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Supplementary Materials for

The protein kinase activity of fructokinase A specifies the antioxidant responses of tumor cells by phosphorylating p62

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This PDF file includes:

Fig. S1. KHK-A is required for oxidative stress–enhanced p62 oligomerization.
Fig. S2. KHK-A is required for Nrf2 activation upon oxidative stress.
Fig. S3. AMPK phosphorylates KHK-A and promotes the association between KHK-A and p62.
Fig. S4. AMPK-mediated KHK-A S80 phosphorylation inhibits the interaction between KHK-A and PRPS1 and PRPS1 T225 phosphorylation.
Fig. S5. KHK-A acts as a protein kinase and phosphorylates p62 at S28.
Fig. S6. KHK-A is required for oxidative stress–induced but not proteasomal stress–induced p62 aggregation.
Fig. S7. KHK-A–mediated p62 S28 phosphorylation is required for oxidative stress–enhanced p62 oligomerization and Nrf2 activation.
Fig. S8. KHK-A mediated p62 S28 phosphorylation reduces ROS production and promotes cancer cell survival without altering autophagy initiation.
Fig. S9. The phosphorylation-mimicking KHK-A S80E and p62 S28E mutations promote p62 oligomerization and Nrf2 activation.
Fig. S10. KHK-A–mediated p62 S28 phosphorylation promotes hepatocellular tumorigenesis and is associated with the clinical aggressiveness of human HCC.

SUPPLEMENTARY FIGURES

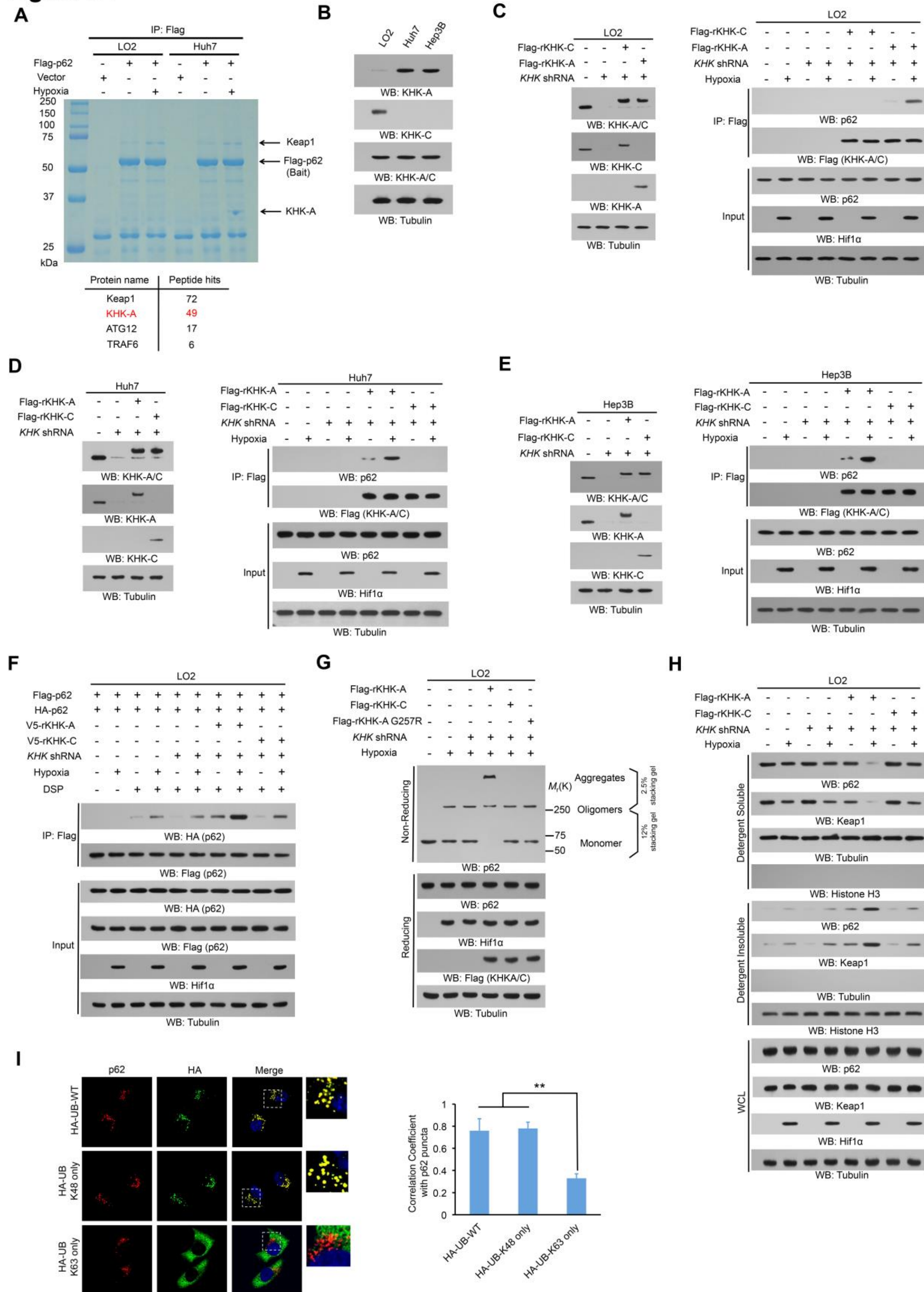

Fig. S1. KHK-A is required for oxidative stress–enhanced p62 oligomerization. (A) LO2 and Huh7 cells with or without Flag-p62 expression were treated with or without hypoxia for 6 h in the presence of 10 μM lysosome inhibitor chloroquine (CQ). Flag-p62 immunoprecipitated with an anti-Flag antibody was separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and stained with coomassie brilliant blue. The indicated protein bands were excised for mass spectrometric analysis and were identified as Keap1 and KHK-A. **(B)** The cell lysates of LO2, Huh7, and Hep3B cells were analyzed by immunoblotting with the indicated antibodies. **(C, D, E)** LO2 (**C**), Huh7 (**D**), and Hep3B (**E**) cells with or without expressing *KHK* shRNA and with or without reconstituted expression of Flag-tagged rKHK-A or rKHK-C were stimulated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). Immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. **(F)** LO2 cells with or without expressing *KHK* shRNA and with or without reconstituted expression of the indicated proteins were transfected with vectors expressing Flag-p62 and HA-p62 and treated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). After incubation with dithiobis succinimidyl propionate (DSP) (0.4 mg/ml) for 2 h, the cells were lysed in a buffer containing 1% SDS to solubilize all proteins. The lysates were subjected to immunoprecipitation analyses with an anti-Flag antibody after diluting SDS to 0.1%. **(G)** LO2 cells with or without expressing *KHK* shRNA and with or without reconstituted expression of the indicated proteins were treated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM) and analyzed by reducing and non-reducing SDS-PAGE. Immunoblot analyses were performed with the indicated antibodies. **(H)** LO2 cells with or without expression of *KHK* shRNA were reconstituted with or without expression of the indicated KHK proteins. After stimulation with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM), the cells were lysed in a lysis buffer with 1% Triton X-100. The insoluble fraction was lysed in a lysis buffer with 1% SDS. Immunoblotting analyses were

performed with the indicated antibodies. **(I)** Huh7 cells expressing HA-ubiquitin, HA-ubiquitin K48 (ubiquitin with only K48), and HA-ubiquitin K63 (ubiquitin with only K63) were treated with hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). Immunofluorescent analyses were performed with the indicated antibodies (left panel). The colocalization coefficients between the indicated proteins are presented (right panel). At least 100 cells from each independent experiment were quantified. Similar results were obtained from three repeated experiments. Values are presented as means ± SD. $^{**}P < 0.01$ (two-tailed Student *t*-test).

Figure S2

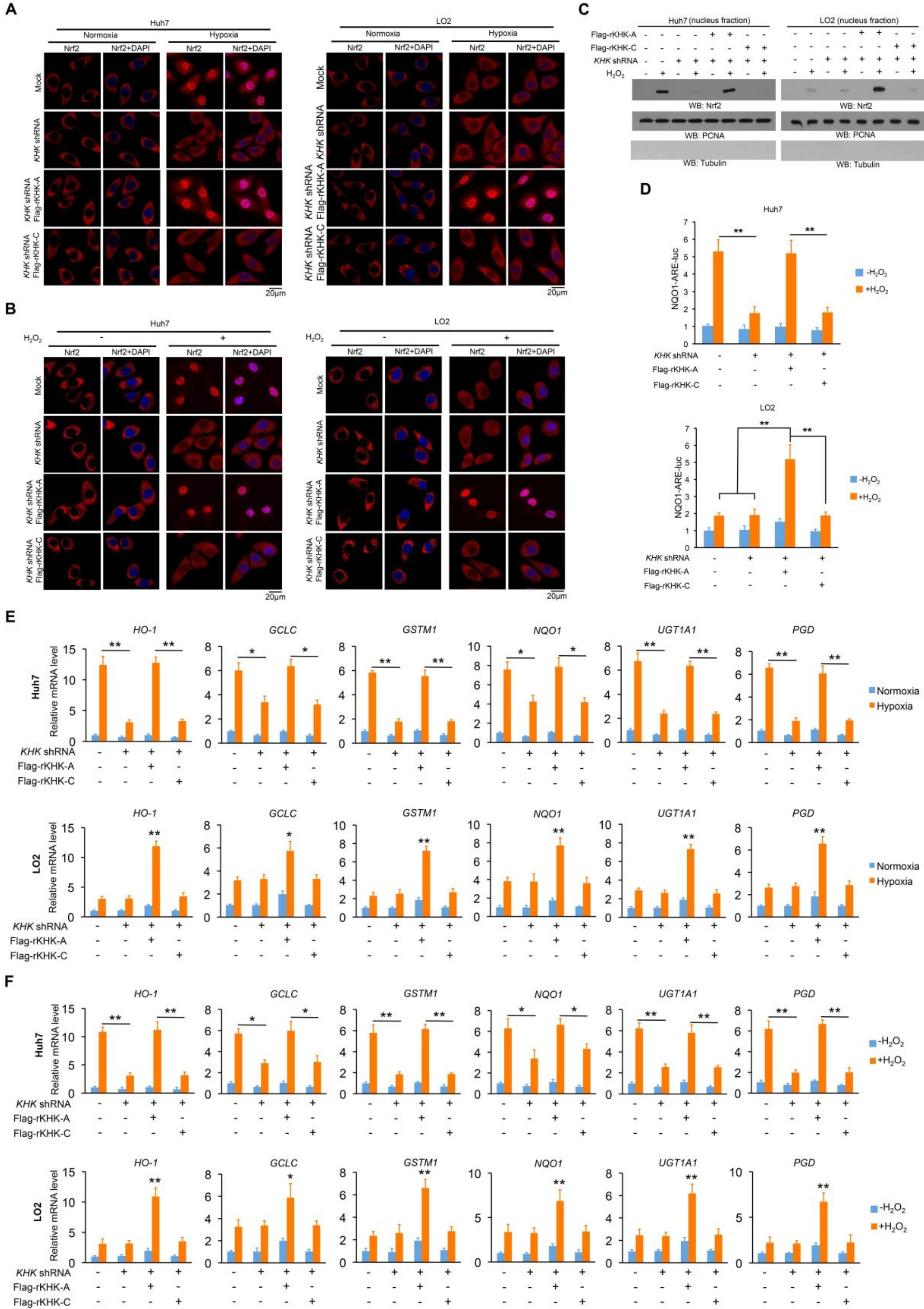

Fig. S2. KHK-A is required for Nrf2 activation upon oxidative stress. (A) Huh7 and LO2 cells with or without expressing *KHK* shRNA and with or without reconstituted expression of the indicated proteins were treated with or without hypoxia for 12 h. Immunofluorescence analyses were performed with an anti-Nrf2 antibody. **(B)** Huh7 and LO2 cells with or without expressing *KHK* shRNA and with or without reconstituted expression of the indicated proteins were treated with or without H_2O_2 (0.5 mM) for 12 h. Immunofluorescence analyses were performed with an anti-Nrf2 antibody. **(C)** Huh7 and LO2 cells with or without expressing *KHK* shRNA and with or without reconstituted expression of the indicated proteins were treated with or without H_2O_2 (0.5 mM) for 12 h. The nuclear fractions were prepared. **(D)** Huh7 and LO2 cells with or without expressing *KHK* shRNA and with or without reconstituted expression of the indicated proteins were transfected with NQO1-ARE-luc and pRL-TK plasmids. Eighteen hours after transfection, cells were treated with or without H_2O_2 (0.5 mM) for 12 h and harvested for luciferase activity analyses. The data are presented as means ± SD from triplicate samples. $^{**}P < 0.01$. **(E)** Huh7 and LO2 cells with or without expressing *KHK* shRNA and with or without reconstituted expression of the indicated proteins were treated with or without hypoxia for 12 h. The mRNA levels of Nrf2 target genes were measured by quantitative polymerase chain reaction (PCR). The data are presented as means ± SD from triplicate samples. $^{*}P < 0.05$, $^{**}P < 0.001$. **(F)** Huh7 and LO2 cells with or without expressing *KHK* shRNA and with or without reconstituted expression of the indicated proteins were treated with or without H_2O_2 (0.5 mM) for 12 h. The mRNA levels of Nrf2 target genes were measured by quantitative PCR. The data are presented as means ± SD from triplicate samples. $^{*}P < 0.05$, $^{**}P < 0.01$.

Figure S3

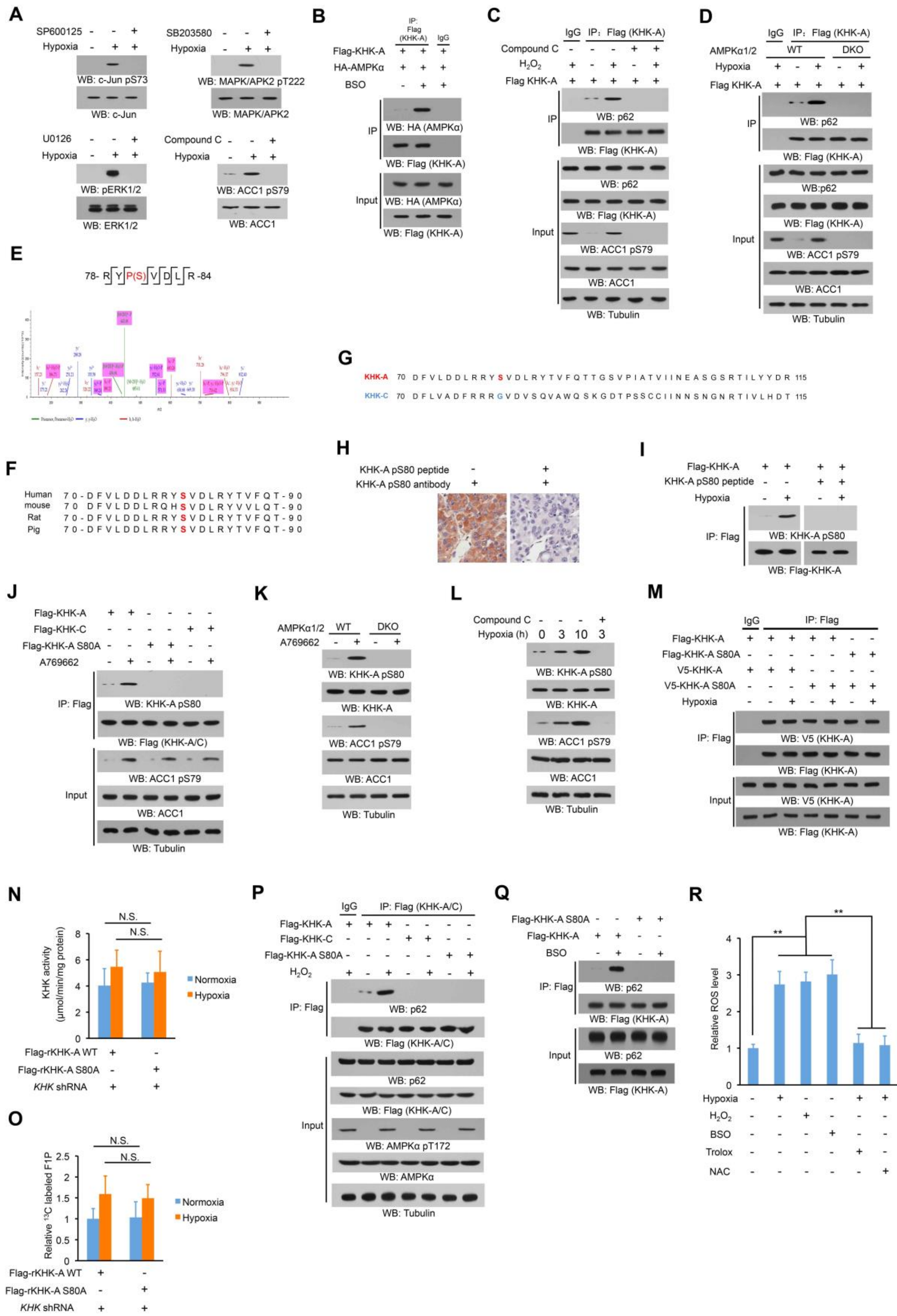

Fig. S3. AMPK phosphorylates KHK-A and promotes the association between KHK-A and p62. A-D, I-M, P, Q, Immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. **(A)** Huh7 cells were pretreated with SP600125 (25 μM), SB203580 (10 μM), U0126 (20 μM), or Compound C (5 μM) for 30 min before hypoxia stimulation for 6 h. **(B)** Huh7 cells expressing Flag-KHK-A and HA-AMPKα were treated with or without BSO (100 μM) for 12 h. **(C)** Huh7 cells expressing Flag–KHK-A were pretreated with or without compound C (5 μM) for 30 min before being treated with or without H_2O_2 (0.5 mM) for 1 h in the presence of lysosome inhibitor CQ (10 μM). **(D)** WT and AMPKα1/2 double-knockout (DKO) mouse embryonic fibroblasts (MEFs) infected with the lentivirus expressing Flag–KHK-A were treated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). **(E)** Purified GST–KHK-A was phosphorylated by AMPK *in vitro* and analyzed by mass spectrometry. A tryptic fragment at m/z 494.73422 Da (-0.02 mmu/-0.03 ppm) was matched to the +2 charged peptide 78-RYSVDLR-84; the results suggested that S80 was phosphorylated. The Mascot score was 28, and the expectation value was 3.7e-001. **(F)** Alignment of protein sequences spanning KHK-A S80 from different species. **(G)** Comparison of the amino acid sequences in exon 3A of KHK-A and exon 3C of KHK-C. **(H)** Immunohistochemistry (IHC) analyses of human HCC tissues were performed with the indicated antibodies in the presence or absence of a blocking peptide of KHK-A pS80. **(I)** Huh7 cells expressing Flag–KHK-A were treated with or without hypoxia for 6 h. Immunoprecipitation and immunoblot analyses were performed with the indicated antibodies in the presence or absence of a blocking peptide of KHK-A pS80. **(J)** Huh7 cells expressing WT Flag–KHK-A, Flag–KHK-C, or Flag–KHK-A S80A were treated with or without 0.5 mM A769662 for 4 h in the presence of lysosome inhibitor CQ (10 μM). **(K)** WT and AMPKα1/2 DKO MEFs were cultured with or without A769662 (0.5 mM) for 4 h in the presence of lysosome inhibitor CQ (10 μM). **(L)** Huh7 cells were pretreated with or without compound C (5 μM) for 30 min before hypoxia stimulation for

the indicated period of time. **(M)** Huh7 cells expressing Flag-KHK-A WT, V5-KHK-A WT, Flag-KHK-A S80A, or V5-KHK-A S80A were treated with or without hypoxia for 6 h. **(N)** Huh7 cells with depletion of endogenous KHK and reconstituted expression of WT Flag-rKHK-A or Flag-rKHK-A S80A were treated with or without hypoxia for 6 h. The KHK activity was measured. A two-tailed Student *t* test was used. N.S., not significant. **(O)** Huh7 cells with depletion of endogenous KHK and reconstituted expression of WT Flag-rKHK-A or Flag-rKHK-A S80A were cultured with a medium supplied with ^{13}C-labeled fructose with or without hypoxia for 6 h. Relative amount of ^{13}C-labeled F1P production was measured. A two-tailed Student *t* test was used. N.S., not significant. **(P)** Huh7 cells expressing Flag–KHK-A, Flag–KHK-C, or Flag–KHK-A S80A were treated with or without H_2O_2 (0.5 mM) for 1 h. **(Q)** Huh7 cells expressing Flag–KHK-A or Flag–KHK-A S80A were treated with or without BSO (100 μM) for 12 h in the presence of lysosome inhibitor CQ (10 μM). **(R)** Huh7 were treated with or without hypoxia for 6 h, H_2O_2 (0.5 mM) for 2 h, BSO (100 μM) for 12 h, or pretreated with 5 mM NAC or 100 μM Trolox for 30 min before hypoxia stimulation for 6 h. The intercellular ROS levels were measured. The data are presented as means ± SD from triplicate samples. $^{**}P < 0.001$.

Figure S4

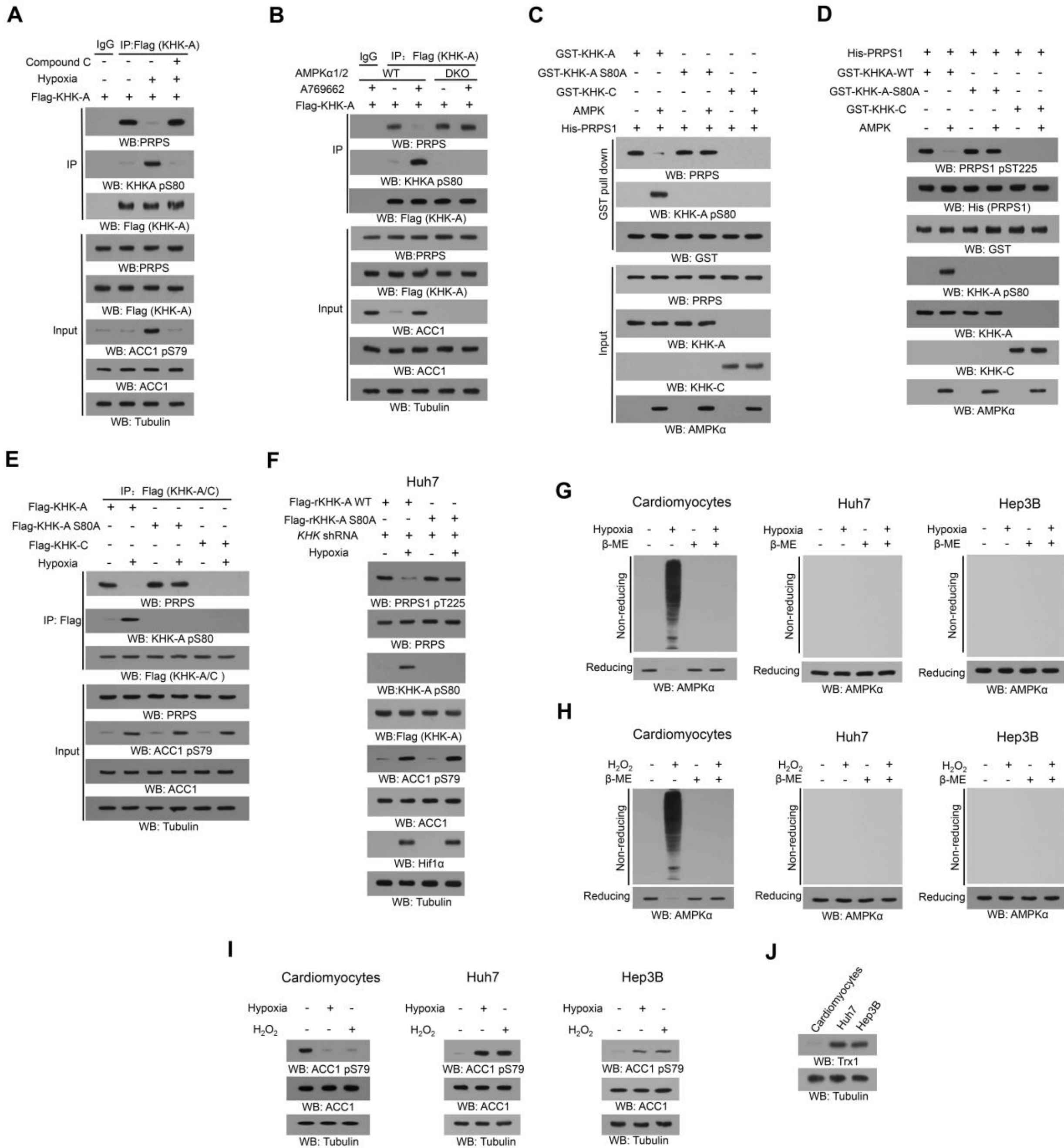

Fig. S4. AMPK-mediated KHK-A S80 phosphorylation inhibits the interaction between KHK-A and PRPS1 and PRPS1 T225 phosphorylation. Immunoprecipitation or immunoblot analyses were performed with the indicated antibodies. **(A)** Huh7 cells expressing Flag–KHK-A were pretreated with or without compound C (5 μM) for 30 min before being treated with or without hypoxia for 6 h. **(B)** WT and AMPKα1/2 DKO MEFs expressing Flag–KHK-A were

treated with or without A769662 (0.5 mM) for 4 h. **(C)** An *in vitro* AMPK kinase assay was performed by mixing bacterially purified WT GST–KHK-A, GST–KHK-A S80A, or GST–KHK-C on glutathione agarose beads with or without purified active AMPK in the presence of AMP and ATP for 1 h. The glutathione agarose beads were then washed and incubated with purified His-PRPS1 for a GST pulldown analysis. **(D)** An *in vitro* AMPK kinase assay was performed by mixing bacterially purified WT GST–KHK-A, GST–KHK-A S80A, or GST–KHK-C on glutathione agarose beads with or without purified active AMPK in the presence of AMP and ATP for 1 h. The glutathione agarose beads were then washed and incubated with or without bacterially purified His-PRPS1 in the presence of ATP.

(E) Huh7 cells expressing Flag–KHK-A, Flag-KHK-A S80A, or Flag-KHK-C were treated with or without hypoxia for 6 h. **(F)** Endogenous KHK-depleted Huh7 cells with reconstituted expression of WT Flag–rKHK-A or Flag-rKHK-A S80A were stimulated with or without hypoxia for 6 h. **(G, H)** Neonatal cardiomyocytes, Huh7 and Hep3B cells were treated with or without hypoxia for 6 h (**G**) or H_2O_2 (0.5 mM) for 2 h (**H**). SDS-PAGE under reducing or non-reducing conditions and immunoblot analysis were performed. **(I)** Neonatal cardiomyocytes, Huh7 and Hep3B cells were treated with or without hypoxia for 6 h or H_2O_2 (0.5 mM) for 1 h. **(J)** Neonatal cardiomyocytes, Huh7 and Hep3B cells were harvested for immunoblot analysis.

Figure S5

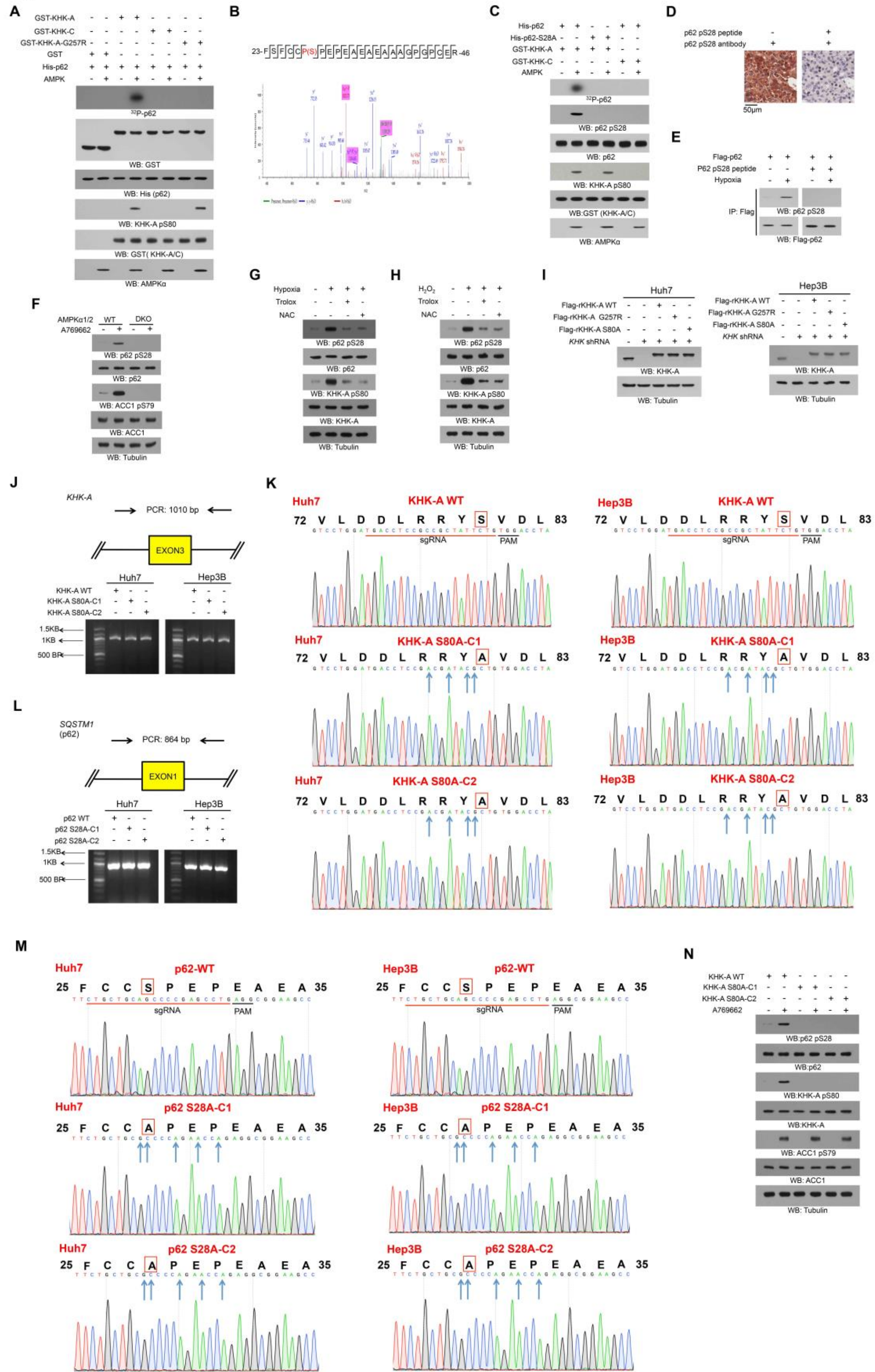

Fig. S5. KHK-A acts as a protein kinase and phosphorylates p62 at S28.

(A) An *in vitro* AMPK kinase assay was performed by mixing bacterially purified WT GST–KHK-A, GST–KHK-A G257R, or GST–KHK-C on glutathione agarose beads with or without purified active AMPK in the presence of AMP and ATP for 1 h. The glutathione agarose beads were then washed and incubated with purified His-p62 in the presence of [γ-^{32}P] ATP. Autoradiography was performed. **(B)** Purified His-p62 was phosphorylated by KHK-A *in vitro* and analyzed by mass spectrometry. A tryptic fragment at m/z 1353.51953 Da (+0.51 mmu/+0.37 ppm) was matched to the +2 charged peptide 23-FSFCCSPEPEAEAEAAAGPGPCER-46; the results suggested that S28 was phosphorylated. The Mascot score was 30, and the expectation value was 1.6e-001. **(C)** An *in vitro* AMPK kinase assay was performed by mixing bacterially purified WT GST–KHK-A or GST–KHK-C on glutathione agarose beads with or without purified active AMPK in the presence of AMP and ATP for 1 h. The glutathione agarose beads were then washed and incubated with purified His-p62 or His-p62 S28A in the presence of [γ-^{32}P] ATP. Autoradiography and immunoblot analyses were performed. **(D)** IHC analyses of human HCC tissues were performed with the indicated antibodies in the presence or absence of a specific blocking peptide of p62 pS28. **(E)** Huh7 cells expressing Flag-p62 were treated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). Immunoprecipitation and immunoblot analyses were performed with the indicated antibodies in the presence or absence of a specific blocking peptide of p62 pS28. **(F)** WT and AMPKα1/2 double-knockout (DKO) MEFs were treated with or without A769662 (0.5 mM) for 4 h in the presence of lysosome inhibitor CQ (10 μM). Immunoblot analyses were performed with the indicated antibodies. **(G)** Huh7 cells were pretreated with or without NAC (5 mM) or Trolox (100 μM) for 30 min before hypoxia stimulation for 6 h. Immunoblot analyses were performed with the indicated antibodies. **(H)** Huh7 cells were pretreated with or without NAC (5 mM) or Trolox (100 μM) for 30 min before H_2O_2 (0.5 mM) stimulation for 2 h.

Immunoblot analyses were performed with the indicated antibodies. **(I)** Huh7 and Hep3B cells with or without expression of *KHK* shRNA were reconstituted with or without expression of the indicated KHK proteins. Immunoblot analyses were performed with the indicated antibodies. **(J)** Genomic DNA was extracted from two individual clones of Huh7 and Hep3B cells with knock-in of KHK-A S80A expression. PCR products amplified from the indicated DNA fragment were separated on an agarose gel. KHK-A S80A-C1, clone 1; KHK-A S80A-C2, clone 2. **(K)** Sequencing of parental and two individual clones of parental Huh7 and Hep3B cells with knock-in of KHK-A S80A expression. The red line indicates the sgRNA-targeting sequence. The black line indicates the protospacer adjacent motif (PAM). The mutated nucleotides are highlighted by the blue arrows. The mutated amino acid and its WT counterpart are highlighted by the solid red box. **(L)** Genomic DNA was extracted from two individual clones of Huh7 and Hep3B cells with knock-in of p62 S28A expression. PCR products amplified from the indicated DNA fragment were separated on an agarose gel. p62 S28A-C1, clone 1; p62 S28A-C2, clone 2. **(M)** Sequencing of parental and two individual clones of Huh7 and Hep3B cells with knock-in of p62 S28A expression. The red line indicates the sgRNA-targeting sequence. The black line indicates the PAM. The mutated nucleotides are highlighted by the blue arrows. The mutated amino acid and its WT counterpart are highlighted by the solid red box. **(N)** Parental Huh7 cells and the indicated clones of Huh7 cells with knock-in of KHK-A S80A expression were stimulated with or without A769662 (0.5 mM) for 4 h in the presence of lysosome inhibitor CQ (10 μM). Immunoblot analyses were performed with the indicated antibodies.

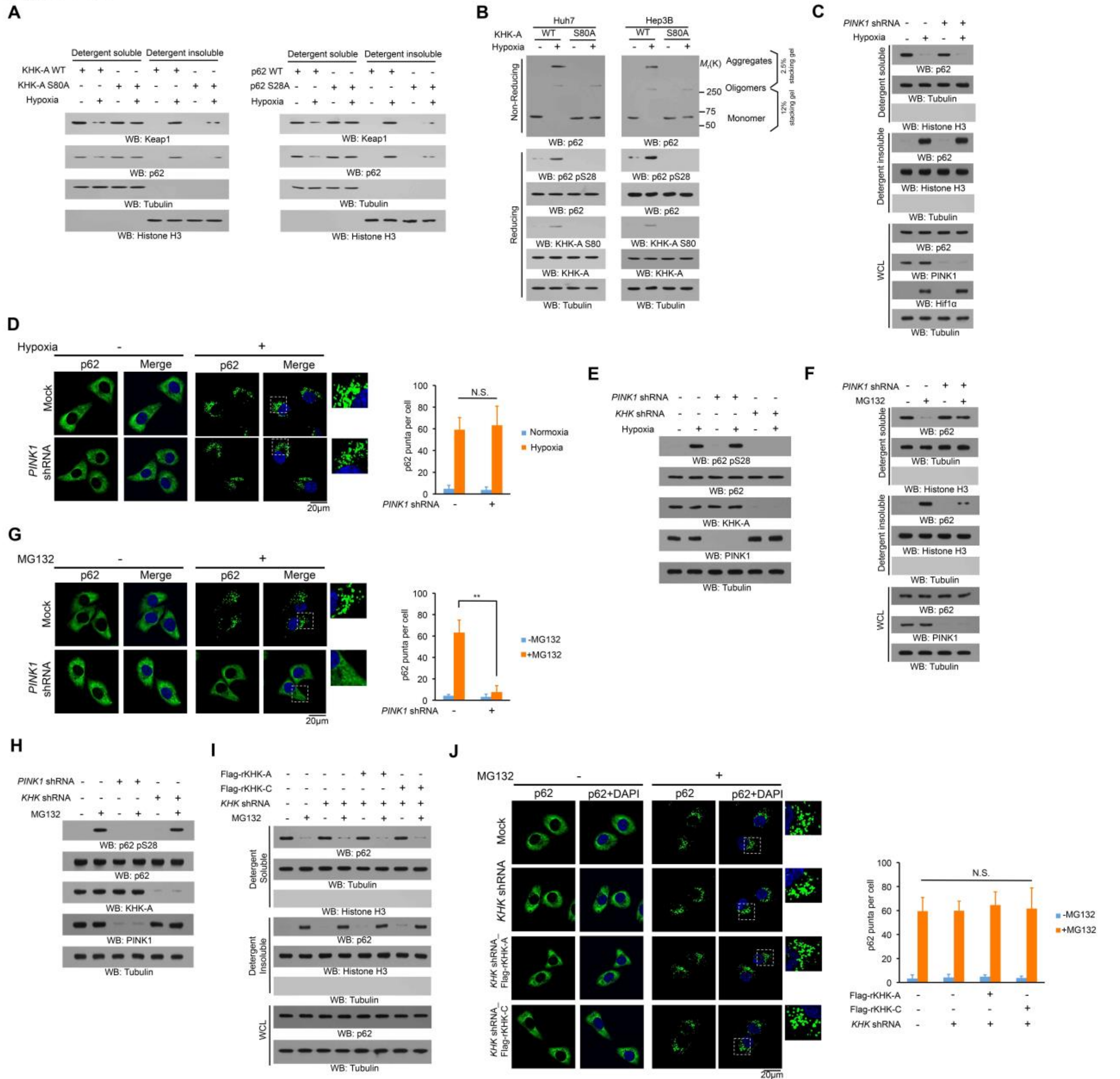

Fig. S6. KHK-A is required for oxidative stress–induced but not proteasomal stress–-induced p62 aggregation. (A) Huh7 cells with or without knock-in of KHK-A S80A (left panel) or p62 S28A (right panel) expression were stimulated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). The cells were lysed in a lysis buffer with 1% Triton X-100. The insoluble fraction was lysed in a lysis buffer with 1% SDS. Immunoblotting analyses were performed with the indicated antibodies. **(B)** Huh7 and Hep3B cells with or without knock-in of KHK-A S80A expression were treated with or without hypoxia for 6 h in the

presence of lysosome inhibitor CQ (10 μM). The whole-cell lysates were analyzed by reducing and nonreducing SDS-PAGE to detect p62 aggregation. **(C)** Huh7 cells with or without *PINK1* shRNA expression were treated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). The cells were lysed in a lysis buffer with 1% Triton X-100. The insoluble fraction was lysed in a lysis buffer with 1% SDS. Immunoblotting analyses were performed with the indicated antibodies. **(D)** Huh7 cells with or without *PINK1* shRNA expression were treated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). Immunofluorescence analyses were performed with an anti-p62 antibody (left panel). The numbers of puncta in 100 cells were counted and quantified (right panel). Data are shown as mean ± SD of 100 cells per group. A two-tailed Student *t* test was used. N.S., not significant. **(E)** Huh7 cells expressing *PINK1* shRNA or *KHK* shRNA were treated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). Immunoblotting analyses were performed with the indicated antibodies. **(F)** Huh7 cells with or without *PINK1* shRNA expression were treated with or without MG132 (0.25 μM) for 12 h. The cells were lysed in a lysis buffer with 1% Triton X-100. The insoluble fraction was lysed in a lysis buffer with 1% SDS. Immunoblotting analyses were performed with the indicated antibodies. **(G)** Huh7 cells with or without *PINK1* shRNA expression were treated with or without MG132 (0.25 μM) for 12 h. Immunofluorescence analyses were performed with an anti-p62 antibody (left panel). The numbers of puncta in 100 cells were counted and quantified (right panel). Data are shown as mean ± SD of 100 cells per group. A two-tailed Student *t* test was used. $^{**}P < 0.01$. **(H)** Huh7 cells expressing *PINK1* shRNA or *KHK* shRNA were treated with or without MG132 (0.25 μM) for 12 h. Immunoblotting analyses were performed with the indicated antibodies. **(I)** Huh7 cells with or without *KHK* shRNA expression were reconstituted with or without expression of the indicated KHK protein and treated with or without MG132 (0.25 μM) for 12 h. The cells were lysed in a lysis buffer with 1% Triton X-100. The insoluble fraction was lysed in a lysis buffer

with 1% SDS. Immunoblotting analyses were performed with the indicated antibodies. **(J)** Huh7 cells with or without *KHK* shRNA expression were reconstituted with or without expression of the indicated KHK protein and treated with or without MG132 (0.25 μM) for 12 h. Immunofluorescence analyses were performed with an anti-p62 antibody (left panel). The numbers of puncta in 100 cells were counted and quantified (right panel). Data are shown as mean ± SD of 100 cells per group. A two-tailed Student *t* test was used. N.S., not significant.

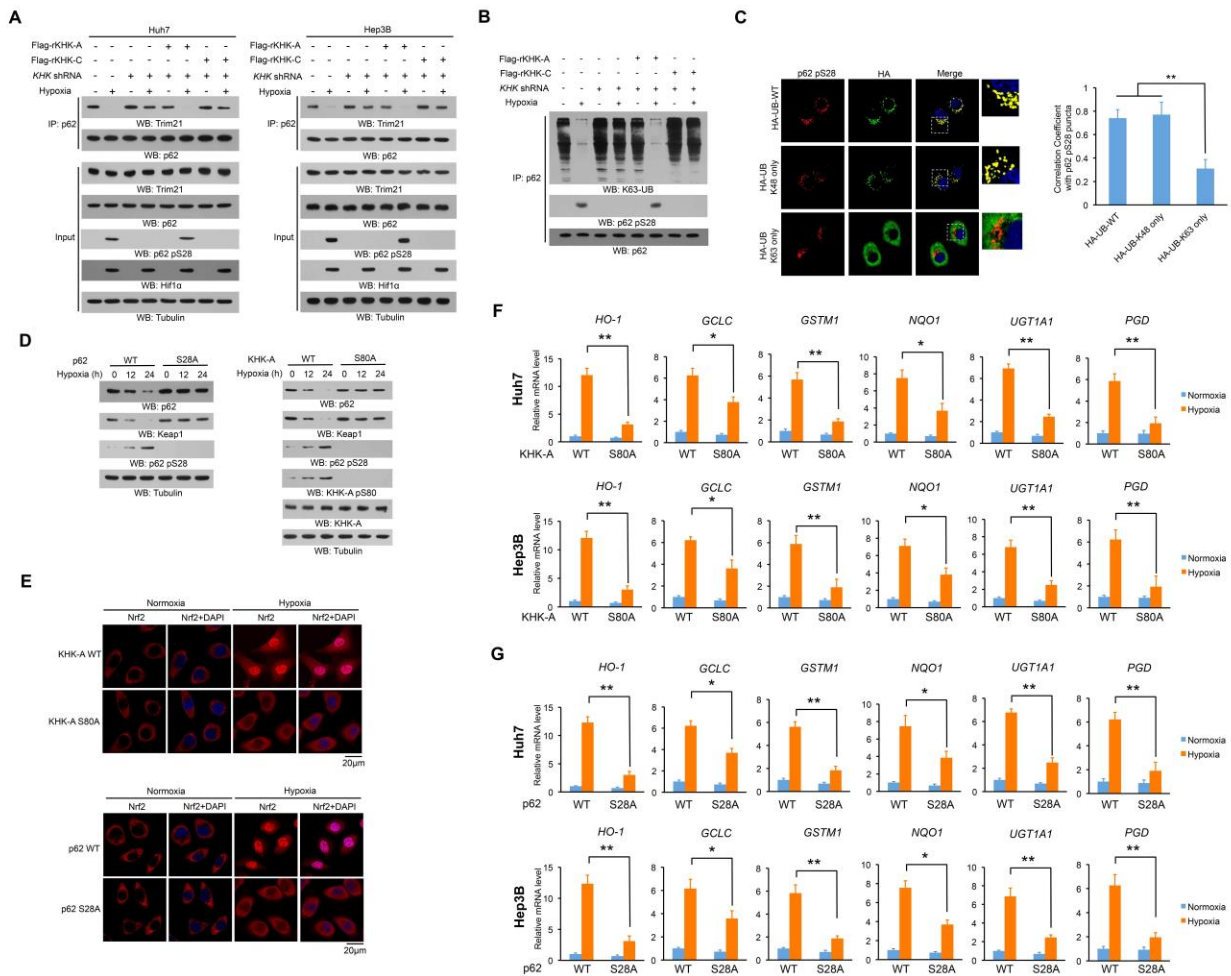

Fig. S7. KHK-A–mediated p62 S28 phosphorylation is required for oxidative stress–enhanced p62 oligomerization and Nrf2 activation. (A) Huh7 and Hep3B cells expressing *KHK* shRNA with or without reconstituted expression of the indicated proteins were treated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM).

Immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. **(B)** Huh7 cells expressing *KHK* shRNA with or without reconstituted expression of the indicated proteins were treated with or without hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). Immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. **C)** Huh7 cells expressing WT HA-ubiquitin, HA-ubiquitin K48 (ubiquitin with only K48), or HA-ubiquitin K63 (ubiquitin with only K63) were treated with hypoxia for 6 h in the presence of lysosome inhibitor CQ (10 μM). Immunofluorescent analyses were performed with the indicated antibodies (left panel). The colocalization coefficients between the indicated proteins are presented (right panel). At least 100 cells from each independent experiment were quantified. Similar results were obtained from three repeated experiments. Values are presented as means ± SD. $^{**}P < 0.01$ (two-tailed Student *t*-test). **(D)** Huh7 cells with or without knock-in of p62 S28A (left panel) or KHK-A S80A (right panel) expression were treated with hypoxia for the indicated time period in the presence of lysosome inhibitor CQ (10 μM). Immunoblot analyses were performed with the indicated antibodies. **(E)** Huh7 cells with or without knock-in of KHK-A S80A (upper panel) or p62 S28A (lower panel) expression were treated with or without hypoxia for 12 h. Immunofluorescence analyses were performed with an anti-Nrf2 antibody. **(F, G)** Huh7 and Hep3B cells with or without KHK-A S80A (**F**) or p62 S28A (**G**) knock-in expression were treated with or without hypoxia for 12 h. The mRNA was extracted and Nrf2 target gene expression levels were measured by quantitative PCR. The data are presented as means ± SD from triplicate samples. $^{*}P < 0.05$, $^{**}P < 0.001$.

Figure S8

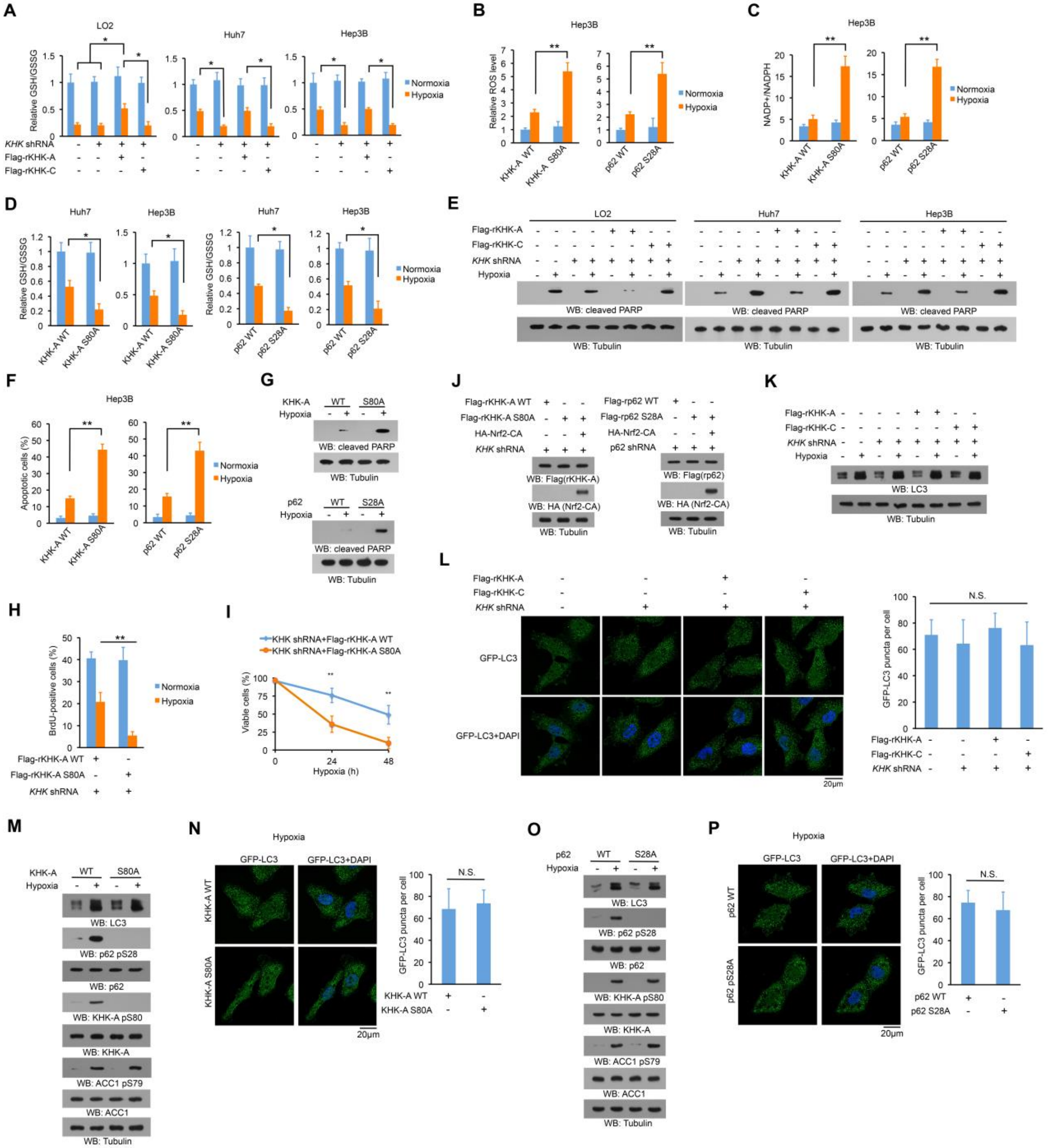

Fig. S8. KHK-A mediated p62 S28 phosphorylation reduces ROS production and promotes cancer cell survival without altering autophagy initiation. **(A)** LO2, Huh7, and Hep3B cells with or without expression of *KHK* shRNA and reconstituted expression of the indicated KHK proteins were treated with or without hypoxia for 36 h. The GSH/GSSG ratio was measured. The data are presented as the mean ± SD from three independent experiments. $^*P < 0.05$. A two-

tailed Student *t* test was used. **(B, C)** Hep3B cells with or without knock-in of KHK-A S80A (left panel) or p62 S28A (right panel) expression were treated with or without hypoxia for 36 h. ROS levels (**B**) and intracellular NADP+ and NADPH levels (**C**) were measured. The data are presented as the mean ± SD from three independent experiments. **$P < 0.001$. A two-tailed Student *t* test was used. **(D)** Huh7 and Hep3B cells with or without knock-in of KHK-A S80A (left panel) or p62 S28A (right panel) expression were treated with or without hypoxia for 36 h. GSH/GSSG ratio was measured. The data are presented as the mean ± SD from three independent experiments. *$P < 0.05$. A two-tailed Student *t* test was used. **(E)** LO2, Huh7, and Hep3B cells with or without expression of *KHK* shRNA and reconstituted expression of the indicated KHK proteins were treated with or without hypoxia for 36 h. Immunoblot analyses were performed with the indicated antibodies. **(F)** Hep3B cells with or without knock-in expression of KHK-A S80A (left panel) or p62 S28A (right panel) were treated with or without hypoxia for 36 h. The percentages of apoptotic cells were measured. The data are presented as the mean ± SD from three independent experiments. **$P < 0.001$. A two-tailed Student *t* test was used. **(G)** Huh7 cells with or without knock-in of KHK-A S80A (upper panel) or p62 S28A (lower panel) expression were treated with or without hypoxia for 36 h. Immunoblot analyses were performed with the indicated antibodies. **(H)** *KHK* depleted Huh7 cells reconstituted with Flag-rKHK-A WT or Flag-rKHK-A S80A were treated with or without hypoxia for 24 h. Cellular proliferation rates were examined by BrdU incorporation assay. The data are presented as the mean ± SD from three independent experiments. **$P < 0.001$. A two-tailed Student *t* test was used. **(I)** *KHK* depleted Huh7 cells reconstituted with Flag-rKHK-A WT or Flag-rKHK-A S80A were treated with hypoxia as the indicated time period. The viable cells were measured. The data are presented as the mean ± SD from three independent experiments. **$P < 0.01$. A two-tailed Student *t* test was used. **(J)** Huh7 cells reconstituted by the indicated KHK-A proteins (left panel) or p62 proteins (right panel) were stably transfected with or without HA tagged

constitutively active Nrf2. Immunoblot analyses were performed with the indicated antibodies. **(K)** Huh7 cells expressing *KHK* shRNA with or without reconstituted expression of the indicated proteins were treated with or without hypoxia for 12 h. Immunoblot analyses were performed with the indicated antibodies. **(L)** Huh7 cells expressing *KHK* shRNA with or without reconstituted expression of the indicated proteins were transfected with GFP-LC3 and treated with hypoxia for 12 h. Representative images of GFP-LC3 puncta are shown (left panel). The numbers of puncta in 100 cells were counted and quantified (right panel). Data are shown as mean ± SD of 100 cells per group. A two-tailed Student *t* test was used. N.S., not significant. **(M)** Huh7 cells with or without KHK-A S80A knock-in expression were stimulated with or without hypoxia for 12 h in the presence of lysosome inhibitor CQ (10 μM). Immunoblot analyses were performed with the indicated antibodies. **(N, P)** Huh7 cells with or without KHK-A S80A (**N**) or p62 S28A (**P**) knock-in expression were transfected with GFP-LC3 and treated with hypoxia for 12 h. Representative images of GFP-LC3 puncta are shown (left panel). The numbers of puncta in 100 cells were counted and quantified (right panel). Data are shown as mean ± SD of 100 cells per group. A two-tailed Student *t* test was used. N.S., not significant. **(O)** Huh7 cells with or without p62 S28A knock-in expression were stimulated with or without hypoxia for 12 h in the presence of lysosome inhibitor CQ (10 μM). Immunoblot analyses were performed with the indicated antibodies.

Figure S9

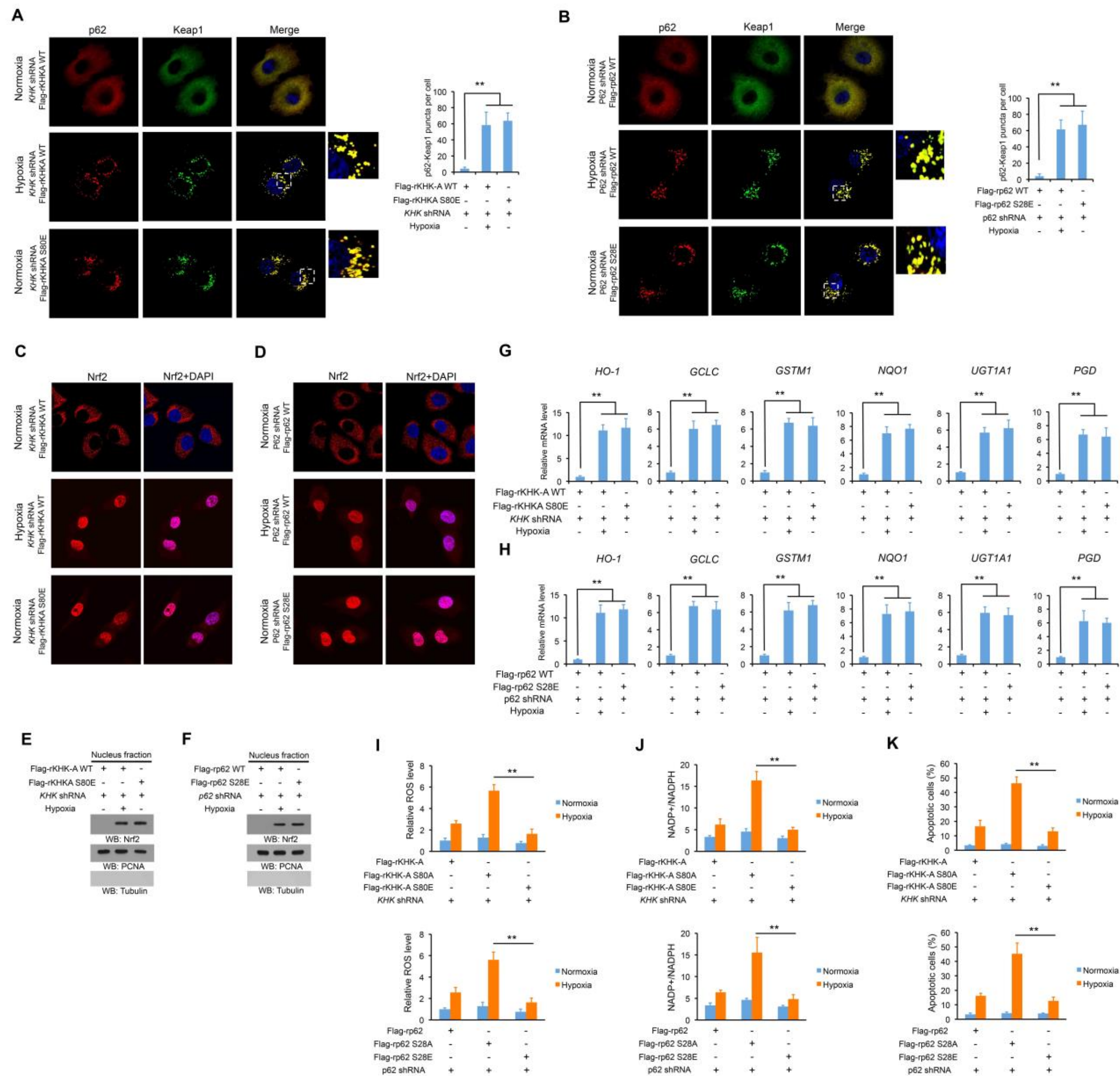

Fig. S9. The phosphorylation-mimicking KHK-A S80E and p62 S28E mutations promote p62 oligomerization and Nrf2 activation. (A) Huh7 cells with endogenous KHK-depletion and reconstituted expression of WT Flag-rKHK-A or Flag-rKHK-A S80E were stimulated with or without hypoxia for 6 h. Immunofluorescent analyses were performed with the indicated antibodies (left panel). The numbers of puncta in 100 cells were counted and quantified (right panel). Data are shown as mean ± SD of 100 cells per group. A two-tailed Student *t* test was used. $^{**}P < 0.01$. **(B)** Huh7 cells with endogenous p62 depletion and reconstituted expression of WT

Flag-rp62 or Flag-rp62 S28E were stimulated with or without hypoxia for 6 h. Immunofluorescent analyses were performed with the indicated antibodies (left panel). The numbers of puncta in 100 cells were counted and quantified (right panel). Data are shown as mean ± SD of 100 cells per group. A two-tailed Student *t* test was used. $^{**}P < 0.01$. **(C)** Huh7 cells with endogenous KHK-depletion and reconstituted expression of WT Flag-rKHK-A or Flag-rKHK-A S80E were stimulated with or without hypoxia for 12 h. Immunofluorescence analyses were performed with an anti-Nrf2 antibody. **(D)** Huh7 cells with endogenous p62 depletion and reconstituted expression of WT Flag-rp62 or Flag-rp62 S28E were stimulated with or without hypoxia for 12 h. Immunofluorescence analyses were performed with an anti-Nrf2 antibody. **(E)** Huh7 cells with endogenous KHK-depletion and reconstituted expression of WT Flag-rKHK-A or Flag-rKHK-A S80E were stimulated with or without hypoxia for 12 h. The nuclear fractions were prepared. Immunoblot analyses were performed with the indicated antibodies. **(F)** Huh7 cells with endogenous p62 depletion and reconstituted expression of WT Flag-rp62 or Flag-rp62 S28E were stimulated with or without hypoxia for 12 h. The nuclear fractions were prepared. Immunoblot analyses were performed with the indicated antibodies. **(G)** Huh7 cells with endogenous KHK-depletion and reconstituted expression of WT Flag-rKHK-A or Flag-rKHK-A S80E were stimulated with or without hypoxia for 12 h. The mRNA levels of Nrf2 target genes were measured by quantitative PCR. The data are presented as means ± SD from triplicate samples. $^{**}P < 0.01$. **(H)** Huh7 cells with endogenous p62 depletion and reconstituted expression of WT Flag-rp62 or Flag-rp62 S28E were stimulated with or without hypoxia for 12 h. The mRNA levels of Nrf2 target genes were measured by quantitative PCR. The data are presented as means ± SD from triplicate samples. $^{**}P < 0.01$. **(I, J, K)** Huh7 cells with reconstituted expression of the indicated KHK-A proteins (upper panel) or p62 proteins (lower panel) were treated with or without hypoxia for 36 h. ROS levels (**I**), intracellular NADP+ and NADPH levels (**J**), and the percentages of apoptotic cells (**K**) were measured. The

data are presented as means ± SD from triplicate samples. $**P < 0.01$. A two-tailed Student t test was used.

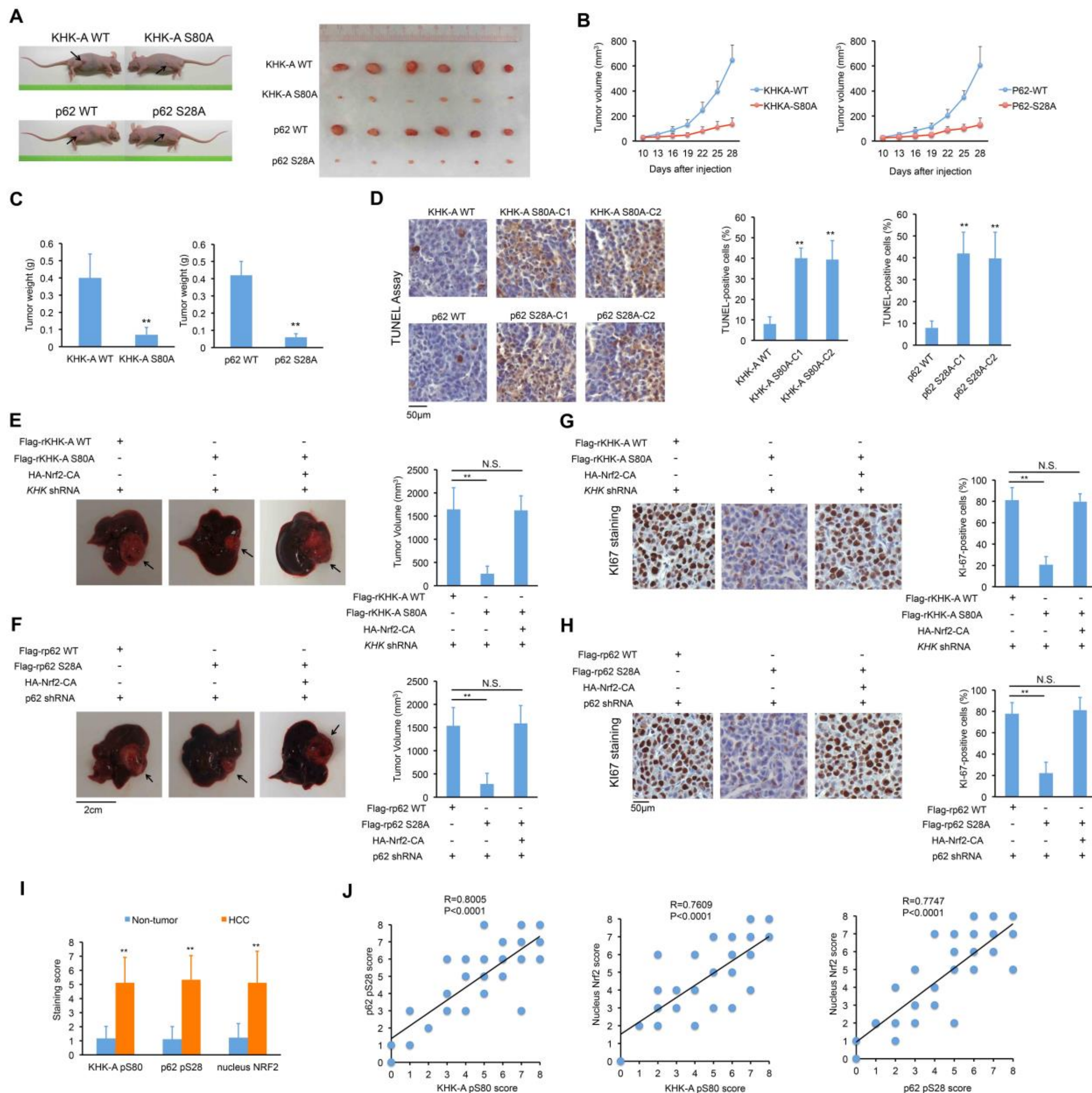

Fig. S10. KHK-A–mediated p62 S28 phosphorylation promotes hepatocellular tumorigenesis and is associated with the clinical aggressiveness of human HCC. (A, B, C) 1 × 10^6 Huh7 cells and Huh7 cells with or without knock-in of KHK-A S80A or p62 S28A expression were subcutaneously injected into the left and right flanks of athymic nude mice, respectively (n = 7 per group) (**A**, left panel). These tumors were resected 28 days after injection

(**A**, right panel). The growth of xenograft tumors was calculated (**B**). Tumor weight was measured (**C**). Data represent the mean ± SD. $^{**}P < 0.01$ by a two-tailed Student *t* test. **(D)** TUNEL analyses of the indicated tumor tissues were performed. Apoptotic cells were stained brown. Apoptotic cells were quantified in 10 microscope fields. $^{**}P < 0.01$ by a two-tailed Student *t* test. **(E, F)** 1×10^6 Huh7 cells with reconstituted expression of the indicated KHK-A proteins (**E**) or the indicated p62 proteins (**F**), and with or without expression of constitutively active Nrf2 were intrahepatically injected into athymic nude mice (n = 7 per group). The mice were euthanized and examined for tumor growth 28 days after injection. The arrows point to the tumors. Tumor volumes were calculated. Data represent the mean ± SD of seven mice. A two-tailed Student *t* test was used. N.S., not significant. $^{**}P < 0.01$. **(G, H)** Representative IHC staining of the indicated tumor tissues were performed with an anti-Ki67 antibody (left panel). Ki67-positive cells were quantified in 10 microscope fields (right panel). A two-tailed Student *t* test was used. N.S., not significant. $^{**}P < 0.01$. **(I)** The indicated staining scores of KHK-A pS80, p62 pS28, and nuclear Nrf2 levels between HCC and matched non-tumor liver samples were compared using a paired Student *t* test. The data represent the mean ± SD. $^{**}P < 0.01$. **(J)** The IHC staining in 90 patient HCC samples were scored, and correlation analyses were performed. A Pearson correlation test was used. Note that the scores of some samples are overlapped.