Figure S1, related to Figure 1

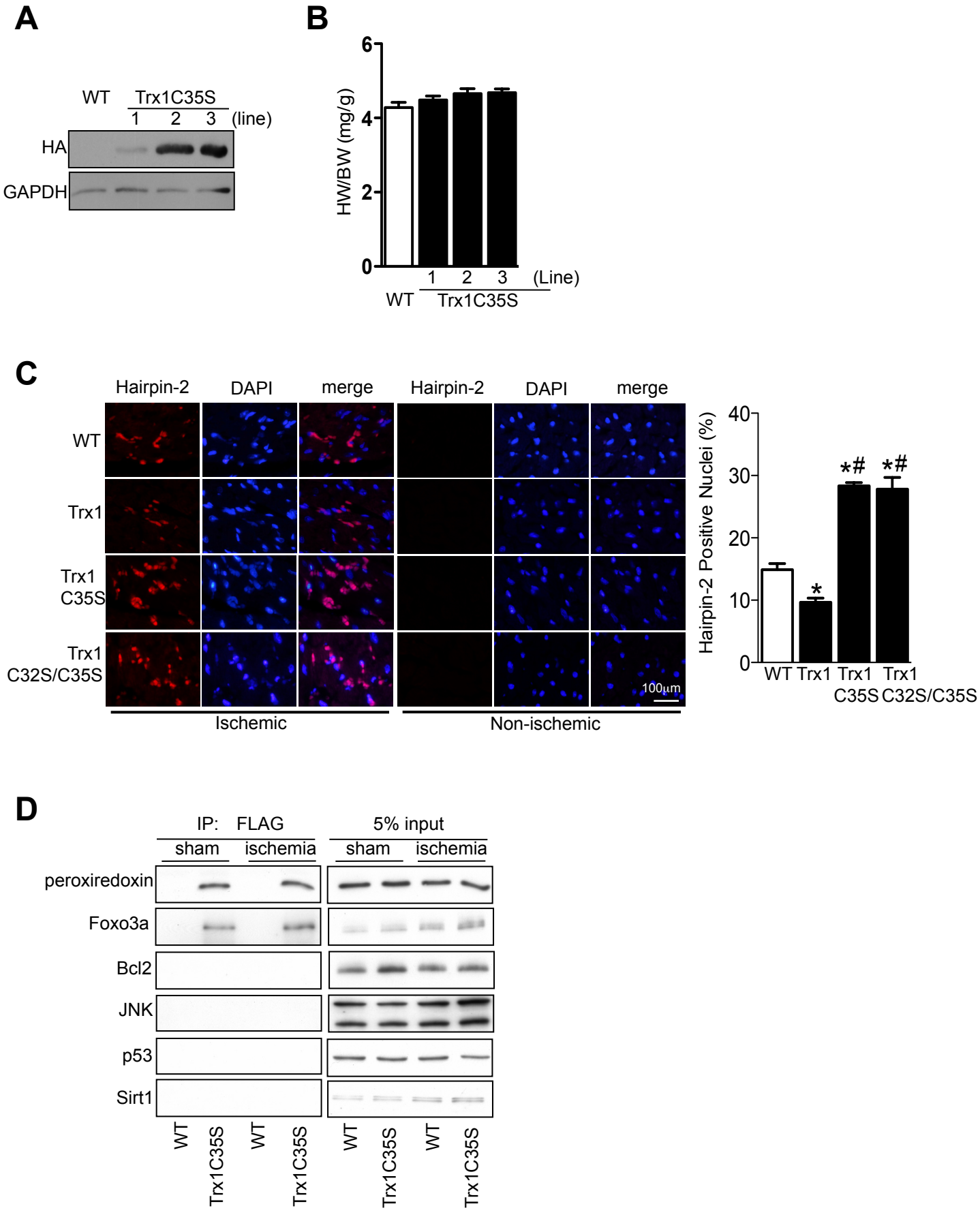

Figure S2, related to Figure 2

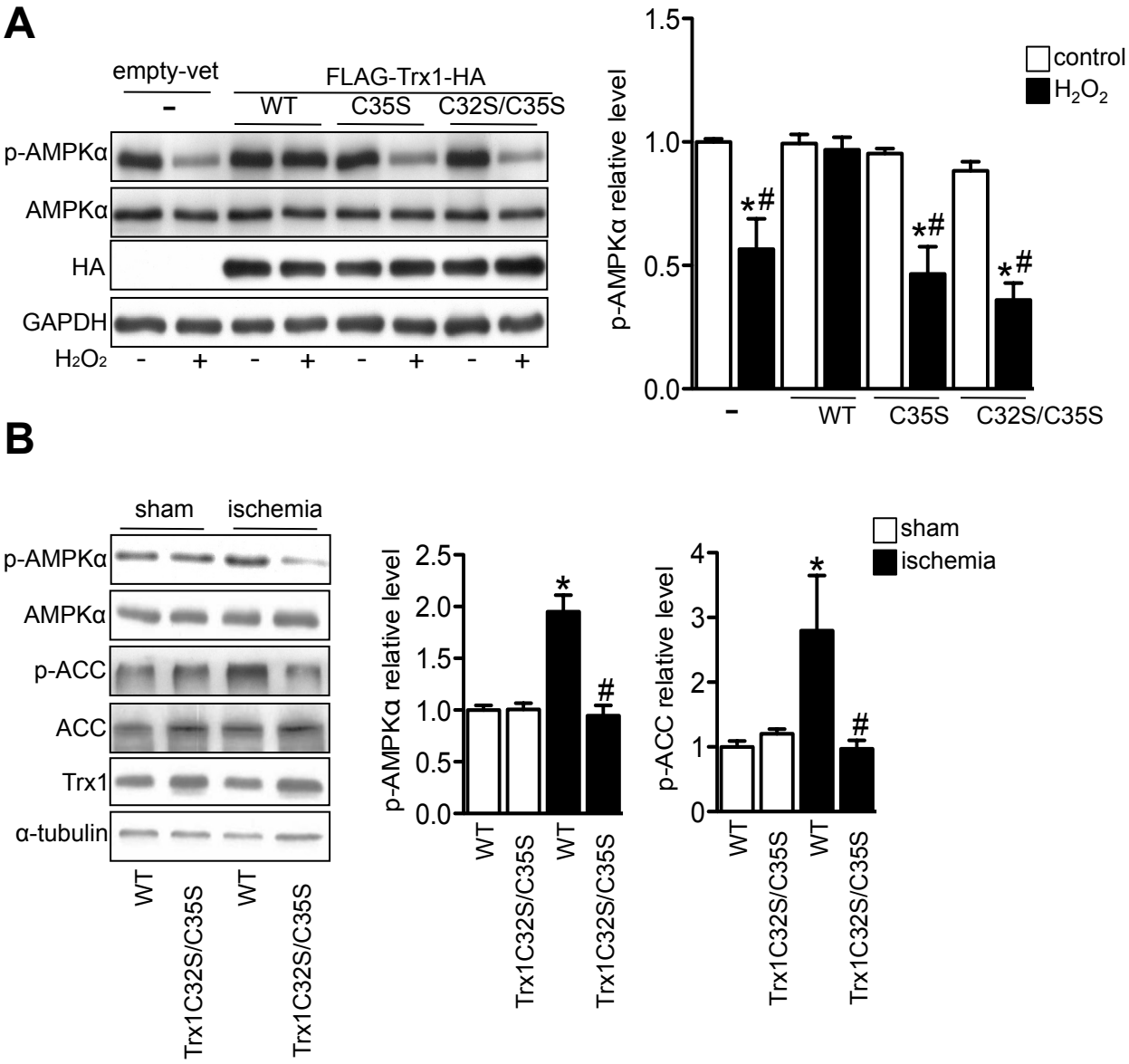

Figure S3, related to Figure 3

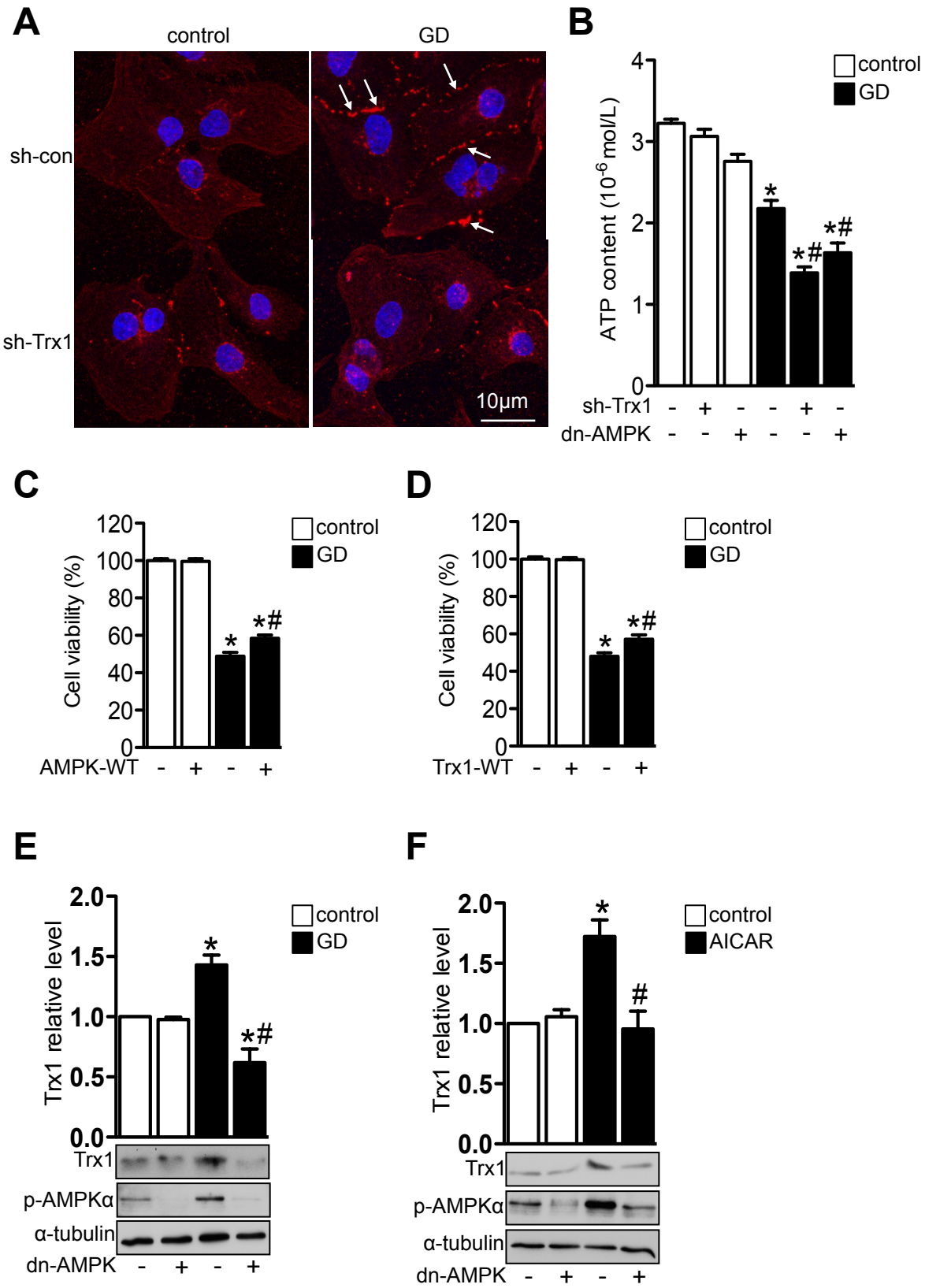

Figure S4, related to Figure 4

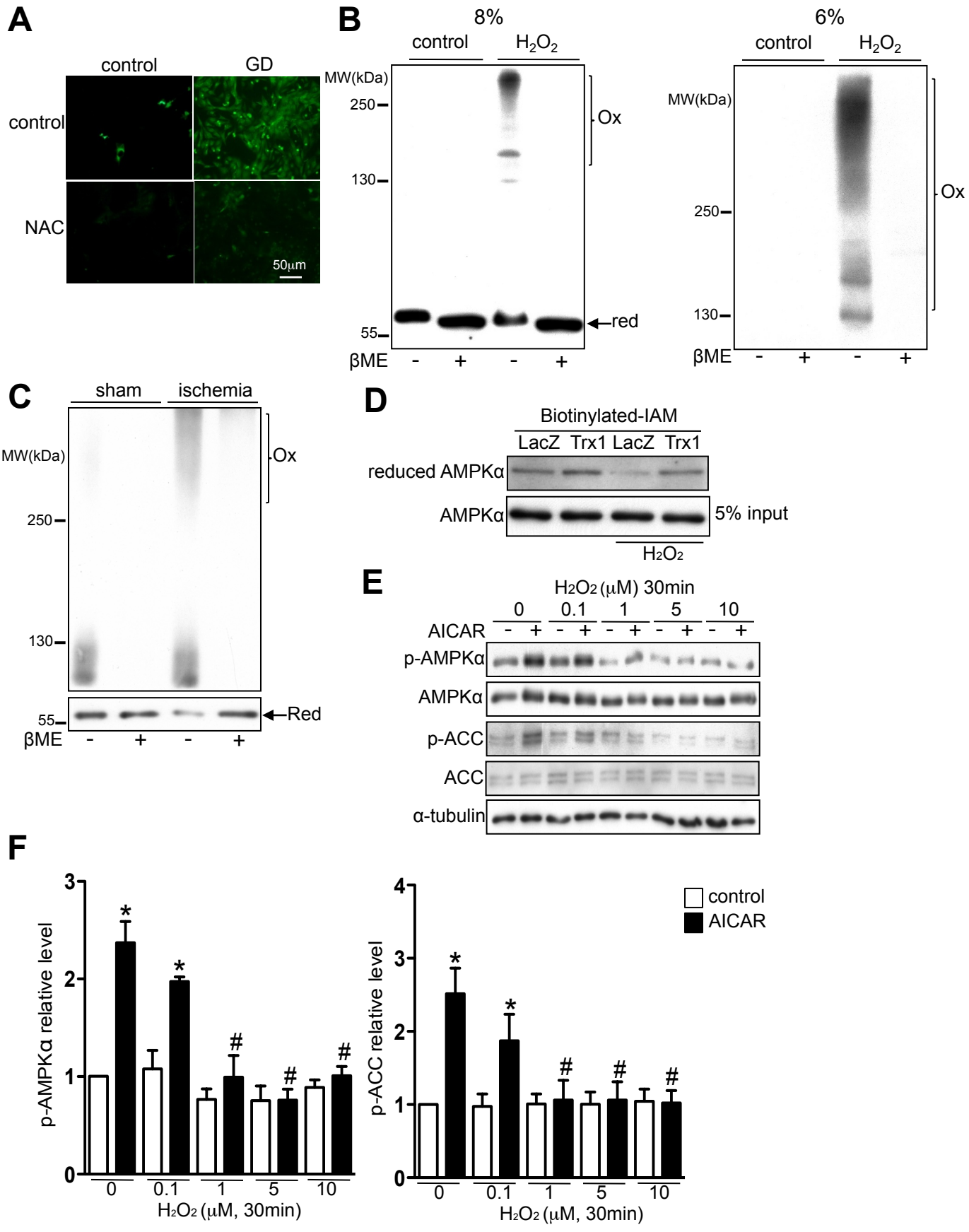

Figure S4, related to Figure 4, continued

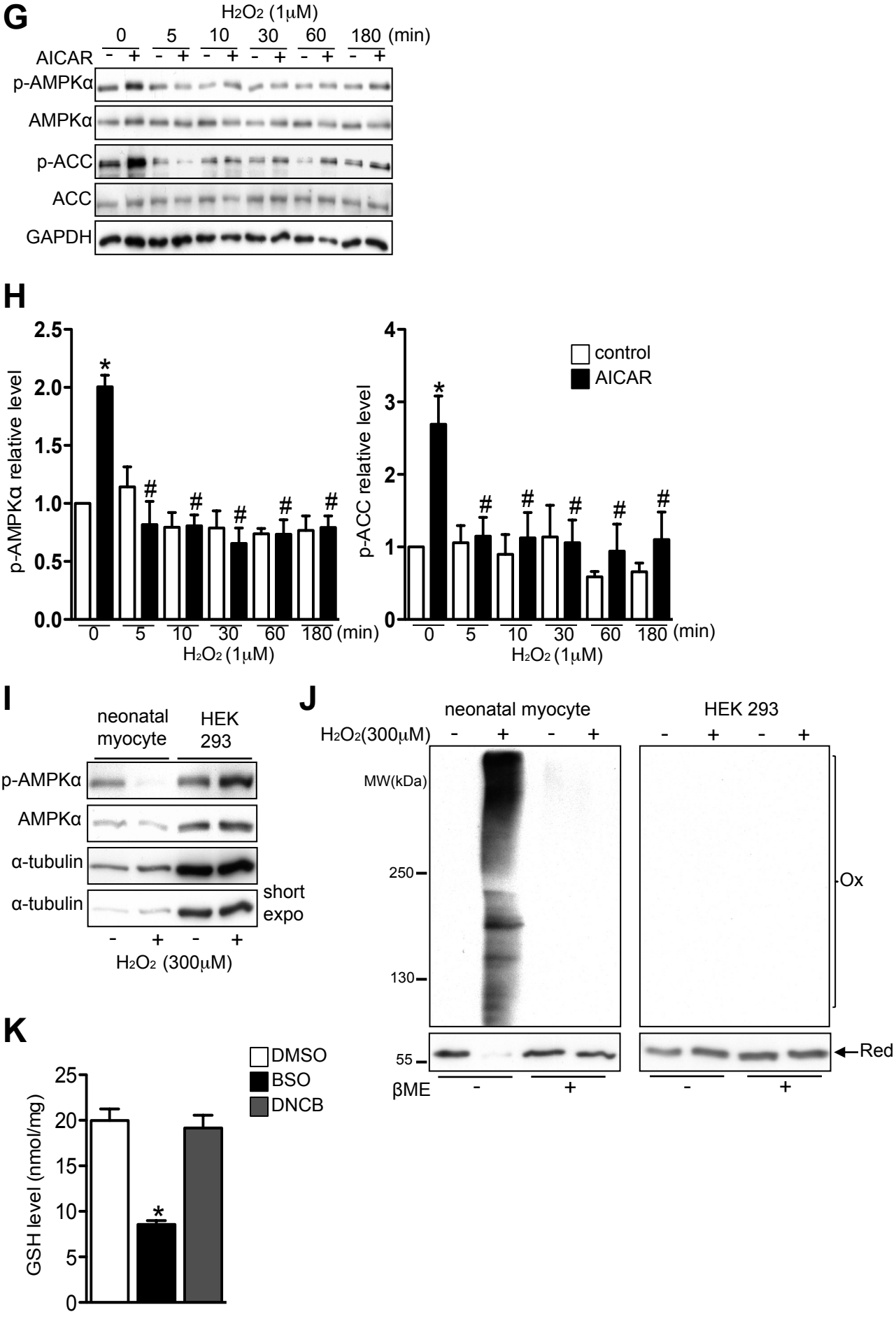

Figure S5, related to Figure 5

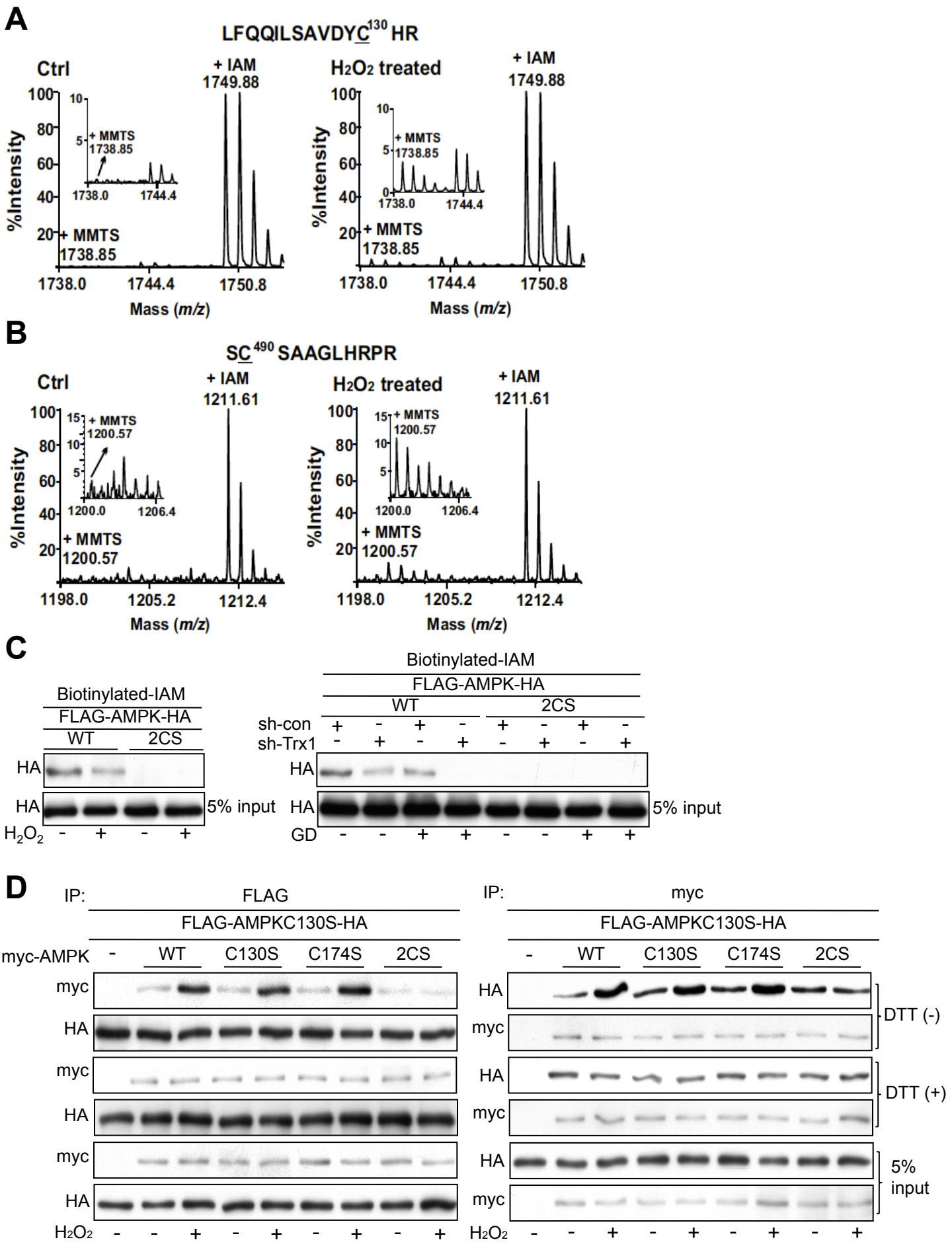

Figure S5, related to Figure 5, continued

E

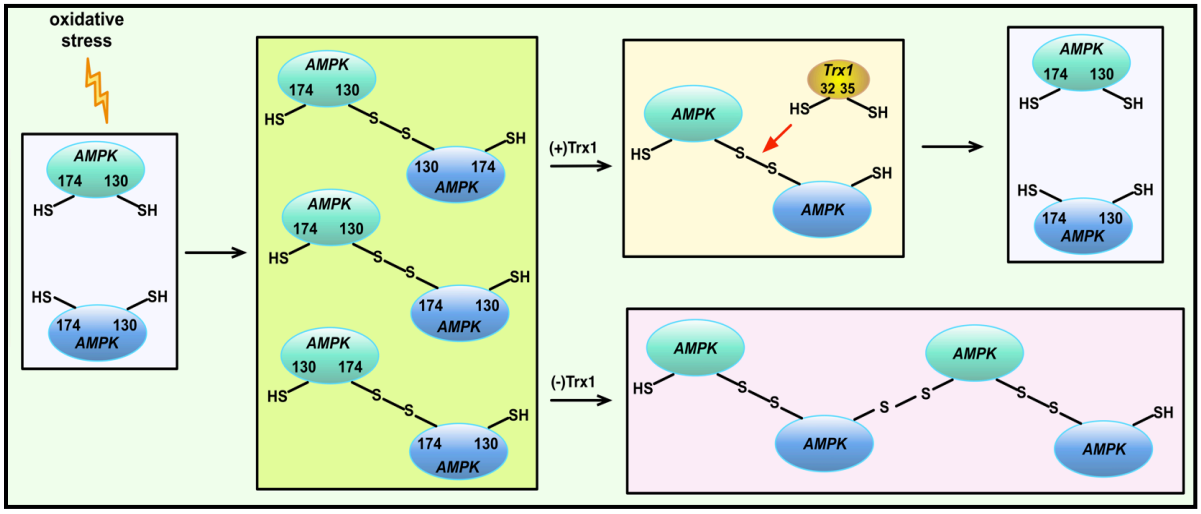

Figure S6, related to Figure 6

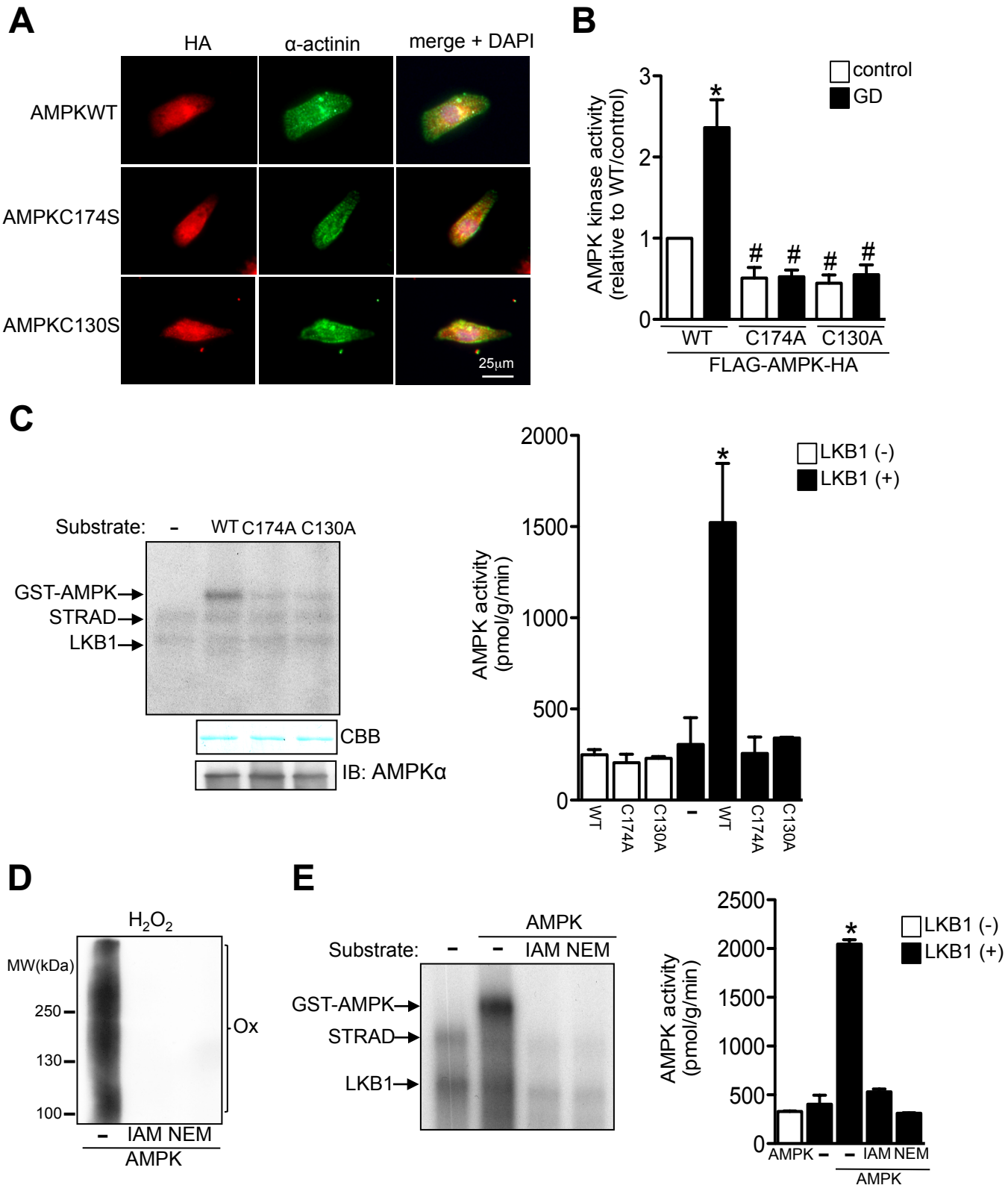

Figure S7, related to Figure 7

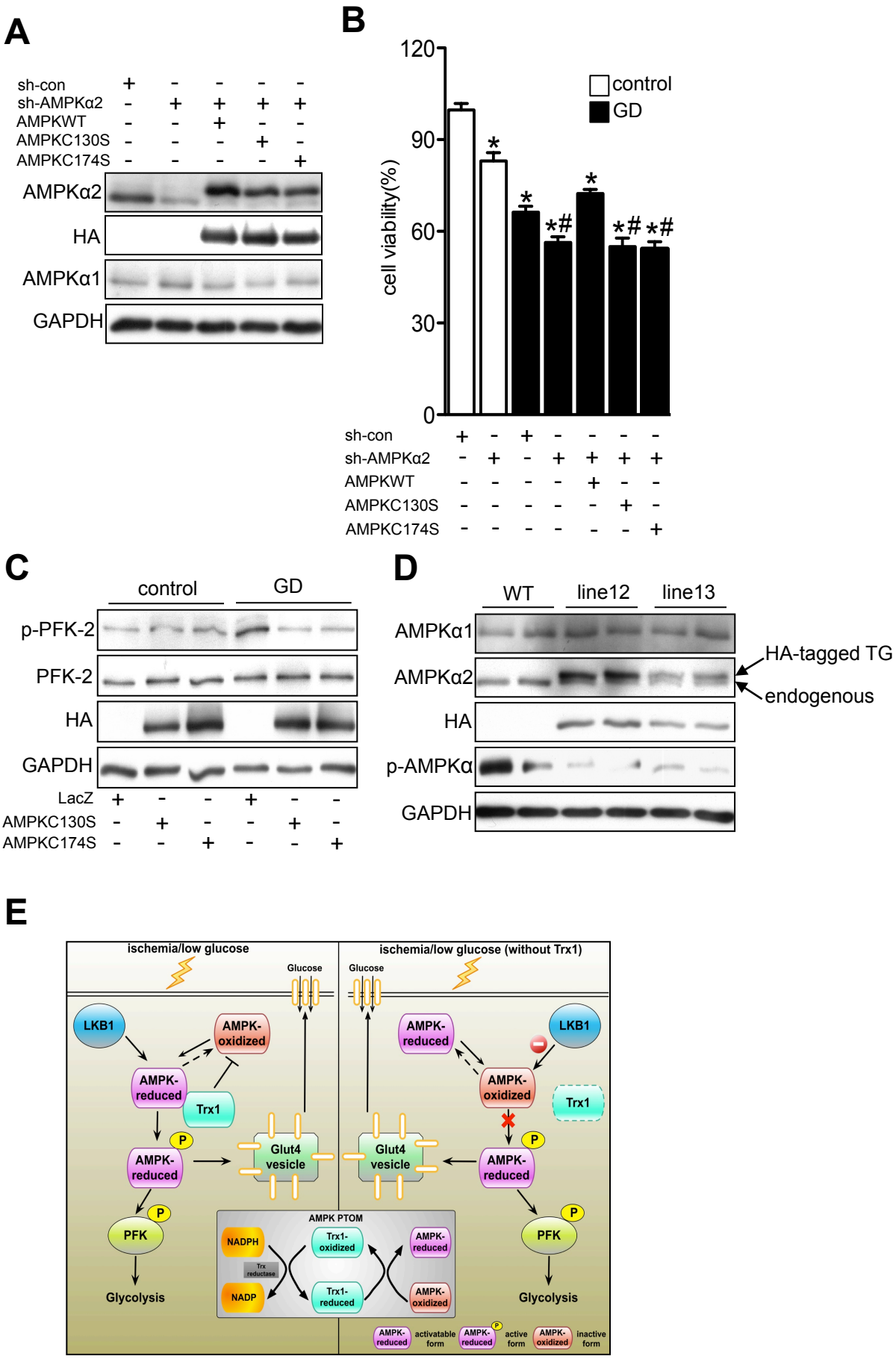

Table S1, related to Figure 1

Apoptosis: ASK1, Bax, Bcl2, Bcl-XL, BID, Bim, Caspase3, Caspase6, Caspase8, Caspase9, Caspase12, Cytochrome C
Kinase: Akt, AMPKα, CamKII, eEF2K, ERK, GSK3α, GSK3β, JNK, ,Lats1, Lats2, LKB1, MEK1/2, MEKK1, MEKK3, Mst1, Mst2, P38 MAPK, P70S6K, PDK1, PI3K p110, PI3K p85, PKA, PKCα, PKCε, PKN, Src
Phosphatase: PP2A A Subunit, PP2A C Subunit, PP2Cα
Signal transduction: ACC, CoxIV, eEF2, eNOS, HDAC3, HDAC4, HDAC5, iNOS, mTOR, Nampt, Nox1, Nox2, Nox4, PTEN, Rheb, Sirt1, Sirt3, YAP
Transcriptional factor: CREB, c-Jun, ERα, Foxo1, Foxo3, Foxp1, GATA4, HIF1α, NRF1, P53, PGC1α,PPARα, PPARγ, RXRα, tFAM
ER stress related genes: ATF6, Chop, Grp78, PERK, XBP-1
Heat shock protein: HSP25, HSP40, HSP70, HSP90
Autophagy related genes: Atg4B, Atg5, Atg7, Beclin1, P62, ULK1, UVRAG, VPS34
Other: Catalase, GAPDH, MnSOD, Peroxiredoxin, α-tubulin

No interaction
Interaction

Table S2, related to Figure 6

cysteine	Description (alignment comparison)	MS Identification (high dose H_2O_2)	Phosphorylation change	Kinase activity change
106	non-consensus	No	partial	20% reduction
130	identical	Yes	Yes	Yes
174	identical	Yes	Yes	Yes
200	consensus	No	No	No
209	consensus	No	No	No
297	consensus	Yes	No	No
302	non-consensus	No	No	No
382	non-consensus	Yes	No	No
490	non-consensus	Yes	No	No
543	consensus	Yes	No	No

Supplemental Figure Legends

Figure S1, related to Figure 1

(S1A-B) Characterization of Tg-Trx1C35S transgenic mice. (S1A) Exogenous Trx1C35S expression was examined using an HA antibody. (S1B) The effect of Trx1C35S overexpression on cardiac hypertrophy. Different lines of Tg-Trx1C35S mice were sacrificed at 3 months of age. Heart weight to body weight ratios (HW/BW (mg/g)) are shown (n=3-7). (S1C) Tg-Trx1, Tg-Trx1C35S, Tg-Trx1C32S/C35S, and WT mice were subjected to 3 h of ischemia. Representative images of Hairpin-2 staining of cardiac tissue sections are shown. The percentages of Hairpin-2 positive nuclei are shown (*$p<0.05$ vs. WT, #<0.05 vs. Tg-Trx1, n=3). (S1D) Tg-Trx1C35S and control WT mice were subjected to ischemia for 20 min. Homogenates prepared from sham-operated hearts and ischemic areas were used for co-immunoprecipitation using FLAG agarose beads and immunoblots for the indicated molecules were performed. Immunoblots of input lysate controls (5% of inputs) are also shown. Error bars represent SEM.

Figure S2, related to Figure 2

(S2A) Cos7 cells transfected with WT, C35S or C32S/C35S FLAG-Trx1-HA plasmids were treated with or without 300 μM H_2O_2 for 30 min. Lysates were subjected to immunoblot analysis of p-AMPKα, AMPKα, HA, and GAPDH. A statistical analysis of densitometric measurements of p-AMPKα is shown (*$p<0.05$ vs. (-)/control, #$p<0.05$ vs. WT/H_2O_2, n=3). (S2B) Tg-Trx1C32S/C35S and control WT mice were subjected to ischemia for 20 min. Homogenates prepared from sham-operated hearts and the

ischemic areas were used for immunoblot analyses of p-AMPKα, AMPKα, p-ACC, ACC, Trx1, and α-tubulin. Statistical analyses of densitometric measurements of p-AMPKα and p-ACC are shown (*p<0.05 vs. WT/sham, #p<0.05 vs. WT/ischemia, n=3-4). Error bars represent SEM.

Figure S3, related to Figure 3

(S3A-B) Cardiomyocytes transduced with the indicated adenovirus were incubated in normal or glucose-free medium. (S3A) Immunostaining was performed with Glut4 antibody (red) and DAPI (blue). Results are representative of 3 individual experiments. (S3B) Intracellular ATP content was measured (*p<0.05 vs. control, #p<0.05 vs. GD, n=3). (S3C-D) Cardiomyocytes transduced with the indicated adenovirus were incubated with normal or glucose-free medium for 24 h. Cell viability was measured (*p<0.05 vs. control, #p<0.05 vs. GD, n=3). (S3E-F) Cardiomyocytes transduced with the indicated adenovirus were incubated with glucose-free medium or AICAR (0.5 μM) for 6 h. Lysates were subjected to immunoblot analysis of Trx1, p-AMPKα, and α-tubulin. Statistical analyses of densitometric measurements of Trx1 are shown (*p<0.05 vs. control, #p<0.05 vs. GD or AICAR, n=3). Error bars represent SEM.

Figure S4, related to Figure 4

(S4A) Measurement of the intracellular ROS level. Cardiomyocytes were loaded with CM-H_2DCFDA for 15 min, washed and incubated with normal or glucose-free medium in the presence or absence of N-acetylcysteine (NAC) pretreatment for 30 min. Representative images obtained using fluorescence microscopy are shown. (S4B)

Oxidation of AMPK was examined. Cardiomyocytes were incubated with or without H_2O_2. Duplicate lysate samples were separated by SDS-PAGE on 8% and 6% gels under reducing or non-reducing conditions and subjected to immunoblot analysis using an AMPKα antibody. The high molecular weight proteins reactive with the AMPKα antibody that were present after incubation with H_2O_2 barely entered the 8% SDS-PAGE gel, whereas the 6% gel better represents the mobility shift pattern of the high molecular weight complex. (S4C) WT mouse hearts were subjected to ischemia for 30 min. The ischemic area was subjected to immunoblot analysis using an AMPKα antibody under reducing and non-reducing conditions. Results are representative of 3 individual experiments. (S4D) Cardiomyocytes transduced with the indicated adenovirus were treated with or without 10 μM H_2O_2 for 10 min. The extent of cysteine reduction in AMPKα was detected. Results are representative of 3 individual experiments. (S4E-H) Neonatal cardiomyocytes were pretreated with the indicated doses of H_2O_2 for the indicated times and incubated with AICAR for 2 h. Lysates were subjected to immunoblot analysis of p-AMPKα, AMPKα, p-ACC, ACC, α-tubulin, and GAPDH. Statistical analyses of densitometric measurements of p-AMPKα and p-ACC are shown (*$p<0.05$ vs. untreated control, #$p<0.05$ vs. AICAR alone, n=3). (I-J) Neonatal cardiomyocytes and HEK 293 cells were treated with 300 μM H_2O_2 for 30 min. (I) Lysates were subjected to immunoblot analysis of p-AMPKα, AMPKα, and α-tubulin. (J) The oxidation of AMPK was examined. Lysate samples from the two different cell types were separated by SDS-PAGE under reducing or non-reducing conditions and subjected to immunoblot analysis using an

AMPKα antibody. Results are representative of 3 individual experiments. (K) The effect of BSO or DNCB on GSH level was examined. Cardiomyocytes were treated with either DMSO vehicle alone, DMSO+BSO (100 μM) for 24 h or DMSO+DNCB (100 μM) for 15 min. Intracellular GSH level was measured (*p<0.05 vs. DMSO, n=3). BSO but not DNCB selectively suppressed GSH level in cardiomyocytes. Error bars represent SEM.

Figure S5, related to Figure 5

(S5A) The MS spectra of AMPKα2 peptide [117-132]. The peptide in the control group contained oxidized Cys130 covalently linked to MMTS (m/z 1894.93) as well as reduced Cys130 alkylated with IAM (m/z 1905.96). In the H_2O_2-treated group, the intensity of the peak representing the peptide containing oxidized Cys130 was increased. (S5B) The MS spectra of AMPKα2 peptide [489-499]. The peptide in the control group only contained reduced Cys490 bound to IAM (m/z 1211.61). In the H_2O_2-treated group, the peptide contained oxidized Cys490 linked to MMTS (m/z 1200.57) as well as reduced Cys490 alkylated with IAM (m/z 1211.61). (S5C) Examination of AMPK 2CS oxidation. Cardiomyocytes transduced with the indicated adenovirus were treated with H_2O_2 for 30 min or incubated with glucose-free medium for 2 h. The extent of cysteine reduction in AMPKα2 WT or 2CS was detected. Results are representative of 3 individual experiments. (S5D) The intermolecular disulfide bond of AMPK was examined. Cos7 cells transfected with FLAG-AMPKC130S-HA and Myc-tagged AMPK WT or mutant plasmids were treated with or without 100 μM H_2O_2 for 30 min and then were lysed with or without 1 mM DTT. After immunoprecipitation with anti-FLAG agarose beads (left) or

anti-myc agarose beads (right), immunoblot analyses with myc and HA antibodies were performed. Immunoblots of input lysate controls (5% of inputs) are also shown. Results are representative of 3 individual experiments. (S5E) A scheme showing the hypothetical model of intermolecular disulfide bond formation. In response to oxidative stress, the redox active Cys130 or Cys174 in one AMPK molecule (green) forms an intermolecular disulfide bond with Cys130 or Cys174 in another AMPK molecule (blue). The black rectangle highlights the three possible disulfide combinations: Cys130-Cys130, Cys130-Cys174, and Cys174-Cys174. The remaining free Cys130 or Cys174 in either AMPK molecule can form additional disulfide bonds, such that multiple disulfide linking induces high molecular weight complex formation. Trx1 catalyzes reduction of the disulfide and prevents AMPK oxidative aggregation.

Figure S6, related to Figure 6

(S6A) Localization of WT or mutant HA-tagged AMPK was evaluated in cardiomyocytes. Cells were stained with an HA antibody (red), an α-actinin antibody (green) and DAPI (blue). (S6B) Cos7 cells transfected with either FLAG-AMPK WT-HA or the indicated cysteine mutants were incubated with glucose-free medium for 2 h. After immunoprecipitation with FLAG agarose beads, the immunocomplexes were subjected to AMPK kinase assay using SAMS peptide as a substrate (*$p<0.05$ vs. WT/control, #$p<0.05$ vs. WT/GD, n=3). (S6C) *In vitro* kinase assays with recombinant AMPK mutants. GST-AMPKα2 WT or mutants were incubated with the LKB1 complex. The phosphorylation of AMPK was detected by autoradiography. Coomassie brilliant blue

(CBB) dye staining and an anti-AMPKα immunoblot of the recombinant substrates are also shown. A standard AMPK kinase assay was performed using SAMS peptide as a substrate (*p<0.05 vs. WT/LKB1(-), n=3). (S6D-E) GST-AMPKα2WT was pretreated with or without either IAM or NEM. (S6D) AMPK oxidation was evaluated in response to H_2O_2 treatment under non-reducing conditions. IAM- and NEM-alkylated AMPK was resistant to H_2O_2-induced oxidation. (S6E) The phosphorylation of AMPK was detected by autoradiography. Standard AMPK kinase assays were performed using SAMS peptide as a substrate (*p<0.05 vs. AMPK alone, n=3). Error bars represent SEM.

Figure S7, related to Figure 7

(S7A) Cardiomyocytes were transduced with the indicated adenoviruses and the lysates were used for immunoblot analysis of AMPKα1, AMPKα2, HA, and GAPDH. Exogenous AMPK WT or mutant expression was examined using an HA antibody. (S7B) The effect of mutant AMPK on GD-induced cell death. Cardiomyocytes were first transduced with Ad-sh-AMPKα2 and then with WT or mutant AMPK adenovirus. GD-induced cell death was measured by CellTiter-Blue assay (*p<0.05 vs. sh-con/control, #p<0.05 vs. sh-con/GD, n=3). (S7C) Cardiomyocytes transduced with the indicated adenovirus were incubated with normal or glucose-free medium for 2 h. Whole myocyte lysates were subjected to immunoblot analysis for p-PFK-2, PFK-2, HA, and GAPDH. Results are representative of 3 individual experiments. (S7D) Characterization of Tg-AMPKC174S transgenic mice. Different lines of Tg-AMPKC174S mice were sacrificed at 3 months of age. Exogenous AMPKC174S expression was examined using an HA antibody. The

heart homogenates were used for immunoblot analysis of AMPKα1, AMPKα2, HA, p-AMPKα, and GAPDH. (S7E) A scheme showing redox regulation of AMPK by Trx1. During energy stress, AMPK is phosphorylated and activated when present in its reduced state, which is maintained through a Trx1-dependent mechanism. This in turn mediates adaptive responses such as increasing glucose metabolism. Without Trx1, the reduced form of AMPK undergoes oxidation. The switch from reduced state to oxidized state induces conformational changes and prevents AMPK phosphorylation by LKB1, thereby blocking AMPK activation. We here propose that AMPK has three different statuses: oxidized (inactive form), reduced (activatable form), and reduced and phosphorylated (active form). Endogenous Trx1 acts as an essential co-factor to mediate AMPK activation by preventing AMPK oxidation. PTOM: post-translational oxidative modification. Error bars represent SEM.

Table S1, related to Figure 1

Summary of potential substrates identified for Trx1. Tg-Trx1C35S and control WT mice were subjected to ischemia for 20 min. The ischemic area was used for co-immunoprecipitation. After immunoprecipitation with FLAG antibody, immunoblots with the indicated antibodies were performed. Red color indicates interaction and black color indicates no interaction.

Table S2, related to Figure 6

The characteristics of cysteine mutants of the AMPK α2 subunit. Alignment was done to find conserved cysteines. Individual cysteines' effects on oxidation, AMPK

phosphorylation and kinase activity are summarized.

Supplemental Experiment Procedures

Transgenic Mice

Transgenic mice expressing either hTrx1C32S/C35S (designated as Tg-Trx1C32S/C35S) or wild-type hTrx1 mice (designated as Tg-Trx1) were generated on an FVB background as described previously (Yamamoto et al., 2003a). The shuttle and mammalian expression vectors for N-terminal FLAG-, and C-terminal HA-tagged Trx1 (pDC316-FLAG-Trx1-HA) were generated by insertion of human Trx1 cDNA lacking start and stop codons into pDC316-FLAG-HA, which contains a DNA fragment encoding the FLAG (ACCATGGACTACAAAGACGATGACGACAAGAATTC; MDYKDDDDK) and HA (GGATCCTACCCATACGATGTTCCGGATTACGCTAGTCTCTAGTCGAC; YPYDVPDYASL) tags inserted downstream of the CMV promoter of pDC316 (Microbix). Rat AMPKα2 cDNA was inserted into the pDC316 shuttle vector containing a C-terminal HA tag (pDC316-AMPK-HA). Site-directed mutagenesis was performed to generate pDC316-FLAG-Trx1C35S-HA and pDC316-AMPKC174S-HA using the QuikChange mutagenesis kit (Agilent Technologies). The cDNA encoding FLAG-Trx1C35S-HA or AMPKC174S-HA was inserted downstream of the α-myosin heavy chain promoter in order to achieve cardiac-specific expression (courtesy of J. Robbins, University of Cincinnati, Cincinnati, Ohio, USA). Tg-FLAG-Trx1C35S-HA and Tg-AMPKC174S-HA mice were generated on the FVB background. Tg-Trx1 mice on a C57BL/6 background were used in the high-fat diet study. All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee at the New Jersey Medical School,

Rutgers Biomedical and Health Sciences.

Ischemia Surgery *in Vivo*

Pathogen-free mice were housed in a temperature-controlled environment with 12-h light/dark cycles, where they received food and water *ad libitum*. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). A rodent ventilator (model 683; Harvard Apparatus Inc.) was used with 65% oxygen during the surgical procedure. The animals were kept warm using heat lamps and heating pads. Rectal temperature was monitored and maintained between 36.8 and 37.2°C. The chest was opened by a horizontal incision through the muscle between the ribs (third intercostal space). Ischemia was achieved by ligating the anterior descending branch of the left coronary artery (LAD) using an 8-0 nylon suture, with a silicon tubing (1 mm OD) placed on top of the LAD, 2 mm below the border between left atrium and left ventricle (LV). Regional ischemia was confirmed by ECG change (ST elevation).

Assessment of Area at Risk and Infarct Size

After prolonged ischemia, the animals were reanesthetized and intubated, and the chest was opened. After arresting the heart at the diastolic phase by KCl injection, the ascending aorta was cannulated and perfused with saline to wash out blood. The LAD was occluded with the same suture, which had been left at the site of the ligation. To demarcate the ischemic area at risk, Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into 1-mm-thick cross sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride

(TTC) solution at 37°C for 10 min. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured using Adobe Photoshop (Adobe Systems Inc.), and the values obtained were averaged. The percentages of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from all sections. AAR/LV and infarct area/AAR were expressed as percentages.

Cell Cultures

HEK 293 and Cos7 cells (American Type Culture Collection) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM)/F12 (Life Technologies, Inc.) with 10% FBS. Primary cultures of ventricular cardiomyocytes were prepared from 1-day-old Crl: (WI) BR-Wistar rats (Harlan Laboratories). A cardiomyocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient. Cells were cultured in complete medium containing DMEM/F-12 supplemented with 5% horse serum, 4 μg/ml transferrin, 0.7 ng/ml sodium selenite (Life Technologies, Inc.), 2 g/L bovine serum albumin (fraction V), 3 mM pyruvic acid, 15 mM HEPES, 100 μM ascorbic acid, 100 μg/ml ampicillin, 5 μg/ml linoleic acid, and 100 μM 5-bromo-2'-deoxyuridine (Sigma). Myocytes were changed to serum-free medium for 24 h before any experiment.

Antibodies

Antibodies used for immunoblots were purchased from the indicated companies. Akt, AMPKα, AMPKα1, AMPKα2, AMPKβ2, AMPKγ1, p-AMPKα, p-ACC, ACC, FoxO3a, GAPDH, Histone H3, JNK, LKB1, p53 (Cell Signaling Technology), HA, myc, p-PFK-2,

PFK (Santa Cruz Biotechnology), α-tubulin, FLAG (Sigma), Glut4, Hsp25, pan-Cadherin, peroxiredoxin, Sirt1 (abcam), Bcl2, and Trx1 (BD Biosciences).

Construction of Adenoviral Expression Vectors

Briefly, pBHGloxΔE1,3Cre (Microbix), including the ΔE1 adenoviral genome, was co-transfected with pDC shuttle vector containing the gene of interest into 293 cells using Lipofectamine 2000 (Invitrogen). Through homologous recombination, the test genes were integrated into the E1-deleted adenoviral genome. The viruses were propagated in 293 cells as described (Yamamoto et al., 2003b). Generation of adenovirus harboring a dominant negative form of the α2 subunit of AMPK has been described (Ad-dn-AMPK) (Sakoda et al., 2002). AMPK and Trx1 mutants were generated using the QuikChange mutagenesis kit. Adenovirus harboring β-galactosidase (Ad-LacZ) was used as a control.

Construction of Short Hairpin RNA (shRNA) Adenoviral Expression Vectors

Adenoviruses harboring shRNA for Trx1 (Ad-sh-Trx1) or AMPKα2 (Ad-sh-AMPKα2) were generated using the following hairpin forming oligos:

Trx1:(5'-AGTATTCCAATGTGGTGTTTTCAAGAGAAACACCACATTGGAATACTCTTTTTTTT-3')

AMPKα2:(5'-GGCCATAGGGTCTTTAAATATTCAAGAGATATTTAAAGACCCTATGGCCTTTTTT-3').

These oligos and their corresponding antisense oligos with ApaI and Hind III overhangs were synthesized, annealed, and subcloned into the pDC311 vector. The loop sequences

are underlined. Recombinant adenoviruses were generated using homologous recombination in 293 cells as described above.

Immunocytochemistry

Neonatal cardiomyocytes in 4-well chambers were washed with phosphate-buffered saline (PBS) three times, fixed with 4% paraformaldehyde for 15 min, permeabilized in 0.3% Triton X-100 for 10 min and blocked with 3% bovine serum albumin for 1 h at room temperature. The following antibodies were used as primary antibodies: Glut4 (abcam), AMPKα (Santa Cruz Biotechnology), Trx1 (BD Biosciences), HA (Santa Cruz Biotechnology), and α-actinin (Sigma). Alexa Fluor 488 Dye- or Alexa Fluor 594 Dye-conjugated secondary antibody (Invitrogen) was used for detecting indirect fluorescence. Slides were mounted using a reagent containing DAPI (VECTASHIELD; Vector Laboratories Inc.).

Glycolysis Measurement

Briefly, cardiomyocytes plated on 6-well plates were washed with 2 ml prewarmed KRH buffer (50 mM HEPES pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, and 0.1% BSA) twice. After that, myoctes were incubated with 2 ml KRH solution with Glucose, D-[5-^{3}H(N)] (1 μCi/ml) (Perkin Elmer) for 30 min. The medium was collected and stored at -20°C for later use. To measure glycolysis, ^3H_2O was separated from [^{3}H]-glucose using an anion exchange column. Dowex® 1-X4 anion exchange resin (200–400 mesh) (Sigma) was suspended in 0.4 M potassium tetraborate (Sigma). Nine g of Dowex® was added to 100 ml of potassium tetraborate and was slowly stirred

overnight at room temperature. Two ml of the Dowex/borate slurry was prepacked into gravity flow affinity columns (Thermo Scientific) and allowed to drain and form a 0.5 cm bed. The Dowex® in the columns was then extensively washed with distilled H_2O before use. A 0.2 ml volume of sample collected as described above, containing a mixture of ^3H_2O and ^{3}H-glucose, was applied to the column. The samples were allowed to interact with the beds for at least 30 min. After that, the ^3H_2O remaining on the columns was eluted with 800 µl ddH_2O and then subjected to scintillation counting (Lopaschuk and Barr, 1997).

CellTiter-Blue Assay

Viability of the cells was measured by CellTiter-Blue (CTB) assays (Promega). In brief, cardiomyocytes ($1X10^5$ per 100 µl) were seeded onto 96-well plates. After 24 h, the cells were transduced with the indicated adenoviruses. After transduction, myocytes were incubated with either complete medium or glucose-free medium. Viable cell numbers were measured on the indicated days by the CTB assay. The CTB assays were performed according to the supplier's protocol.

Recombinant Protein Preparation

rAMPKα2 was subcloned into the pCold-GST vector and expression vectors were transformed into *E. coli* strain BL21(DE3) (Invitrogen). Induction and purification of GST-tagged protein with glutathione Sepharose 4 (GE healthcare) were carried out as described (Hayashi and Kojima, 2008).

AMPK Kinase Assay

For immunocomplexes: Transfection of plasmids into Cos7 cells was performed with Lipofectamine 2000 (Invitrogen). One µg of FLAG-AMPK-HA WT or mutant expression plasmids was transfected into Cos7 cells on 6 cm dishes. After 36 h, cell lysates were prepared using 300 µl Lysis buffer A containing 50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA. After centrifugation, the supernatants were incubated with 30 µl FLAG-Agarose beads (Sigma) for 2 h at 4°C. After immunoprecipitation, the immunocomplexes were washed with 1 ml TBS buffer (50 mM Tris pH 7.4, 150 mM NaCl) three times, and then with 1 ml washing buffer (40 mM HEPES pH 7.0, 80 mM NaCl) twice. AMPK assays were then carried out on the resuspended immunoprecipitates (Hardie et al., 2000).

For GST-recombinant AMPK WT or mutants: One µg of *E. coli*-expressed GST-AMPKα2 was maximally activated by using MgATP and AMPKK in the assay below. Five µl of mixture were subjected to a standard AMPK kinase assay (Hawley et al., 2003).

For AMPK kinase kinase assay: Briefly, GST-AMPKα2 (1 µg), either WT or mutant, was incubated in the presence or absence of recombinant LKB1:STRADα:MO25α complex (10 ng) (Millipore) in 1X reaction buffer containing 50 mM HEPES pH 7.0, 1 mM DTT, 0.02% Brij-35, 5 mM $MgCl_2$, and 1X $MgCl_2$/ATP cocktail (5 mM $MgCl_2$ and 0.1 mM ATP) for 30 min at 30°C.

Phosphorylation of GST-AMPKα2 Using [γ-^{32}P] ATP

GST-AMPKα2 (1 µg), either wild-type or mutant, was incubated for 30 min at 30°C with 5 mM $MgCl_2$ and [γ-^{32}P]ATP (Perkin Elmer) in the presence of the LKB1:STRADα:MO25α

complex (10 ng). The reaction was terminated by the addition of 2X SDS sample buffer. Samples were separated by SDS gel electrophoresis and the dried gel was subjected to autoradiography (Hawley et al., 2003).

Mass Spectrometry

The proteins from immunoprecipitations or *E.coli*-expression systems were separated by SDS-PAGE. The gel band observed in the molecular weight region corresponding to that of AMPKα2 was excised for in-gel trypsin digestion without additional reduction or alkylation. The resulting peptides were subjected to LC-MS/MS analysis on an Ultimate 3000 (Dionex, Synnyvale, CA, USA) LC system coupled with an Orbitrap Velos tandem mass spectrometry instrument (Thermo Fisher Scientific). In brief, the peptides were separated by a C_{18} reversed phase column (75 µm × 150 mm, 3 µm, 100 Å, C_{18}, Dionex, Synnyvale, CA, USA) using an 80 min binary gradient protocol (solvent A (2% acetonitrile (ACN), 0.1% formic acid (FA)), solvent B (85% ACN, 0.1% FA): a 60 min gradient from 3% to 25% B followed by a 15 min gradient from 25% to 45% B and a 5 min gradient from 45% to 95% B). The eluted peptides were directly introduced into a nano-electrospray ionization source on the MS system for LC/MS/MS analyses with a spray voltage of 2 kV, a capillary temperature of 275 ºC and an S-lens voltage of 60%. The automatic gain control (AGC) target was set to 1.0E+6 for full scan in the Orbitrap mass analyzer and 3.0E+4 for MS/MS scans in the ion trap mass analyzer. MS spectra were acquired in a positive ion mode with a resolution of 30,000 FWHM. Four full scans in a range of 390-480 amu, 560-620 amu, 650-810 amu, and 850-900 amu with an inclusion list were

used to monitor the cysteine-containing peptides from AMPKα2. The lock mass feature was used for accurate mass measurements. The selected precursor ions were fragmented once in the collision-induced dissociation (CID) fragmentation mode to confirm the peptide sequence. For MS/MS analysis, the precursor isolation width was set to 2 amu, and the minimum ion threshold count was set to 3,000.

The MS/MS spectra were searched against a Swissprot rat database (7631 sequences) with an MS error window of 10 ppm and an MS/MS error window of 0.5 Da. Methionine oxidation, cysteine methylthio (MMTS), and carbamidomethyl (IAM) modifications were set as variable modifications. The selected ion chromatograms of the peptides identified from AMPKα2 with cysteines modified by either MMTS or IAM were extracted manually. The corresponding ion chromatographic peak areas were used for relative quantification. The median peak areas of two abundant non-cysteine-containing peptides from AMPKα2 were used for normalization.

Hairpin-2 staining

A double stranded DNA fragment with blunt ends was prepared as previously described (Yamamoto et al., 2001). Polymerase chain reaction (PCR) with Pfu Ultra polymerase was performed with 16.6 μM Texas Red-12-dUTP (Molecular Probes), 16.6 μM dTTP, 50 μM dATP, 50 μM dCTP and 50 μM dGTP. Pfu probe recognizes a form of DNA damage characterized by cleavage of multiple DNA fragments with blunt ends, typically observed in necrotic cell death (Guerra et al., 1999; Sciarretta et al., 2012; Zhai et al., 2011). Heart sections were deparaffinized with xylene, rehydrated in graded

alcohol concentrations, briefly washed in water, and then treated with proteinase K (50 μg/ml) in PBS for 45 min at 37°C. After washing with PBS, a mix of 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/mL BSA, 15% polyethylene glycol (8,000 mol wt, Sigma), 1 μg/mL Texas red-labeled DNA fragment, and 250 U/mL DNA T4 ligase (Boehringer Mannheim) was added. Sections were then placed in a humidified box for 16 h. The sections were thoroughly washed in 70°C water and then were observed under a fluorescent microscope immediately after counterstaining with 10 μg/mL 4,6-diamidino-2-phenylindole (DAPI) (Zhai et al., 2011).

Detection of Intracellular ROS Using Fluorescence Staining

5-(and-6)-chloromethyl-2',7'-dichloro-dihydrofluorescein diacetate (CM-H_2DCFDA, Molecular Probes) was used to detect intracellular reactive oxygen species (ROS) generation. Cardiomyocytes were washed with pre-warmed PBS three times. Myocytes were then treated with 5 μM CM-H_2DCFDA and incubated for 15 min at 37°C while protected from light exposure. After that, myoctes were washed with pre-warmed PBS one more time and incubated with glucose-free medium with or without *N*-acetyl-cysteine (NAC) pretreatment for 30 min. The extent of intracellular ROS generation was evaluated by fluorescence microscopy (Yamamoto et al., 2001).

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