Expanded Materials and Methods

Antibodies

Anti-Nampt antibody was purchased from Bethyl and Abcam. Other antibodies include anti-Sirt1 (Upstate Biotechnology), anti-Bcl-xL (Pharmingen), anti-tubulin (Sigma), anti-cleaved-caspase 3 (Cell Signaling), anti-LC3 (MBL), and anti-p62 (American Research Products, Inc.) anti-cytochrome C (Sigma), anti-cytochrome c oxidase I (COX I) (Sigma), anti-hyper-acetylated Histone H4 (Millipore), anti-phospho-AMPKα (Thr172), anti-AMPK, anti-phospho-4E-BP1 (Thr37/46), anti-4E-BP1, anti-phospho-p70 S6K (Thr389), anti-p70 S6K, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-phospho-Akt (Ser473), anti-Akt, anti-acetyl-lysine, anti-acetyl-Histone H3 (Lys 23) (Cell Signaling), anti-Beclin 1 (BD Biosciences), and anti-GAPDH (Chemicon) antibodies.

Adenovirus vectors

We made replication-defective human adenovirus type 5 (devoid of E1) harboring Nampt and shRNA-Nampt. Adenovirus harboring ß-galactosidase or scramble shRNA was used as a control. Adenoviruses harboring Sirt1, shRNA-Sirt1, Bcl-xL, and GFP-LC3 have been described [1, 2].

Primary cultures of neonatal rat ventricular myocytes

Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old Crl: (WI) BR-Wistar rats (Harlan). A cardiac myocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient. Cells were cultured in complete medium (CM) containing Dulbecco's modified Eagle's medium (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 mmol/L pyruvic acid, 15 mmol/L HEPES, 100 μmol/L ascorbic acid, 100 μg/mL ampicillin, 5 μg/mL linoleic acid, and 100 μmol/L 5-bromo-2'-deoxyuridine (Sigma) [3].

TUNEL staining in cultured cardiac myocytes

Myocytes were fixed in PBS containing 3.7% paraformaldehyde. Staining was performed using the In Situ Cell Death Detection kit (Roche) [4].

Hairpin2

A 196 bp double stranded DNA fragment was prepared using primers 5'-CCCTGTAGCCGCTTAAG-3' and 5'-GGTCGAGGTGCCGTAAAGCA-3' complementary to pBluescript SK(−) (Stratagene). Polymerase chain reaction (PCR) with *PfuUltra* polymerase was performed with 16.6 μmol/L Texas Red-12-dUTP (Molecular Probes), 16.6 μmol/L dTTP, 50 μmol/L dATP, 50 μmol/L dCTP and 50 μmol/L dGTP. Agarose gel electrophoresis of an aliquot of the reaction showed a single product. Cells were fixed and permeabilized in methanol and acetone. After washing with PBS, a mix of 50 mmol/L Tris-HCl, pH 7.8, 10 mmol/L $MgCl_2$, 10 mmol/L DTT, 1 mmol/L ATP, 25 μg/mL BSA, 15% polyethylene glycol (8,000 mol wt, Sigma), Texas red-labeled DNA fragment at 1 μg /mL and DNA T4 ligase (Boehringer Mannheim) at 25 U/mL was added. Sections were covered with glass coverslips and placed in a humidified box for 1 h. The sections were thoroughly washed in 70°C water. The samples were observed under a fluorescent microscope immediately after counterstaining with 10 μg/mL 4,6-diamidino-2-phenylindole (DAPI) [5].

NAD^+ measurement

We measured the level of NAD^+ in cardiac myocytes and in the heart, using the EnzyChrom NAD^+/NADH Assay Kit, according to the manufacturer's protocol (#ECND-100, Bioassay Systems, Hayward, CA). The method is based on an alcohol dehydrogenase cycling reaction, in which a tetrazolium dye is reduced by NADH in the presence of phenazine methosulfate. The intensity of the reduced product color, measured at 565 nm, is proportionate to the NAD^+/NADH concentration in the sample. This assay method allows one to measure NAD^+ and NADH from duplicate samples. Although we measured NAD^+ in all samples, we only measured NADH in heart homogenates because the level of NADH was within the detection range of the assay in the *in vivo* heart samples but it was sometimes below the reliable detection range in cardiac myocyte samples. For *in vitro* samples, myocytes cultured on a 10 cm dish were treated with trypsin, washed twice with cold PBS, and pelleted through centrifugation. Myocytes were separated into two parts, one for NAD^+ measurement and the other for protein concentration. Cell pellets were resuspended in 1.5 mL eppendorf tubes with 100 μL NAD^+ extraction buffer (containing 0.40% hydrochloric acid). For the heart samples, the heart (20 mg) was homogenated with 100 μL NAD^+ extraction buffer for NAD^+ determination or with 100 μL NADH extraction buffer for NADH determination. Extracts were heated at 60°C for five minutes. Twenty μL of assay buffer (containing Tris (hydroxymethyl) aminomethane 3.0% and BSA 0.10%) was added to the extracts followed by 100 μL NADH extraction buffer for NAD^+ measurement or 100 μL NAD^+ extraction buffer for NADH measurement (to neutralize the extracts). The samples were vortexed and centrifuged at 13,200 rpm for five minutes. Supernatants (120 μL) were then mixed with Working Reagent, containing 50 μL assay buffer, 1 μL alcohol dehydrogenase, 10 μL 1% (vol) ethanol, 14 μL phenazine methosulfate, and 14 μL tetrazolium dye. Optical density at 565 nm was recorded at time zero (OD_0) and at 15 minutes (OD_{15}) with a 96-well plate reader spectrophotometer. The difference in the absorbances at

time zero and 15 minutes (OD_0-OD_{15}) of the test sample was compared with that of the standard solution to determine the NAD^+ or NADH concentration. Myocytes (the other half) or the heart samples were lysed with RIPA buffer to measure protein concentration. The amount of NAD^+ and NADH was normalized to the protein concentration.

Measurement of intracellular ATP contents

Intracellular ATP contents were measured using an ATP Bioluminescent Assay Kit (Sigma) [2]. Cells were lysed directly in the somatic-cell ATP-releasing agent, and the lysates were assayed according to the manufacturer's instructions, using a 1:625 dilution of the ATP assay mix. Light emitted was measured using a luminometer. ATP contents were calculated by comparison with a standard curve derived from known concentrations of ATP, ranging from 0.01 to 10 pmol/L.

Construction of short hairpin RNA (shRNA) adenoviral expression vectors

p*Silencer* 1.0-U6 expression vector was purchased from Ambion. The U6 RNA polymerase III promoter and the polylinker region were subcloned into the adenoviral shuttle vector pDC311 (Microbix). The hairpin-forming oligo, corresponding to bases 54-72 (5'-CAAGGTTACTCACTATAAATTCAAGAGATTTATAGTGAGTAACCTTGTTTTTT-3') of the rat *nampt* cDNA and its antisense with ApaI and Hind III overhangs were synthesized, annealed, and subcloned distal to the U6 promoter. The loop sequence is underlined. A recombinant adenovirus was generated using homologous recombination in 293 cells.

Immunoblot analysis

For immunoblot analysis, cells were lysed in lysis buffer (50 mmol/L Tris-HCl pH 7.4, 0.1% SDS,

1% IGEPAL CA-630, 0.15 mol/L NaCl, 0.25% Na-deoxycholate, and 1 mmol/L EDTA supplemented with protease inhibitors). Densitometric analysis was performed using Scion Image software (Scion).

Evaluation of GFP-LC3 dots

Cardiac myocytes were grown on gelatinized coverslips. Myocytes were transduced with Ad-GFP-LC3 for 48 hours. Samples were mounted using SlowFade Light Antifade Kit (Molecular Probes) and the fluorescence of GFP-LC3 was observed under a fluorescence microscope. The number of cells with GFP-LC3 dots was counted in five independent visual fields [2].

Reverse Transcription–Polymerase Chain Reaction

Total RNA was subjected to reverse transcription–quantitative polymerase chain reaction (RT-qPCR) as described previously.

Immunohistochemistry

The heart specimens were fixed with formalin, embedded in paraffin, and sectioned at 6-μm thickness. The method of immunostaining has been described [6]

Echocardiography

Mice were anesthetized using 12 μL/g BW of 2.5% avertin (Sigma-Aldrich), and echocardiography was performed using ultrasonography (Acuson Sequoia C256; Siemens Medical Solutions) as previously described [7].

Ischemia/reperfusion and prolonged ischemia

Mice were housed in a temperature-controlled environment with 12-hr light/dark cycles where they received food and water ad libitum. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 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. Rectal temperature was monitored and maintained between 36 and 37°C. The chest was opened by a horizontal incision through the muscle between the ribs (third intercostal space). Ischemia/reperfusion was achieved by ligating the anterior descending branch of the left coronary artery (LAD) using an 8-0 prolene suture, with a silicon tubing (1 mm OD) placed on top of the LAD, 2 mm below the border between left atrium and LV. Regional ischemia was confirmed by ECG change (ST elevation). After occlusion for 45 min, the silicon tubing was removed to achieve reperfusion and the rib space and overlying muscles were closed. When recovered from anesthesia, the mice were extubated and returned to their cages. They were given water and standard mouse food and housed in a climate-controlled environment. Prolonged ischemia was achieved by ligating the anterior descending branch of the left coronary artery (LAD) using an 8-0 prolene suture, and closing the wound. Evaluation of infarction size was done by TTC staining 24 hour after ischemia [2]. In order to evaluate the effect of Nampt upon prolonged ischemia, some mice were subjected to prolonged ischemia (2 hours) and euthanized without reperfusion.

Assessment of area at risk and infarct size

After intervention, 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 canulated 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 (AAR), 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 solution at 37°C for 15 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 percentage 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 [8].

Evaluation of apoptosis in tissue sections

DNA fragmentation was detected *in situ* using TUNEL, as described. [9] Briefly, deparaffinized sections were incubated with proteinase K, and DNA fragments were labeled with fluorescein-conjugated dUTP using TdT (Roche Molecular Biochemicals). Nuclear density was determined by manual counting of DAPI-stained nuclei in six fields for each animal using the 40x objective, and the number of TUNEL-positive nuclei was counted by examining the entire section using the same power objective.

Supplemental Figures

Figure S1

(A) Time course of Nampt expression in response to myocardial ischemia. C57/B6 mice were subjected to myocardial ischemia by coronary ligation. Heart samples were collected at indicated time points. Expression of Nampt and tubulin was evaluated by immunoblot analyses. (B) Reversibility of Nampt downregulation after pressure overload. Mice were subjected to sham operation, aortic banding for one week, or aortic banding for one week followed by debanding for one week. Expression of Nampt and tubulin was evaluated by immunoblot analyses.

Figure S2

Cardiac myocytes were transduced with either Ad-LacZ or Ad-Nampt (A) or Ad-shRNA-scramble (control) or Ad-shRNA-Nampt (B) and cultured for 48 hours. Cell viability was measured after 4-hours of treatment with 1.2 mM methyl methanesulfonate (MMS) or glucose deprivation. Cell viability was evaluated by CellTiter-Blue Cell Viability Assays. Cell viability of myocytes transduced with Ad-LacZ without stress was expressed as 100%.

Figure S3

(A) The effect of Nampt knockdown on necrotic cell death. Neonatal rat cardiac myocytes were transduced with Ad-shRNA-Nampt (shNampt) or Ad-shRNA-scramble (ShControl) (3MOI) and cultured for 5 days. Myocytes treated with chelerythrine (CE, 100 μM for 30 min) were used as a positive control for necrotic cell death. Cells were stained with propidium iodide for 5 min without cell permeabilization. (B) The effect of Nampt knockdown on necrotic cell death in response to glucose deprivation. Cardiac myocytes were transduced with Ad-shRNA-Nampt (shNampt) or Ad-shRNA-scramble (shControl or sh-con) (3MOI) and cultured for 5 days. Myocytes were then incubated with glucose free medium for 4 hours. Cells were stained with propidium iodide for 5

min without cell permeabilization.

Figure S4

The effect of Nampt knockdown on PARP activity. Cultured neonatal rat cardiac myocytes were transduced with Ad-shRNA-scramble (sh-scr) or Ad-shRNA-Nampt (sh-Nampt) (3MOI) and cultured for 5 days. Chelerythrine (100 μM) was used as positive control. Samples were subjected to immunoblot analyses with anti-poly ADP-ribose polymer (PAR) antibody.

Figure S5

(A) Downregulation of Nampt does not affect p62 mRNA expression. Neonatal rat cardiac myocytes (NRCMs) were transduced with either Ad-shRNA-scramble or Ad-shRNA-Nampt at 3 MOI and cultured in serum-free conditions for 96 hours. mRNA expression of p62 was measured by RT-qPCR and normalized by S15RNA. The value in myocytes transduced with Ad-shRNA-scramble was expressed as 1.(B) Downregulation of Nampt does not further increase accumulation of LC3-II in the presence of chloroquine. Cardiac myocytes were transduced with Ad-shRNA-scramble or Ad-shRNA-Nampt at 3 MOI and cultured in serum-free conditions for 96 hours. Some myocytes were incubated with chloroquine (6 μM) for 2 hours. Cell lysates were subjected to immunoblot analyses with anti-LC3 and anti-tubulin antibodies. Results are representative of three experiments.

Figure S6

Downregulation of Nampt does not significantly affect AMPK, p70S6K, 4E-BP1, Akt or mTOR phosphorylation in cardiac myocytes. Cardiac myocytes were transduced with Ad-shRNA-scramble or Ad-shRNA-Nampt at 3 MOI and cultured in serum or serum-free conditions for 96 hours. Cell lysates were subjected to immunoblot analyses with antibodies against phosphor-AMPK, AMPK, phospho-P70, P70, phospho-4E-BP1, 4E-BP1, phospho-mTOR,

mTOR, phospho-Akt, Akt, Beclin-1 and actin. Results are representative of four experiments.

Figure S7

Knockdown of Nampt decreases the activity of Sirt1. Myocytes were transduced with Ad-shRNA-scramble or Ad-shRNA-Nampt. Cell lysates were subjected to immunoblot analyses of acetylated-lysine proteins, acetyl-Histone H3 and hyperacetylated Histone H4 (Ponceau S staining shown as a loading control).

Figure S8

The level of [NAD^+], [NADH], [NAD^+]/[NADH] in the heart was measured using either 1-2 or 12-14 months old mice. Data are mean ± SEM obtained from 5-6 experiments.

References

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Table S1

Gravimetric Parameters of Tg-Nampt (Line 9) at 3 Months of Age

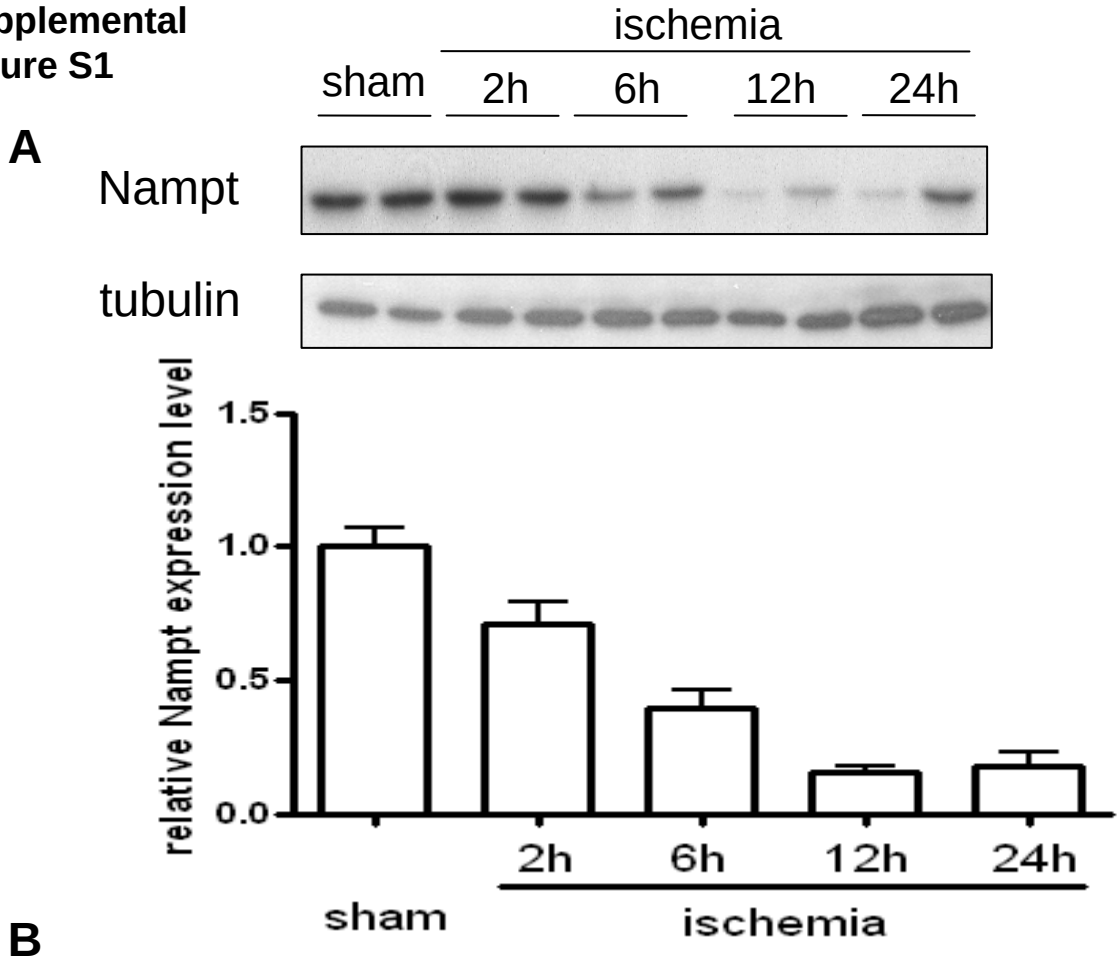
Group	WT (N=5)	Tg (N=5)
HW/BW (mg/g)	3.98±0.02	3.84±0.01
LV/BW (mg/g)	2.75±0.03	2.67±0.05
RV/BW (mg/g)	0.69±0.01	0.66±0.01
Atrium/BW (mg/g)	0.37±0.02	0.31±0.02
Lung/BW (mg/g)	4.99±0.06	4.80±0.07

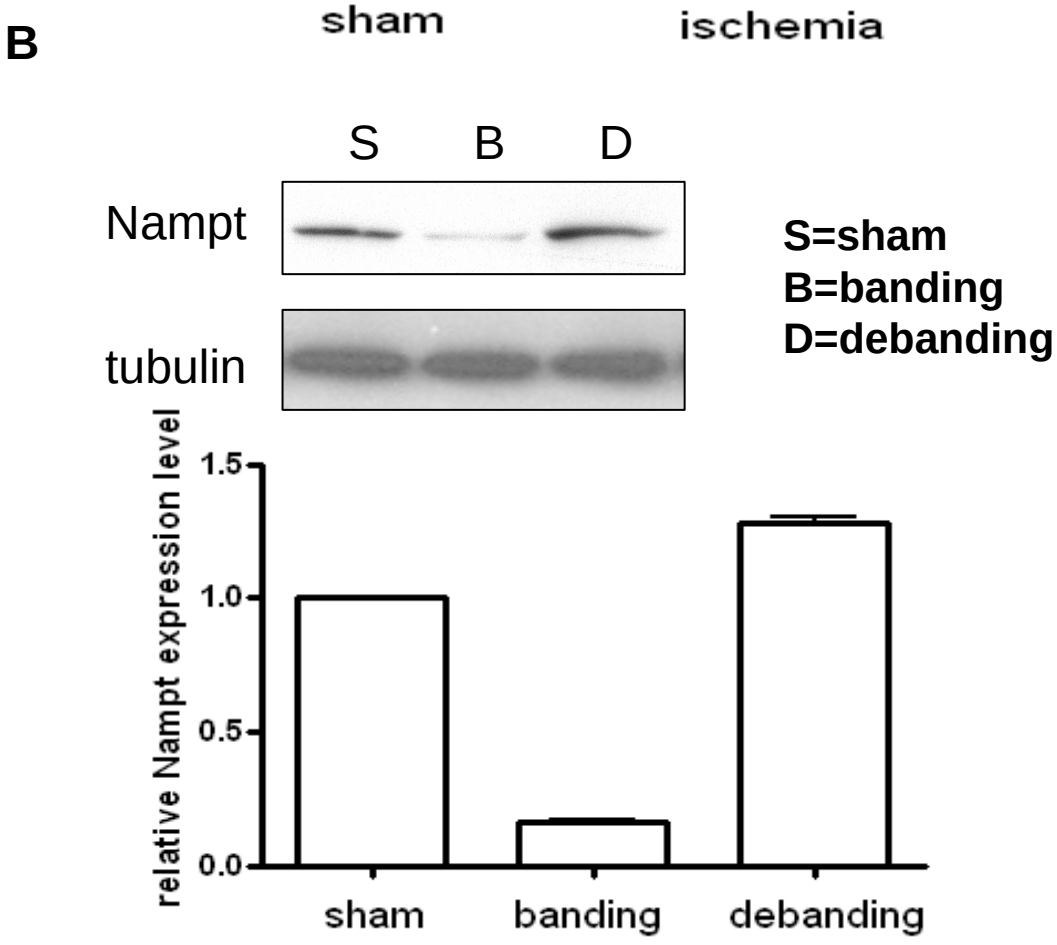
Table S2

Echocardiographic Analysis of Tg-Nampt (Line 9) at 3 Months of Age

Group	WT (N=6)	Tg (N=6)
DSEP WT (mm)	0.85±0.03	0.83±0.03
LVEDD (mm)	3.70±0.08	3.68±0.09
DPW WT (mm)	0.81±0.02	0.78±0.02
SSEP WT (mm)	1.32±0.06	1.30±0.03
LVESD (mm)	2.43±0.06	2.28±0.08
SPW WT (mm)	1.11±0.02	1.10±0.03
EF (%)	71.6±2.1	75.5±2.5
FS (%)	34.5±1.6	37.8±2.2
HR (bpm)	474±31	470±19

Supplemental Figure S1





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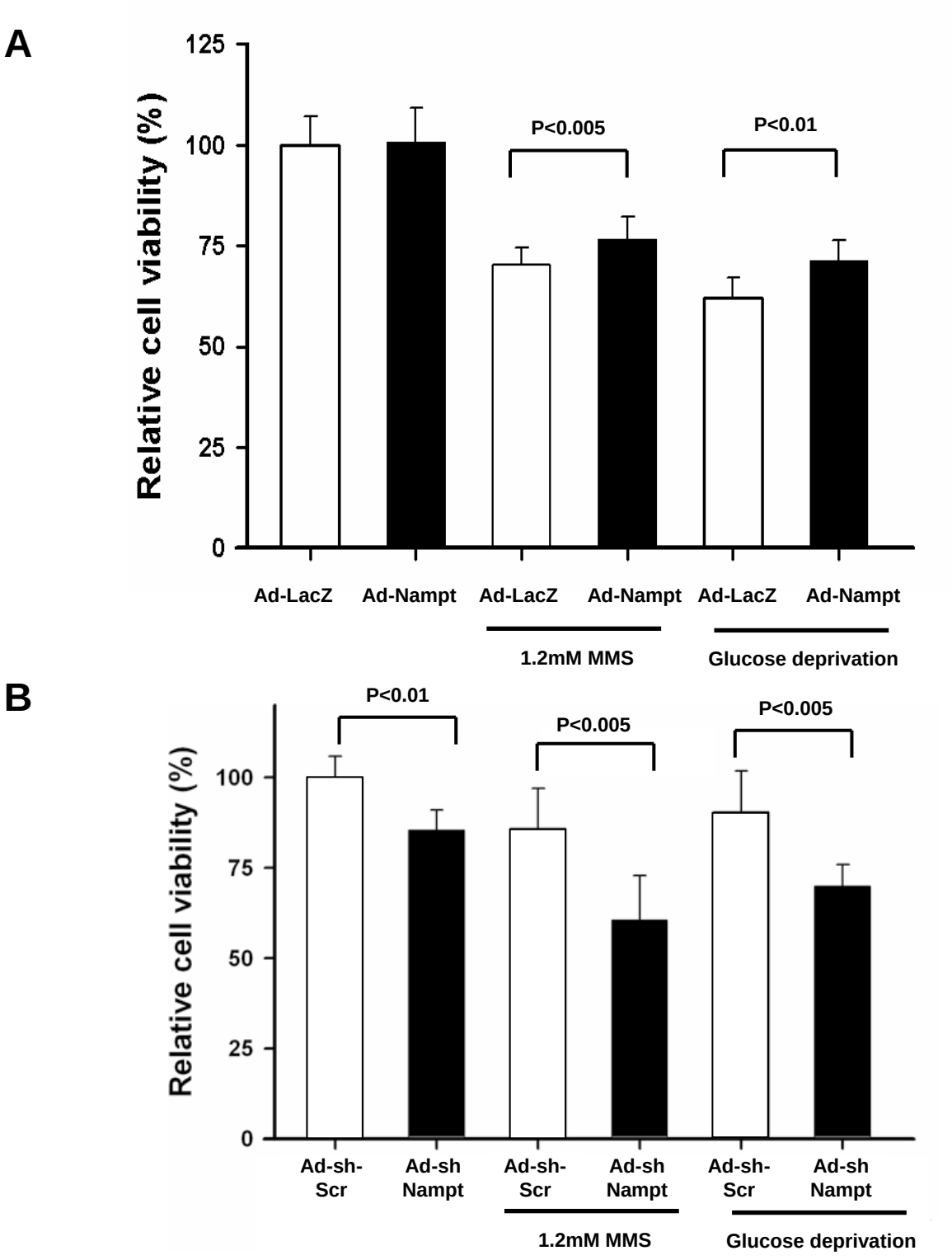

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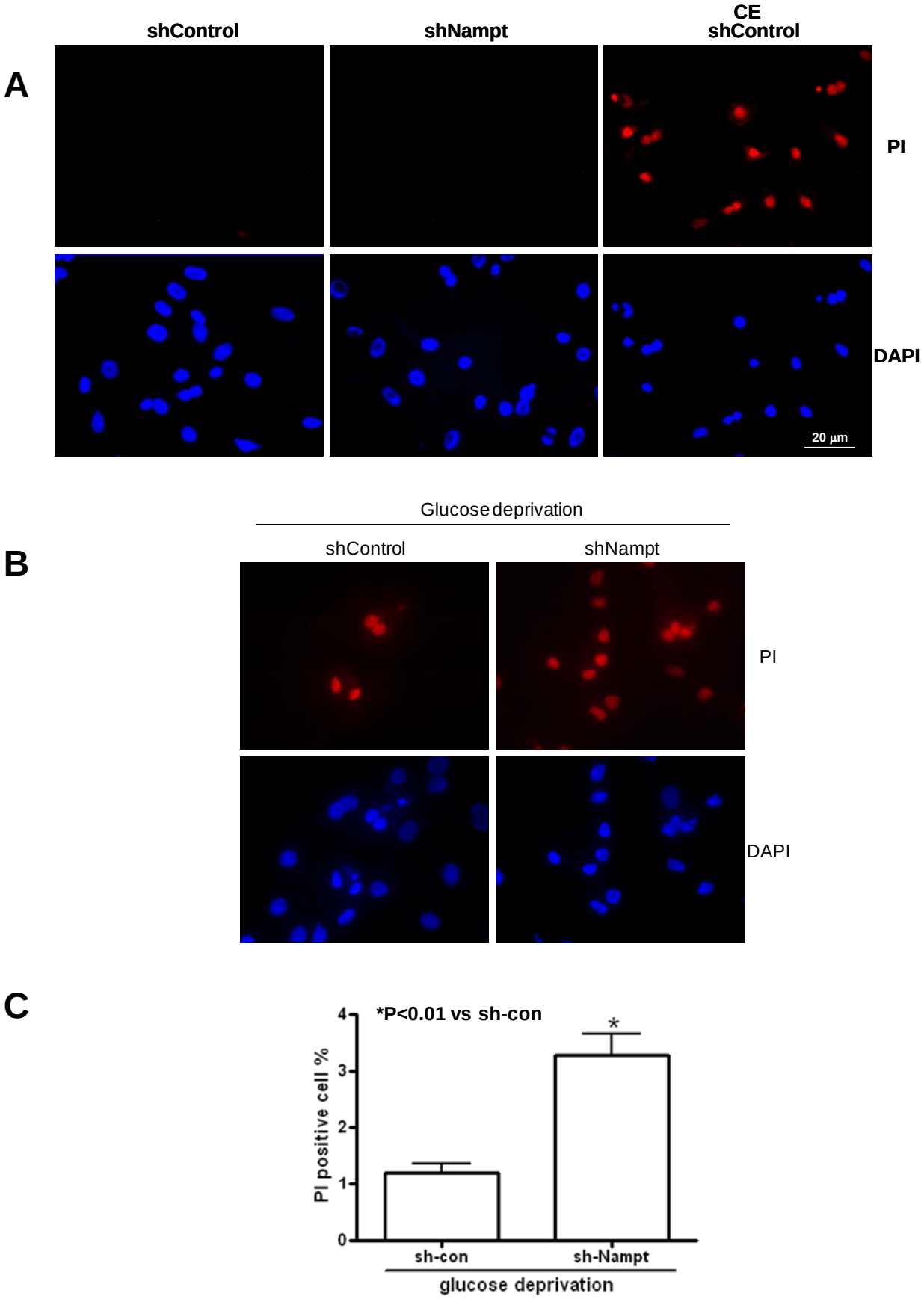

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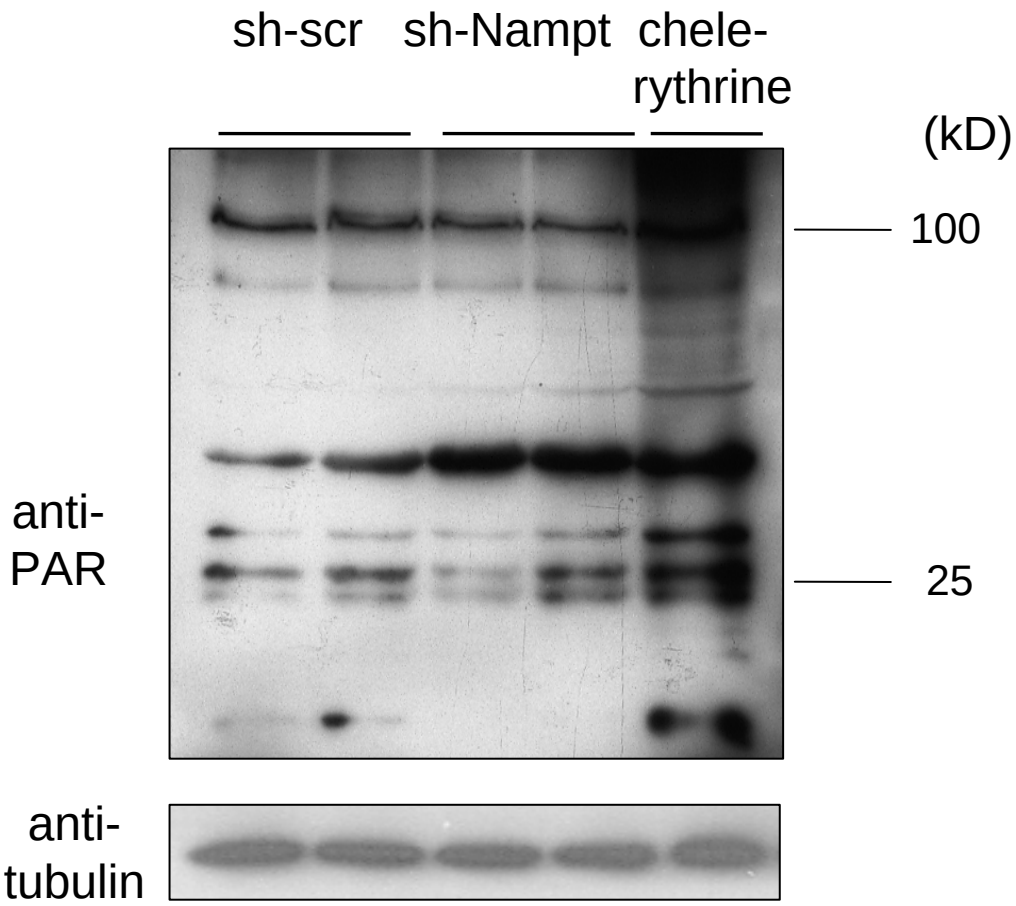

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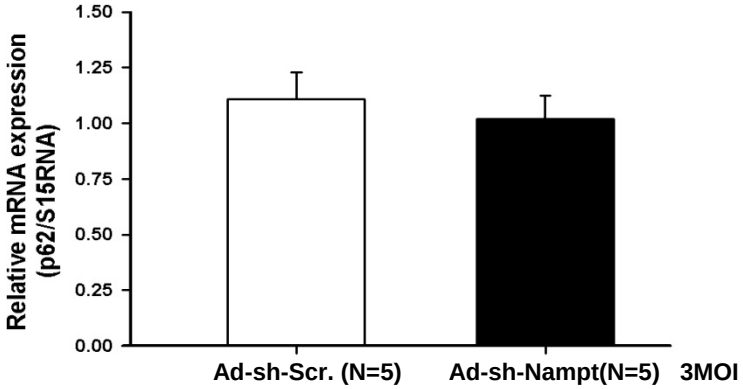
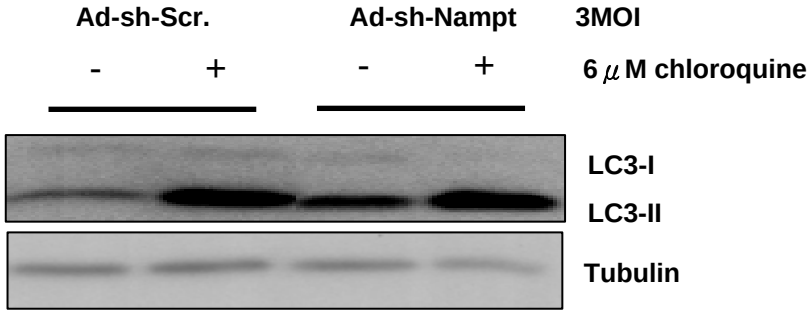
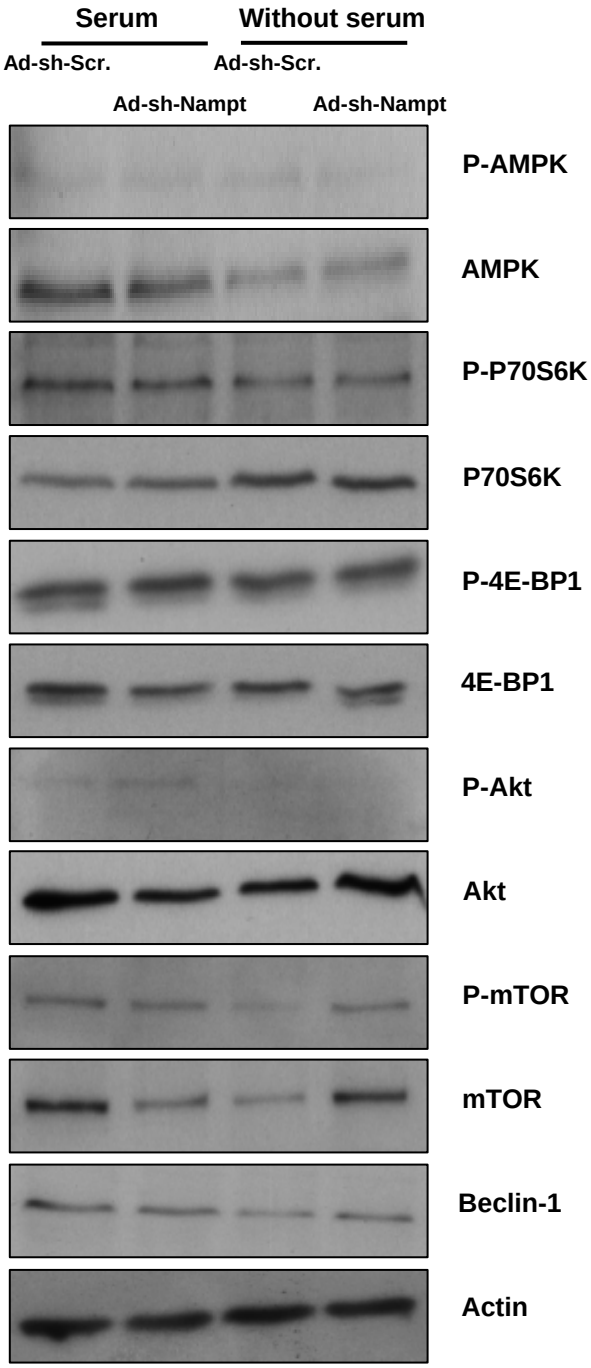
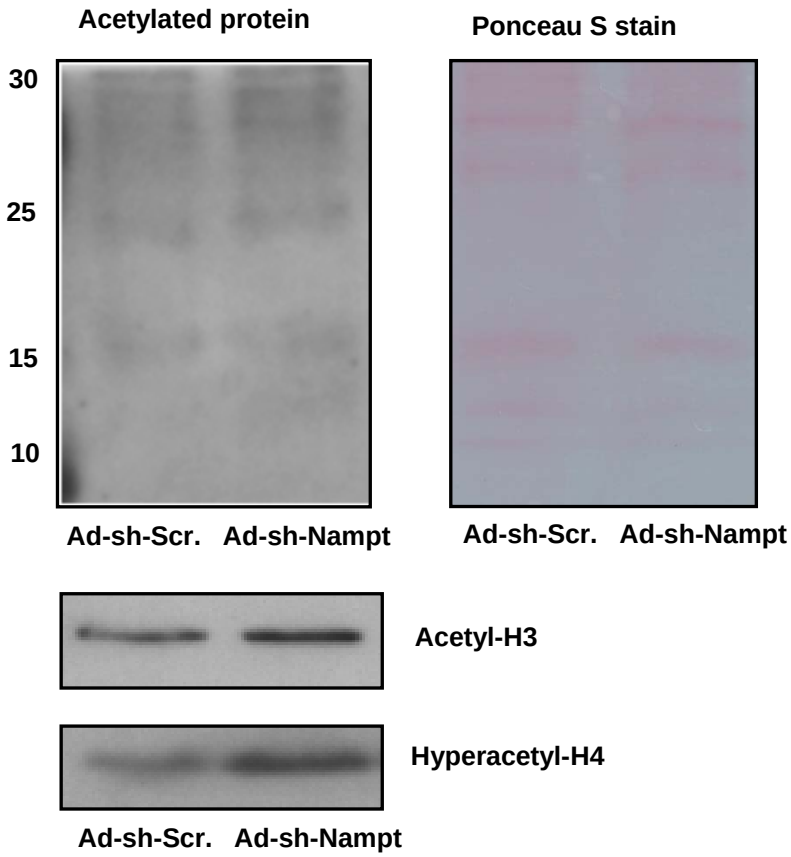




Supplement
Figure S6



Supplemental Figure S7



Supplemental Figure S8