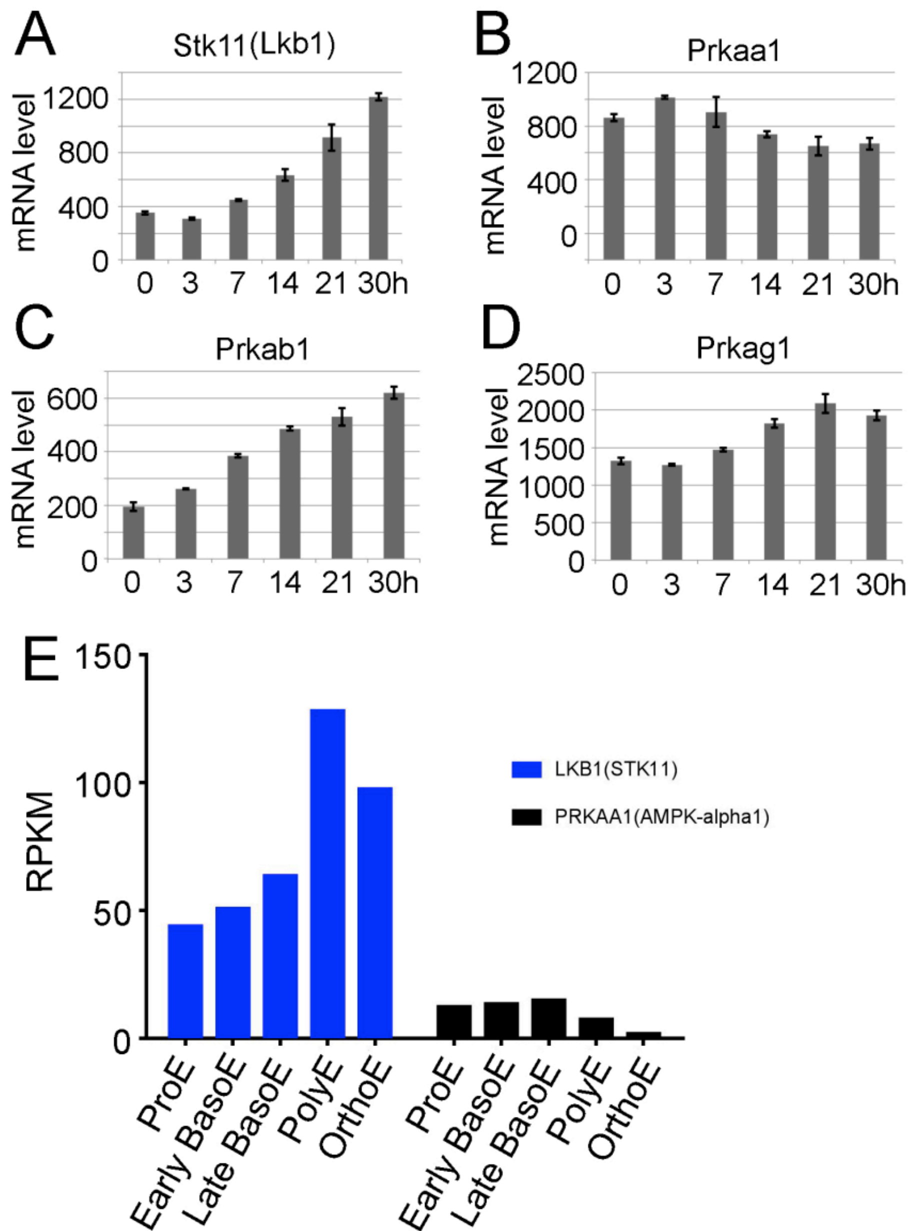
Retroviral shRNA delivery

The following miR-30–adapted shRNA constructs were purchased from GE Dharmacon: Cab39, AMPKα1, and TSC2. Selected hairpins were then subcloned into the MSCV-PIG (puromycin-IRES-GFP) retroviral vector. Cells (5×10^4) from FL cultures were infected with retroviral supernatant from 293T packaging cells plus 8 μg/mL of polybrene. For selection, puromycin was used over a 3-day period. Cells were then analyzed for protein expression and phenotype alteration.

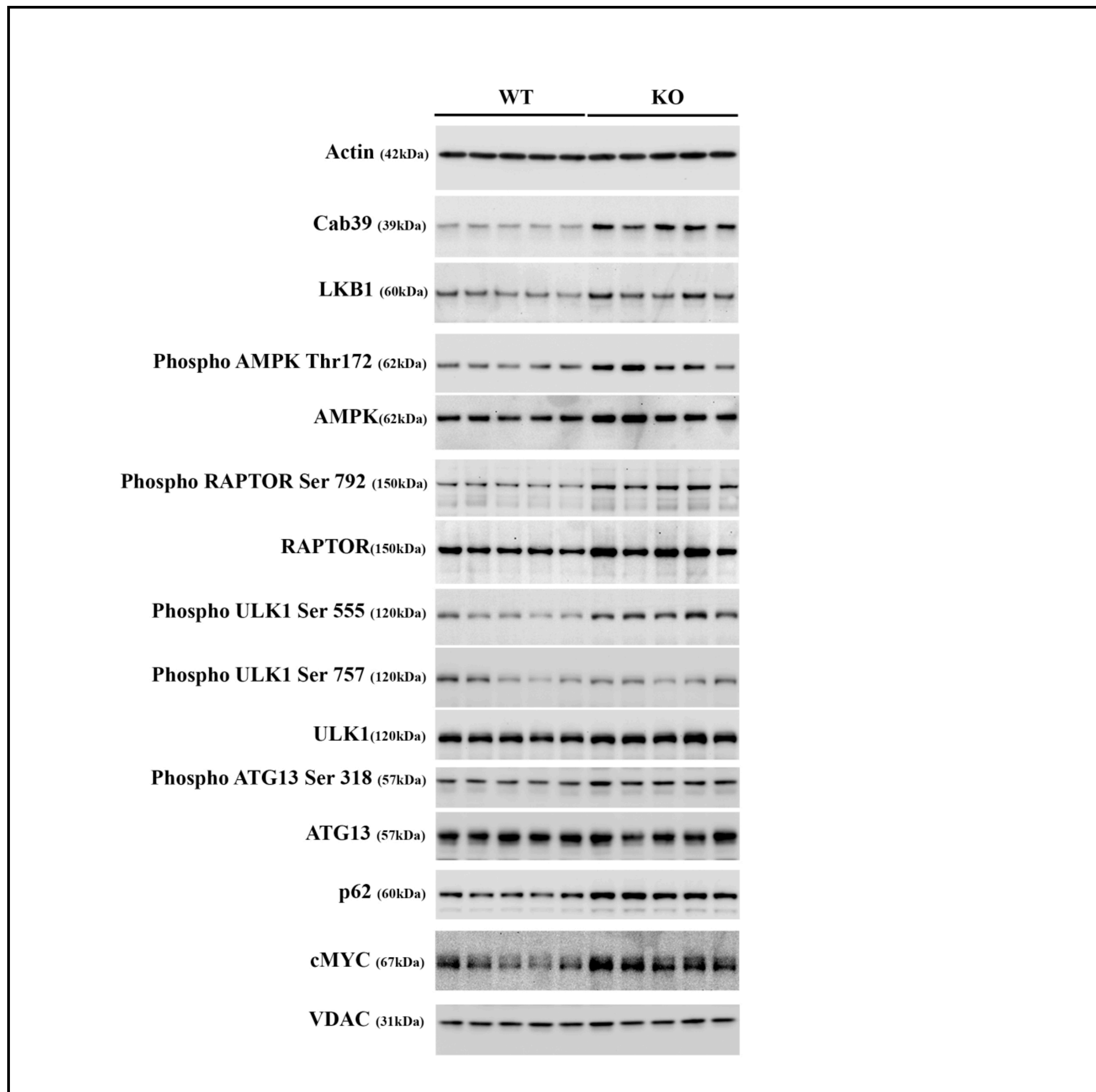
Supplementary figures



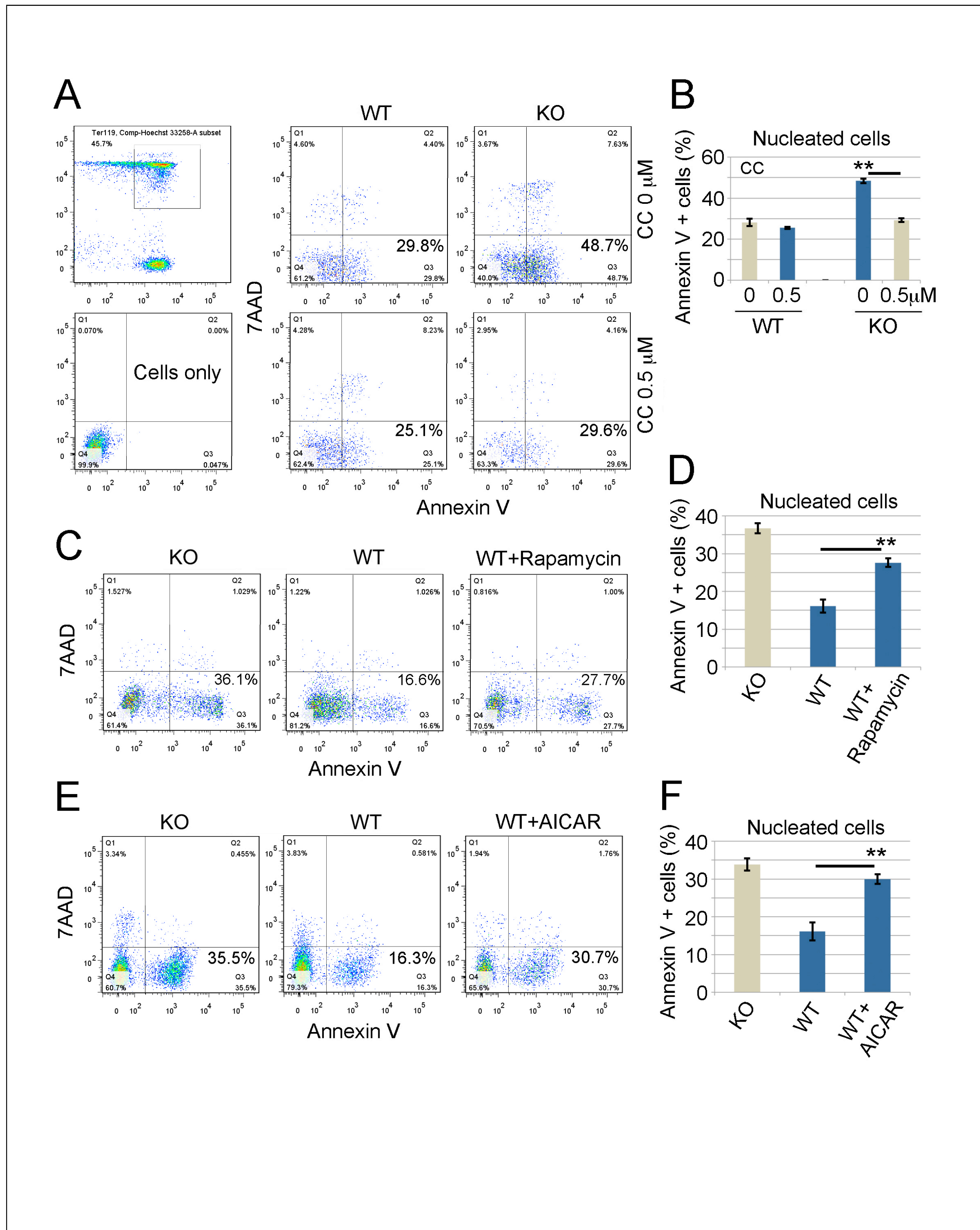
Supplementary Figure 1. Adult *miR-144/451*$^{-/-}$ (KO) mice show no increase in apoptosis of erythroid cells at the baseline when compared with wild-type (WT) mice. Flow cytometric analysis of early apoptosis rates of nucleated erythroblasts from spleen (A) and bone marrow (B) at the baseline. Ter119^{+} represents cells of erythroid origin. Hoechst^{+} indicates nucleated erythroblasts. n=5 mice of each genotype were used. Note: There was no significant change in the apoptotic cell rates for erythroblasts from WT and *miR-144/451* KO mice.



Supplementary Figure 2. *lkb1* and *ampk* mRNA levels during mouse and human erythroid differentiation. Microarray data for mouse G1EJC4 proerythroblasts, [5] showing that *lkb1* (A) and *ampkα* (B, C, D) were mostly transcriptionally upregulated during erythroid differentiation. The x-axis shows the time (in hours) of induction of erythroid differentiation by estradiol. The y-axis shows the absolute fluorescence signal from the microarray. (E) Human RNA sequencing data for *lkb1* and *ampk*. [6]



Supplementary Figure 3. Western blot analysis of the expression of Cab39, LKB1, p-AMPKα, p-RAPTOR, p-ULK1, p-ATG13, p62, cMYC, and VDAC, along with that of their nonphosphorylated counterparts, where applicable, in primary erythroblasts from E14.5 FLs of wild-type (WT) and knockout (KO) mice.



Supplementary Figure 4. Manipulation of mTOR pathway alters the apoptosis rate of FL erythroid cells in culture. (A) Flow cytometric analysis of apoptosis rescued by the AMPK inhibitor Coupon C (CC) in *miR-144/451*$^{-/-}$ erythroblasts. Lineage negative–selected E14.5 FL cells were grown in culture in erythroid maturation medium. After 48 h, the cells were stained with the cell death marker 7AAD and Annexin V. (B) Quantitative analysis of flow cytometric data from panel A, representative of 3 independent experiments. **P<0.01 (*t*-test). The x-axis shows the

concentrations of CC in the culture medium. The y-axis shows the apoptotic cell numbers. Note: There was a significant decrease in apoptotic cells in *miR-144/451*$^{-/-}$ erythroid culture after CC treatment. (C) Flow cytometric analysis of apoptosis accelerated by the mTOR inhibitor rapamycin. Lineage negative–selected wild-type E14.5 FL cells were grown in culture in erythroid maturation medium containing rapamycin at a concentration of 250 nM. After 48 h, the cells were stained with Annexin V and analyzed by flow cytometry. The apoptosis rate of the *miR-144/451*$^{-/-}$ erythroid cells was used as the positive control. (D) Quantitative analysis of flow cytometric data from panel C, representative of 3 independent experiments. ***P*<0.01 (*t*-test). The y-axis shows the percentage of apoptotic cells. Note: There was a significant increase in apoptotic cells in rapamycin-treated wild-type cultures. (E) Flow cytometric analysis of apoptosis accelerated by the AMPK activator 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAR). Lineage negative–selected wild-type E14.5 FL cells were grown in culture in erythroid differentiation medium containing AICAR at a concentration of 0.5 mM. After 48 h, the cells were analyzed by flow cytometry. (F) Quantitative analysis of flow cytometric data from panel E, representative of 3 independent experiments. ***P*<0.01 (*t*-test). Note: There was a significant increase in apoptotic cells in AICAR-treated wild-type cultures.

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