**SUPPLEMENTAL MATERIAL**

## METHODS

### Primary cultures of neonatal rat ventricular cardiomyocytes and reagents

Primary cultures of ventricular cardiomyocytes were prepared from 1-day-old Crl: (WI) BR Wistar rats (Charles River Laboratories), as described [1]. A cardiomyocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium. For glucose deprivation experiments, glucose-free DMEM from GIBCO was used.

### Adenoviruses

Recombinant adenovirus vectors were constructed as described [2]. pBHGloxΔE1,3Cre (Microbix), including the ΔE1 adenoviral genome, was co-transfected with the pDC shuttle vector containing the gene of interest into 293 cells. The method of construction of adenoviruses expressing short hairpin RNA sequences targeting Nox4, Nox2 and PHD4 has been previously described [3-6]. 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). Generation of replication-defective human adenovirus type 5 (devoid of E1) harboring full length cDNA of wild-type Nox4 , Atg7 [7], ER-catalase [8], mito-HyPer [5], mRFP-GFP-LC3 [9], constitutively active IκB (IκB-SA) [5] and beta-galactosidase as a control has been previously described [2]. Adenoviruses expressing wild-type PERK, DSRed-mito and ER-HyPer were generated by subcloning PERK cDNA, DSRed-mito cDNA and ER-HyPer cDNA [8] into pDC316 vector.

### ROS detection

NADPH-dependent $O_2^-$ production was assessed using a lucigenin chemiluminescence assay as previously described [4-6]. Mitochondrial and microsomal fractions from neonatal rat cardiomyocytes and hearts were suspended in 200 µl of an assay buffer composed of 100 mM potassium phosphate (pH 7.0), 10 µM flavin adenine dinucleotide (FAD), 1 mM $NaN_3$, and 1 mM EGTA. After preincubation with 5 µM lucigenin, NADPH was added to a final concentration of 500 µM and the chemiluminescence was continuously monitored using a luminometer.

Cardiomyocyte $O_2^-$ and $H_2O_2$ production were evaluated using dihydroethidium, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester and the Amplex Red kit from Invitrogen (100 µM), as previously described [3, 4, 6]. Incubation time for dihydroethidium and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate was 30 minutes at concentrations of 10 µM and 2.5 µM respectively. Mitochondrial and ER-specific $H_2O_2$ production were evaluated through the expression of compartment-specific HyPer protein, as previously described [5, 10]. Cells were counterstained with DSRed targeted to mitochondria or ER-tracker red from Invitrogen, according to the manufacturer's instructions.

### Subcellular fractionation

Mitochondrial and microsomal fractions were purified through a previously described procedure [5]. Briefly, cardiomyocytes and heart samples were homogenized in a buffer containing 10 mM K-HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.2 mM $Na_3VO_4$ and 1% protease inhibitor. Homogenates were centrifuged at 60 g for 5 minutes to collect unbroken cells, which were then homogenized again. The supernatant and the new homogenate were centrifuged at 1200 g to separate the nuclear fraction from organelles and cytosol (supernatant). The supernatant of the total homogenate was centrifuged at 3500 g for 20 min to separate mitochondrial fractions from cytosolic and microsomal fractions (supernatant). The supernatant was further centrifuged at 100,000 g for 60 minutes to separate microsomal fractions (pelleted in tube) from cytosolic fractions (supernatant). Mitochondrial and microsomal fractions were resuspended in PBS containing protease inhibitors.

### Assessment of cell survival and apoptosis

Viability of the cells was measured by CellTiter Blue (CTB) assays (Promega) according to the supplier's protocol. TUNEL staining for apoptosis was also performed. Cardiomyocytes were fixed in PBS containing 4% paraformaldehyde and staining was performed using the In Situ Cell Death Detection kit (Roche).

**Immunocytofluorescence**

The protocol was previously described [5, 11]. Briefly, neonatal rat cardiomyocytes plated on chamber slides (Lab-Tek) were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100. Cells were then blocked for 1 hour with PBS containing 5% normal goat serum and incubated with primary antibody overnight. Cells were then incubated with Alexa 488- and Alexa 594-conjugated secondary antibodies (Invitrogen) for 3 hours. Nuclei were stained with DAPI. Images were taken with either conventional or confocal microscopy.

***In vivo* procedures**

Cardiac-specific Nox4 conditional knockout mice have been previously described [4, 12]. KO mice (flox/flox plus alpha-MHC-driven CRE transgene) and control mice (flox/flox) were subjected to fasting or to 3 hours of ischemia [7, 9]. For the ischemia procedure, 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 for ventilation. Animal temperature was kept between 36.5 and 37.5°C. The chest was opened by a horizontal incision through the muscle between the ribs (third intercostal space). Ischemia was realized 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). After 3 hours of 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. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised and sliced. Slices were then incubated with a 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 10 min to evaluate infarct size. TTC was shown to be a reliable method to evaluate infarct size in rodents subjected to 3 hours or more of myocardial ischemia without reperfusion [13]. However, we also evaluated the extent of necrosis through Hairpin-2 staining, as previously described [6, 7, 14-17]. Briefly, heart sections were incubated with a mix of 1 μg/ml of Texas red-labeled DNA fragment with blunt ends (generated with PFU polymerase), 50 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA, 15% polyethylene glycol (8,000 mol wt, Sigma) and 250 U/ml DNA T4 ligase (Roche). After 16 hours the sections were thoroughly washed in 70°C water and observed under a fluorescent microscope immediately after counterstaining with 10 μg/ml 4,6-diamidino-2-phenylindole (DAPI).

All experimental procedures with animals were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

**Echocardiography**

Echocardiography was performed after mice were anesthetized with 12 μl/g body weight of 2.5% Avertin, as described previously [18].

**Evaluation of autophagy**

Autophagy was assessed by the evaluation of LC3 isoforms, p62 protein levels and counting of mRFP and GFP dots. mRFP is resistant to lysosomal acids whereas GFP is not. Therefore, autophagosomes detected by both red color (mRFP) and green (GFP) are not yet fused with lysosomes, whereas those detectable only in red are associated with autophagosomes fused with lysosomes (autolysosomes). *In vivo*, LC3II accumulation was evaluated before and after chloroquine administration, which inhibits lysosomal activity. These procedures have been previously described [7, 9].

**Gene expression analysis**

mRNA expression of Nox4 was evaluated with quantitative real time PCR, as described. The following primers were used: Nox4 sense 5'-AGTCAAACAGATGGGATA-3' and antisense 5'-TGTCCCATATGAGTTGTT-3'; GRP78 sense 5'-ACCCATGCAGTTGTGACTGT-3' and antisense 5'-CAGCTGCTGTTGGCTCATTG-3'; GRP94 sense 5'-ACCGAAAAGGACTTGCGACT-3' and antisense 5'-GCTCTCACAAACCCGAAGGT-3'; XBP-1 sense 5'-TTACGAGAGAAAACTCATGGGC-3' and antisense 5'-GCCCAAAAGGATATCAGACTCAG-3'; GAPDH sense 5'-TTCTTGTGCAGTGCCAGCCTCGTC-3' and antisense 5'-TAGGAACACGGAAGGCCATGCCAG-3'.

**Luciferase assay**

CMs were transfected with p5xATF6-GL3 plasmid (Addgene) for evaluation of ATF6 activity, as previously described [19]. Luciferase activity was measured with a luciferase assay system (Promega). The method used for reporter gene assays has been previously described [20].

**Immunoblot analysis, antibodies and reagents**

For specific PERK inhibition, GSK2606414 (Calbiochem) was used, as previously described [21]. For ATF6 inhibition, 4-(2-Aminoethyl)-benzenesulfonylfluoride (AEBSF, Sigma), which is specific at low concentrations, was used as previously shown [22, 23]. For specific IRE-1α inhibition, 4μ8C (Tocris) was used as previously shown [24]. Bafilomycin A1 (50 nM, EMD Millipore) was used as previously described [9]. For immunoblot analyses, heart homogenates and cardiomyocyte lysates were prepared in a RIPA lysis buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 1 mM EDTA, 0.1 mM $Na_3VO_4$, 1 mM NaF, 50 μM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin and 5 μg/ml leupeptin. The primary antibodies used include: Nox4 and p62 (Origene); Nox2 (Abcam); phospho-PERK (Thr980), PERK, phospho-NF-κB (Ser536) , NF-κB, phospho-ERK (Thr202/Tyr204), ERK and Bip (Cell Signaling Technology); phospho-eIF-2α (Ser51, Epitomics); ATF4 (Santacruz); ATF6 (Imgenex); KDEL (Enzo); LC3 (MBL); GAPDH and tubulin (Sigma).

**Measurement of intracellular ATP content**

Heart specimens of equal weights were directly lysed in the somatic-cell ATP-releasing agent, and the ATP content was measured using an ATP Bioluminescent Assay Kit (Sigma), according to the manufacturer's instructions.

**Adenovirus injection**

Adenovirus ($1x10^9$ pfu) was administered by direct injection to the LV free wall (two sites, 25 μl/site), as described. Beta-galactosidase staining of hearts injected with Ad-LacZ has also been previously described [25].

**Statistics**

Data are expressed as (mean ± SEM). The difference in means between 2 groups was evaluated using the t-test. When the analysis compared multiple groups, one-way ANOVA was used. The post hoc comparisons of considered pairs were performed using the Bonferroni post hoc test. Statistical analyses were performed with the use of GraphPad-Prism 5.00 (GraphPad-Software, San Diego, Ca). P values of $<0.05$ were considered statistically significant.

## References


**1.** Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B, Sadoshima J. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circ Res.* 2007;100:914-922.

**2.** Yamamoto S, Yang G, Zablocki D, Liu J, Hong C, Kim SJ, Soler S, Odashima M, Thaisz J, Yehia G, Molina CA, Yatani A, Vatner DE, Vatner SF, Sadoshima J. Activation of Mst1 causes dilated cardiomyopathy by stimulating apoptosis without compensatory ventricular myocyte hypertrophy. *J Clin Invest.* 2003;111:1463-1474.

**3.** Ago T, Kuroda J, Pain J, Fu C, Li H, Sadoshima J. Upregulation of Nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in cardiac myocytes. *Circ Res.* 2010;106:1253-1264.

**4.** Kuroda J, Ago T, Matsushima S, Zhai P, Schneider MD, Sadoshima J. NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart. *Proc Natl Acad Sci U S A.* 2010;107:15565-15570.

**5.** Matsushima S, Kuroda J, Ago T, Zhai P, Park JY, Xie LH, Tian B, Sadoshima J. Increased oxidative stress in the nucleus caused by Nox4 mediates oxidation of HDAC4 and cardiac hypertrophy. *Circ Res.* 2013;112:651-663.

**6.** Matsushima S, Kuroda J, Ago T, Zhai P, Ikeda Y, Oka S, Fong GH, Tian R, Sadoshima J. Broad Suppression of NADPH Oxidase Activity Exacerbates Ischemia/Reperfusion Injury Through Inadvertent Downregulation of Hypoxia-inducible Factor-1alpha and Upregulation of Peroxisome Proliferator-activated Receptor-alpha. *Circ Res.* 2013;112:1135-1149.

**7.** Sciarretta S, Zhai P, Shao D, Maejima Y, Robbins J, Volpe M, Condorelli G, Sadoshima J. Rheb is a Critical Regulator of Autophagy During Myocardial Ischemia: Pathophysiological Implications in Obesity and Metabolic Syndrome. *Circulation.* 2012;125:1134-1146.

**8.** Wu RF, Ma Z, Liu Z, Terada LS. Nox4-derived H2O2 mediates endoplasmic reticulum signaling through local Ras activation. *Mol Cell Biol.* 2010;30:3553-3568.

**9.** Hariharan N, Maejima Y, Nakae J, Paik J, Depinho RA, Sadoshima J. Deacetylation of FoxO by Sirt1 Plays an Essential Role in Mediating Starvation-Induced Autophagy in Cardiac Myocytes. *Circ Res.* 2010;107:1470-1482.

**10.** Belousov VV, Fradkov AF, Lukyanov KA, Staroverov DB, Shakhbazov KS, Terskikh AV, Lukyanov S. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat Methods.* 2006;3:281-286.

**11.** Usui S, Maejima Y, Pain J, Hong C, Cho J, Park JY, Zablocki D, Tian B, Glass DJ, Sadoshima J. Endogenous muscle atrophy F-box mediates pressure overload-induced cardiac hypertrophy through regulation of nuclear factor-kappaB. *Circ Res.* 2011;109:161-171.

**12.** Halkos ME, Zhao ZQ, Kerendi F, Wang NP, Jiang R, Schmarkey LS, Martin BJ, Quyyumi AA, Few WL, Kin H, Guyton RA, Vinten-Johansen J. Intravenous infusion of mesenchymal stem cells enhances regional perfusion and improves ventricular function in a porcine model of myocardial infarction. *Basic Res Cardiol.* 2008;103:525-536.

**13.** Vivaldi MT, Kloner RA, Schoen FJ. Triphenyltetrazolium staining of irreversible ischemic injury following coronary artery occlusion in rats. *Am J Pathol.* 1985;121:522-530.

**14.** Zhai P, Sciarretta S, Galeotti J, Volpe M, Sadoshima J. Differential Roles of GSK-3{beta} During Myocardial Ischemia and Ischemia/Reperfusion. *Circ Res.* 2011;109:502-511.

**15.** Didenko VV, Hornsby PJ. Presence of double-strand breaks with single-base 3' overhangs in cells undergoing apoptosis but not necrosis. *J Cell Biol.* 1996;135:1369-1376.

**16.** Guerra S, Leri A, Wang X, Finato N, Di Loreto C, Beltrami CA, Kajstura J, Anversa P. Myocyte death in the failing human heart is gender dependent. *Circ Res.* 1999;85:856-866.

**17.** Leri A, Claudio PP, Li Q, Wang X, Reiss K, Wang S, Malhotra A, Kajstura J, Anversa P. Stretch-mediated release of angiotensin II induces myocyte apoptosis by activating p53 that enhances the

local renin-angiotensin system and decreases the Bcl-2-to-Bax protein ratio in the cell. *J Clin Invest.* 1998;101:1326-1342.

**18.** Matsui Y, Nakano N, Shao D, Gao S, Luo W, Hong C, Zhai P, Holle E, Yu X, Yabuta N, Tao W, Wagner T, Nojima H, Sadoshima J. Lats2 is a negative regulator of myocyte size in the heart. *Circ Res.* 2008;103:1309-1318.

**19.** Wang Y, Shen J, Arenzana N, Tirasophon W, Kaufman RJ, Prywes R. Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. *J Biol Chem.* 2000;275:27013-27020.

**20.** Oka S, Alcendor R, Zhai P, Park JY, Shao D, Cho J, Yamamoto T, Tian B, Sadoshima J. PPARalpha-Sirt1 complex mediates cardiac hypertrophy and failure through suppression of the ERR transcriptional pathway. *Cell Metab.* 2011;14:598-611.

**21.** Harding HP, Zyryanova AF, Ron D. Uncoupling proteostasis and development in vitro with a small molecule inhibitor of the pancreatic endoplasmic reticulum kinase, PERK. *J Biol Chem.* 2012;287:44338-44344.

**22.** Okada T, Haze K, Nadanaka S, Yoshida H, Seidah NG, Hirano Y, Sato R, Negishi M, Mori K. A serine protease inhibitor prevents endoplasmic reticulum stress-induced cleavage but not transport of the membrane-bound transcription factor ATF6. *J Biol Chem.* 2003;278:31024-31032.

**23.** Toko H, Takahashi H, Kayama Y, Okada S, Minamino T, Terasaki F, Kitaura Y, Komuro I. ATF6 is important under both pathological and physiological states in the heart. *J Mol Cell Cardiol.* 2010;49:113-120.

**24.** Cross BC, Bond PJ, Sadowski PG, Jha BK, Zak J, Goodman JM, Silverman RH, Neubert TA, Baxendale IR, Ron D, Harding HP. The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. *Proc Natl Acad Sci U S A.* 2012;109:E869-878.

**25.** Matsuda T, Zhai P, Maejima Y, Hong C, Gao S, Tian B, Goto K, Takagi H, Tamamori-Adachi M, Kitajima S, Sadoshima J. Distinct roles of GSK-3alpha and GSK-3beta phosphorylation in the heart under pressure overload. *Proc Natl Acad Sci U S A.* 2008;105:20900-20905.

**Supplemental Figure Legends**

**Online Figure I. A.** Cardiomyocytes were cultured with regular medium or in glucose-free conditions for the shown periods of time. Nox4 mRNA levels were measured. N=5. **B-D.** Cardiomyocytes were transduced with Ad-LacZ or Ad-IκB-SA for 48 hours and then subjected to GD. Nox4, phospho-NF-κB and NF-κB levels were evaluated. A representative blot (B) and densitometry analyses (C-D) are presented. **E.** Cardiomyocytes were cultured with regular medium or in glucose-free conditions for the shown periods of time. Amplex Red fluorescence was measured. N=7-8. Data are expressed as percentage of CT. * $p<0.05$ vs. CT.

**Online Figure II. A.** CMs were cultured with normal or glucose-free medium for 4 hours. The mitochondrial fraction was isolated and NADPH-dependent $O2^-$ production was evaluated by lucigenin assay. N=3. **B-C.** Cardiomyocytes were transduced with adenovirus expressing HyPer protein targeted to mitochondria (Ad-Mito-HyPer) and DSRed protein targeted to mitochondria (DSRed-Mito) for 48 hours. Cardiomyocytes were cultured with normal or glucose-free medium for 4 hours. Representative images of Mito-HyPer and DSRed-Mito fluorescence are shown (B) together with quantification (C). Bar=10 μm. N=3. **D.** Cardiomyocytes were cultured with normal or glucose-free medium for 4 hours. Immunocytofluorescence assay of Nox4 and Calreticulin was performed and cells were observed under a conventional microscope. Bar=50 μm.

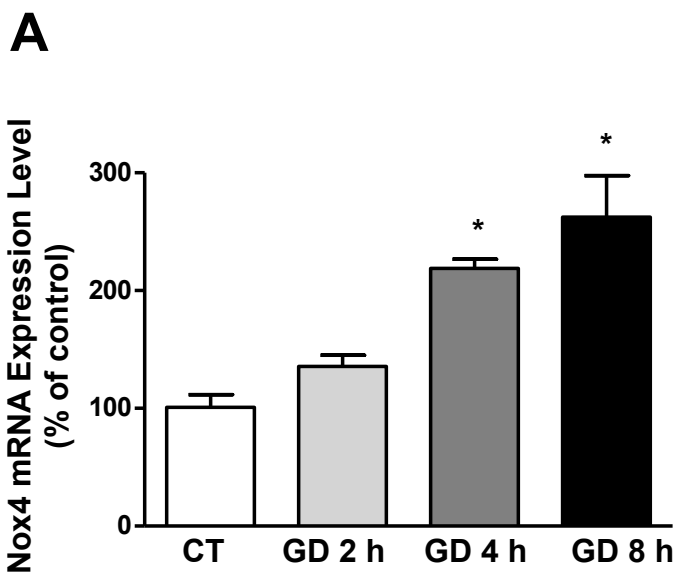
**Online Figure III. A.** Cardiomyocytes with or without Nox4 knock-down were subjected to 4 hours of GD with or without Bafilomycin A1 for the last 2 hours. LC3 levels were evaluated. **B.** Cardiomyocytes were transduced with Ad-LacZ or Ad-Nox4 for 48 hours. Nox4 protein levels were quantified. **C-D.** Cardiomyocytes were transduced with Ad-LacZ or Ad-Nox4, together with Ad-mRFP-GFP-LC3 for 48 hours. Representative images of mRFP and GFP dots are shown (C), together with quantification of autophagosomes and autolysosomes (D). N=3. Bar=10 μm.

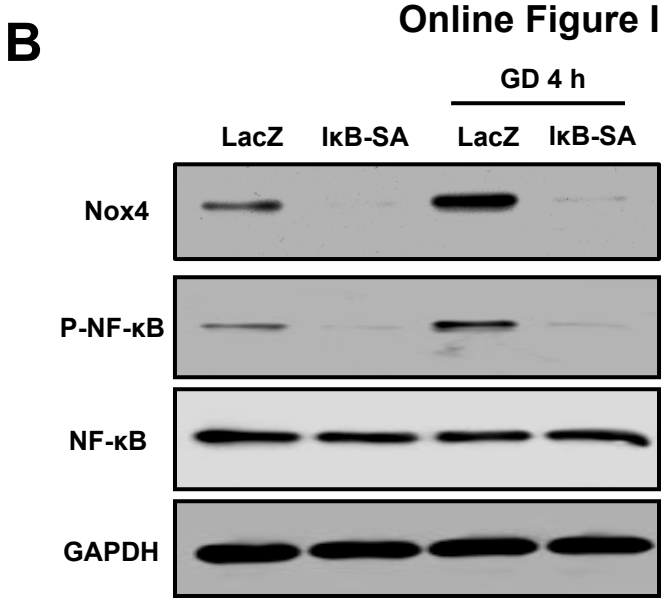
**Online Figure IV. A-B.** Cardiomyocytes were transduced with Ad-LacZ or Ad-ER-Catalase, together with Ad-ER-HyPer for 48 hours, and then cultured with glucose-free medium for 4 hours. CMs were stained with ER-tracker red. Representative images of ER-HyPer and ER-tracker fluorescence are shown (A) together with quantification (B). Bar=50 μm. **C.** Cardiomyocytes were transduced with Ad-LacZ or Ad-PRX-3 for 48 hours, and then cultured with normal or glucose-free medium for 4 hours. PRX-3 and LC3 levels were evaluated. **D.** Cardiomyocytes were transduced with Sh-CT or Sh-PHD4. PHD4 level was evaluated. **E.** Phospho-ERK and ERK levels were evaluated in cardiomyocytes with or without Nox4 deletion cultured with either regular medium or glucose-free medium. **F-K.** Cardiomyocytes with or without Nox4 knockdown were subjected to GD for 4 hours. As a positive control cardiomyocytes were treated with thapsigargin (TG, 1 μM) for 4 hours. ATF6 expression levels (full length and active) were evaluated (F-G). ATF6 reporter luciferase activity was evaluated (H). N=5-6. mRNA levels of GRP78 and GRP94 (downstream targets of ATF6) were measured in order to further assess ATF6 activity (I). N=4. Abundance of unspliced and spliced forms of XBP-1 was evaluated (J-K). N=3. **L.** Cardiomyocytes with or without Nox4 knockdown were subjected to GD for 24 hours. ATF4, ATF6 and XBP-1 isoform levels were evaluated. **M.** Cardiomyocytes were treated with thapsigargin for 4 hours with or without PERK inhibitor (GSK2606414, 1 μM), ATF6 inhibitor (AEBSF, 100 μM) or IRE-1α inhibitor (4μ8C, 32 μM). Phospho-EIF2α, EIF2α, active ATF6 and XBP-1 isoforms were evaluated. **N.** Cardiomyocytes were subjected to 4 hours of GD with or without PERK, ATF6 or IRE-1α inhibitor. P62 and LC3 levels were evaluated. **O-P.** Cardiomyocytes were transduced with Ad-LacZ or Ad-Nox4, with or without Ad-ER-Catalase or Ad-PRX-3 for 48 hours. Phospho-PERK (T980) and LC3 expression levels were evaluated.

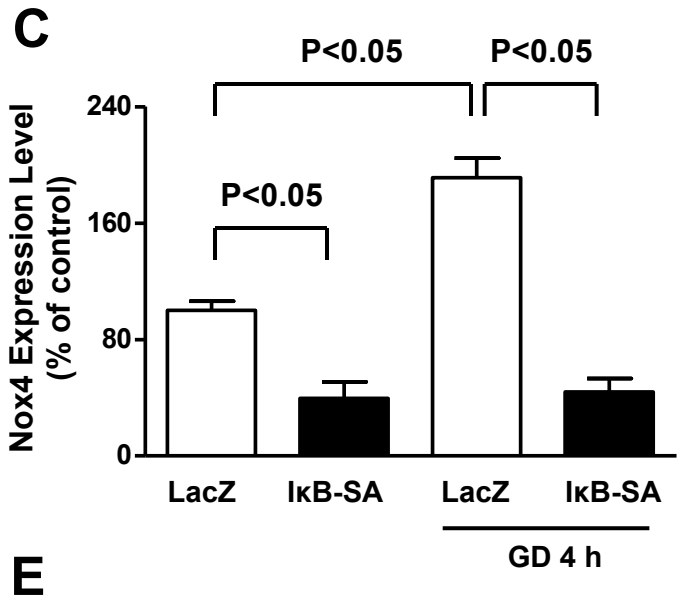
**Online Figure V. A-B.** Cardiomyocytes were transduced with Sh-Nox4 for 96 hours, and with Ad-LacZ or Ad-Atg7, together with Ad-mRFP-GFP-LC3 for the last 48 hours. Representative images of mRFP and GFP dots are shown (A), together with quantification of autophagosomes and autolysosomes (B). N=3. Bar=10 μm

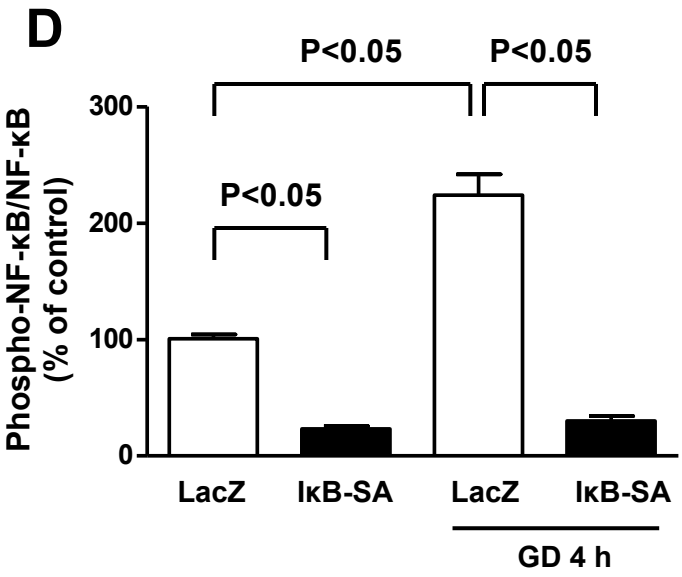
**Online Figure VI. A.** Control mice were subjected to 48 hours of fasting. Cardiac mitochondria were isolated and NADPH-dependent $O_2^-$ production was assessed by lucigenin assay. N=3. **B.** Nox4 levels were evaluated in the mitochondria and microsomes from mouse hearts at baseline and after fasting. **C-E.** Control and Nox4 cKO mice were subjected to 3 hours of ischemia. Nox4 and p62 protein levels were evaluated. Representative immunoblot (C) and densitometric analyses (D-E) are shown. N=4. **F.** Control and Nox4 cKO mice received chloroquine (10 mg/kg i.p) or vehicle 4 hours before they were sacrificed. Representative immunoblot of cardiac LC3 is shown.

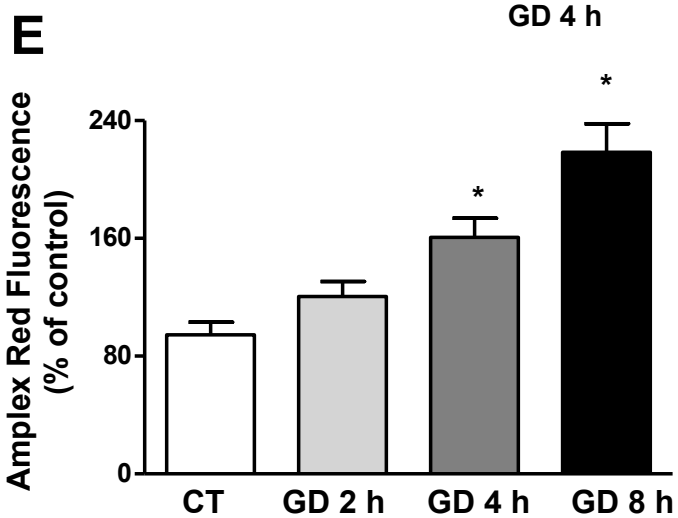
**Online Figure VII. A-B.** Hemodynamic analysis was performed in control and Nox4 cKO mice at baseline and after fasting. dP/dT was measured. **C-D.** Control and Nox4 cKO mice received cardiac injection of Ad-LacZ or Ad-Atg7. Beta-galactosidase staining (C) and LC3 protein levels (D) were evaluated. **E.** Control and Nox4 cKO mice received cardiac injection of Ad-LacZ or Ad-Atg7 and were then subjected to 48 hours of fasting. Myocardial ATP content was evaluated. N=4-7. **F-G.** Control and Nox4 cKO mice were subjected to prolonged ischemia (3 hours). Hairpin-2 staining was performed on myocardial sections. Quantification of the percentage of Hairpin-2-positive nuclei is presented (F) together with representative pictures of ischemic and non-ischemic areas in the two groups (G). N=4. **H.** Representative schema of the hypothesis and findings of the study. In energy-depleted cardiomyocytes, Nox4 is activated in the ER, thereby promoting autophagy and survival.

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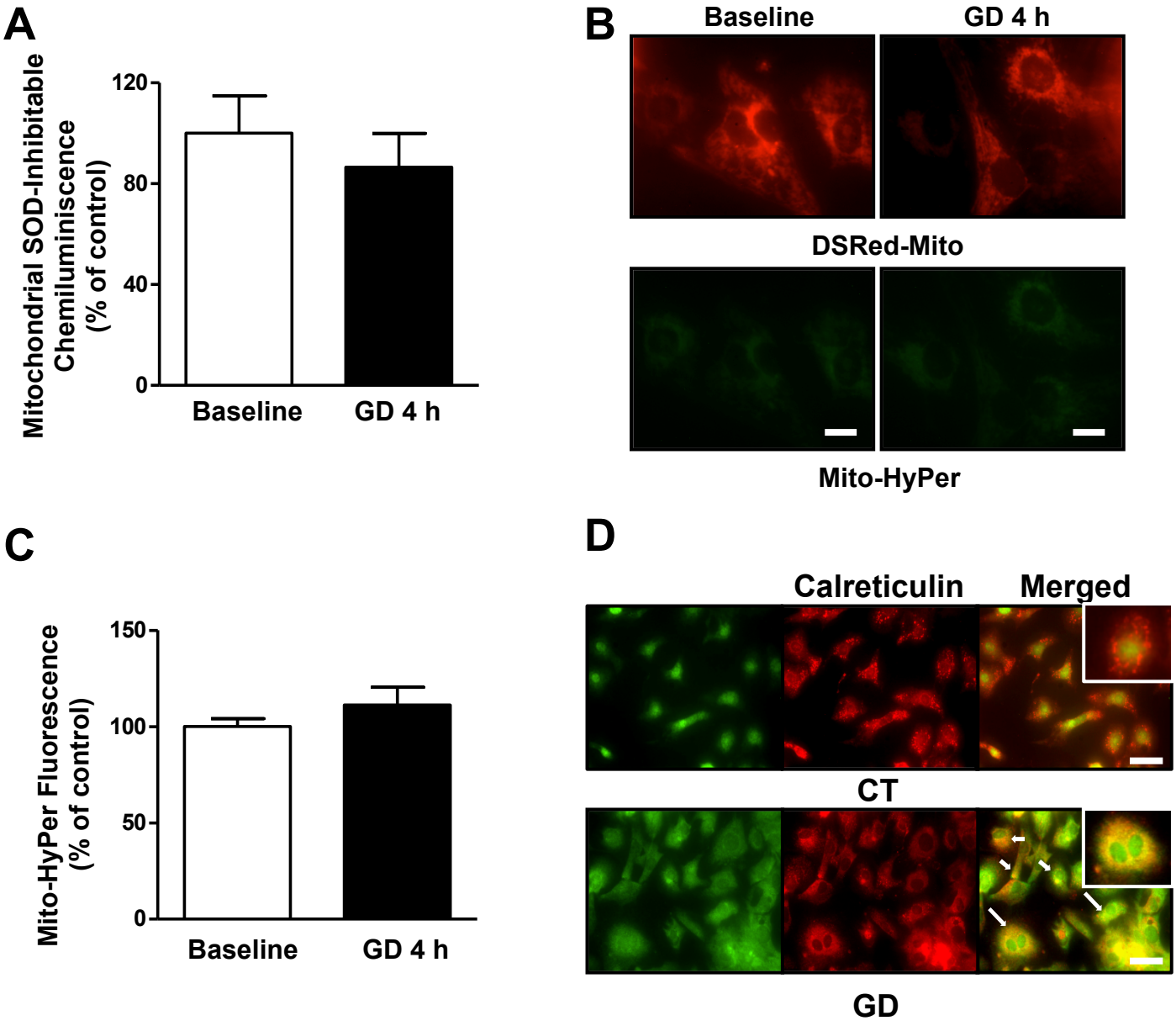
**Online Figure II**

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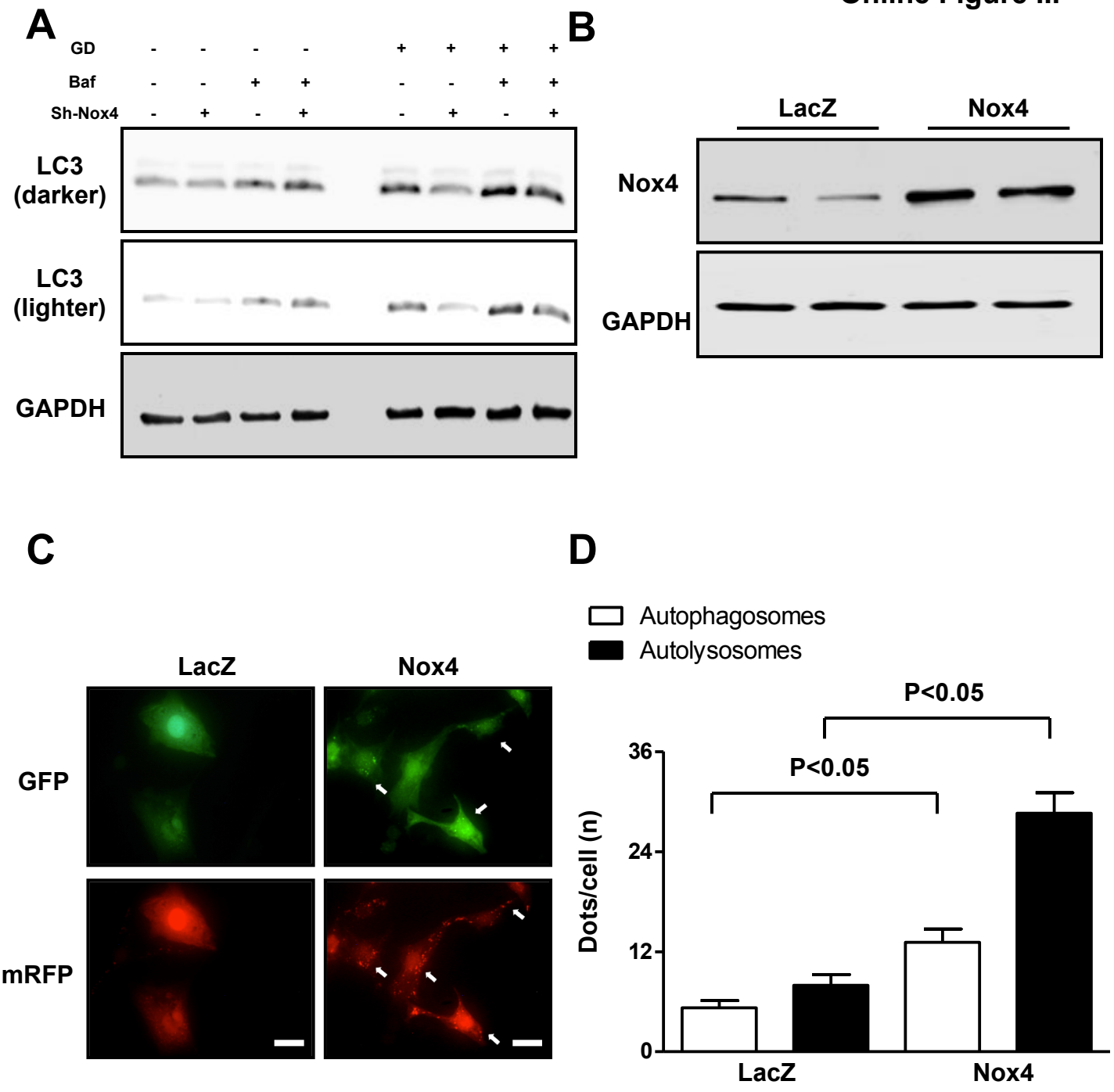
Online Figure III

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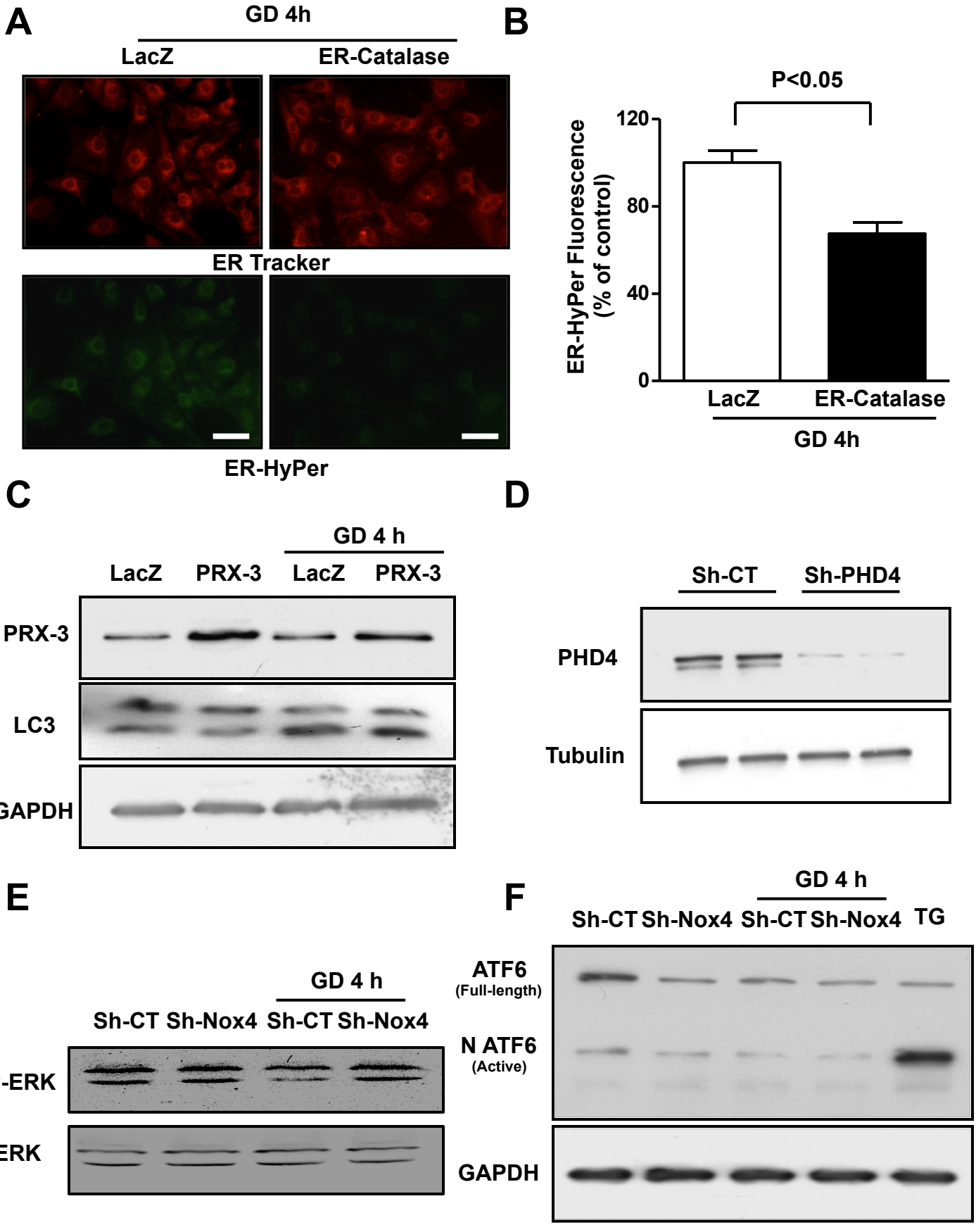
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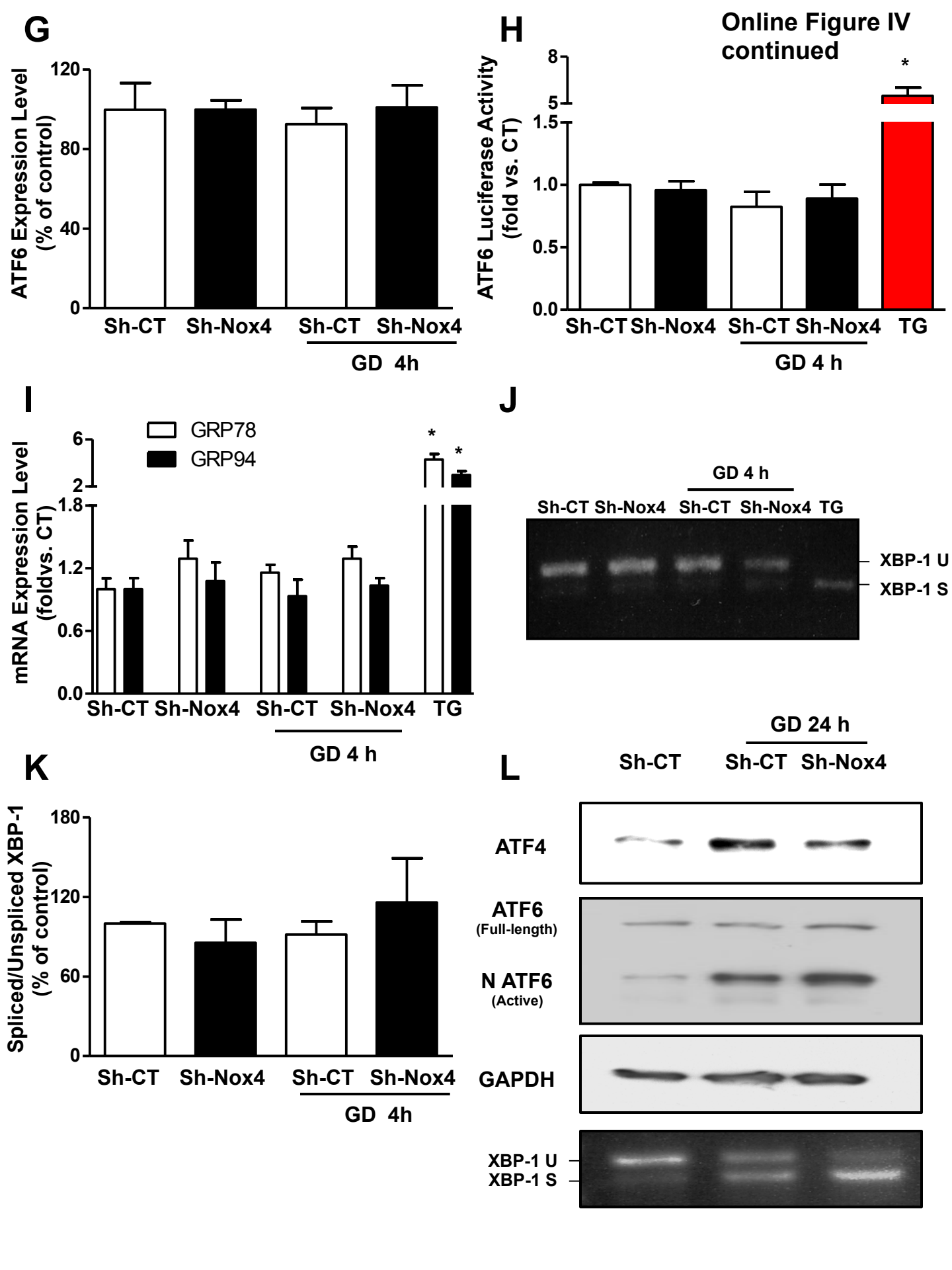
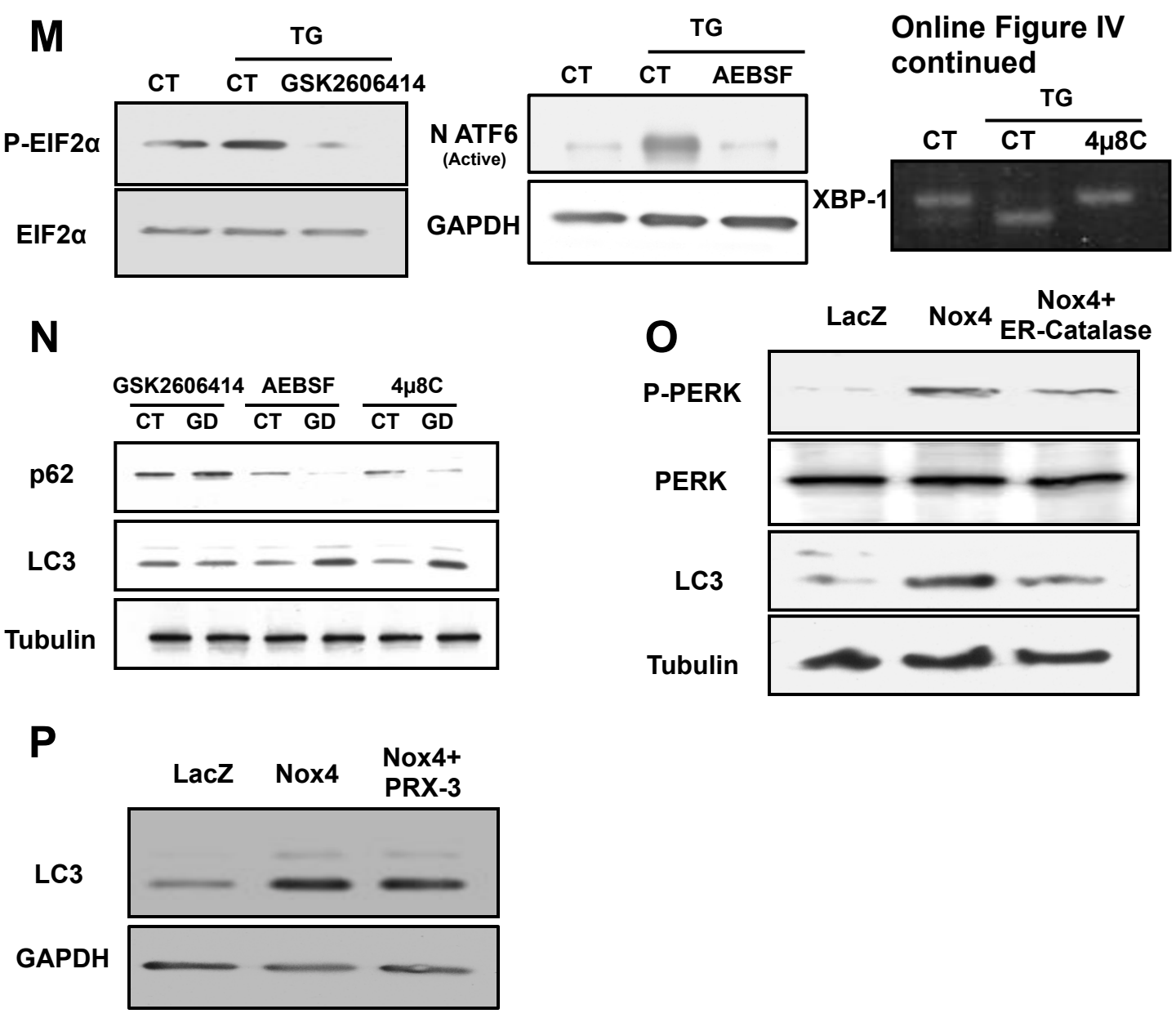
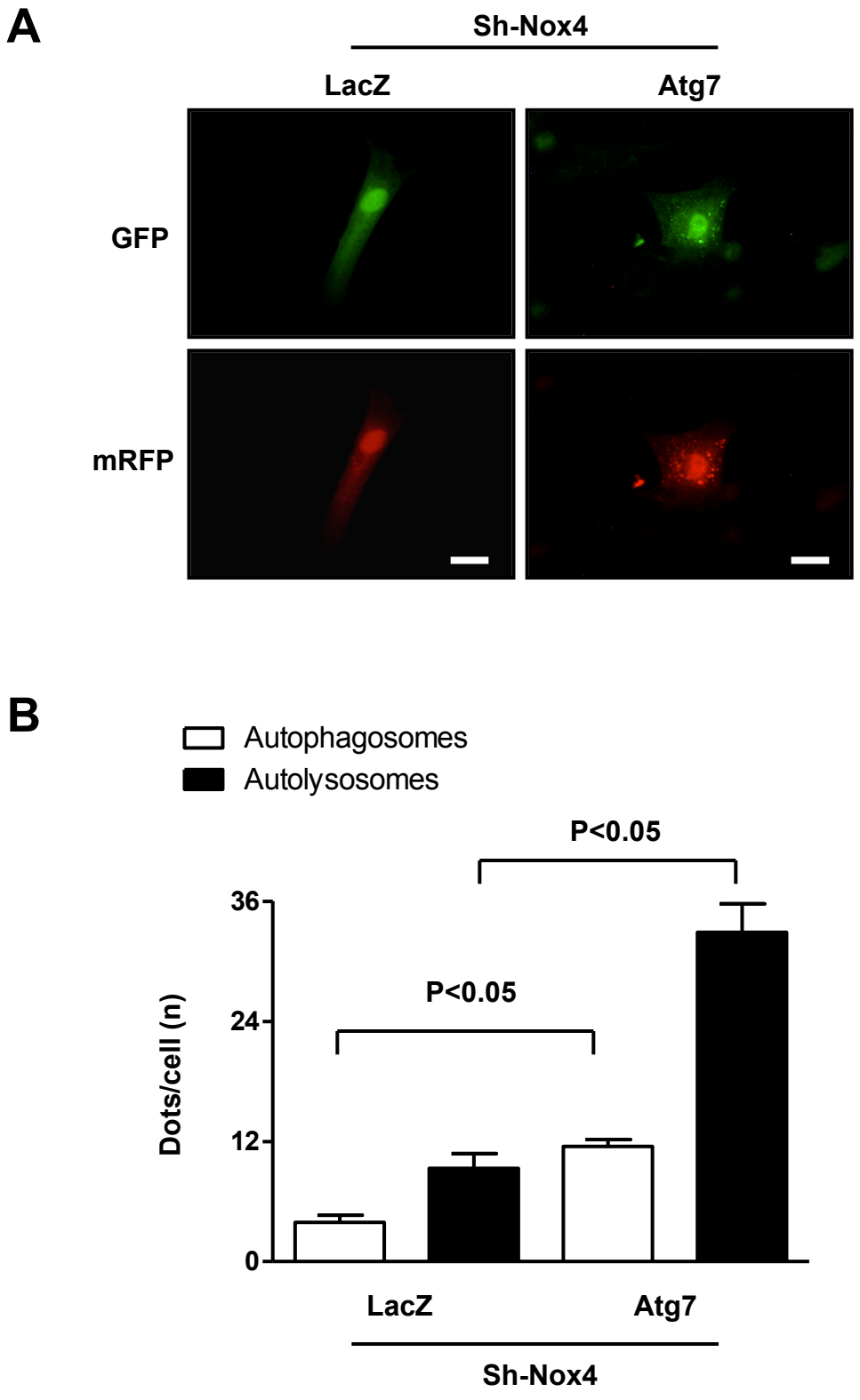
GD 4h
LacZ
ER-Catalase
ER Tracker
ER-HyPer
B
P<0.05
ER-HyPer Fluorescence (% of control)
120
80
40
0
LacZ
ER-Catalase
GD 4h
C
GD 4 h
LacZ
PRX-3
LacZ
PRX-3
PRX-3
LC3
GAPDH
D
Sh-CT
Sh-PHD4
PHD4
Tubulin
E
GD 4 h
Sh-CT
Sh-Nox4
Sh-CT
Sh-Nox4
P-ERK
ERK
F
GD 4 h
Sh-CT
Sh-Nox4
Sh-CT
Sh-Nox4
TG
ATF6
(Full-length)
N ATF6
(Active)
GAPDH


G
ATF6 Expression Level
(% of control)
120
80
40
0
Sh-CT
Sh-Nox4
Sh-CT
Sh-Nox4
GD 4h
H
Online Figure IV
continued
ATF6 Luciferase Activity
(fold vs. CT)
8
5
1.5
1.0
0.5
0.0
*
Sh-CT
Sh-Nox4
Sh-CT
Sh-Nox4
TG
GD 4 h
I
GRP78
GRP94
mRNA Expression Level
(foldvs. CT)
6
2
1.8
1.2
0.6
0.0
*
*
Sh-CT
Sh-Nox4
Sh-CT
Sh-Nox4
TG
GD 4 h
J
GD 4 h
Sh-CT
Sh-Nox4
Sh-CT
Sh-Nox4
TG
XBP-1 U
XBP-1 S
K
Spliced/Unspliced XBP-1
(% of control)
180
120
60
0
Sh-CT
Sh-Nox4
Sh-CT
Sh-Nox4
GD 4h
L
GD 24 h
Sh-CT
Sh-CT
Sh-Nox4
ATF4
ATF6
(Full-length)
N ATF6
(Active)
GAPDH
XBP-1 U
XBP-1 S

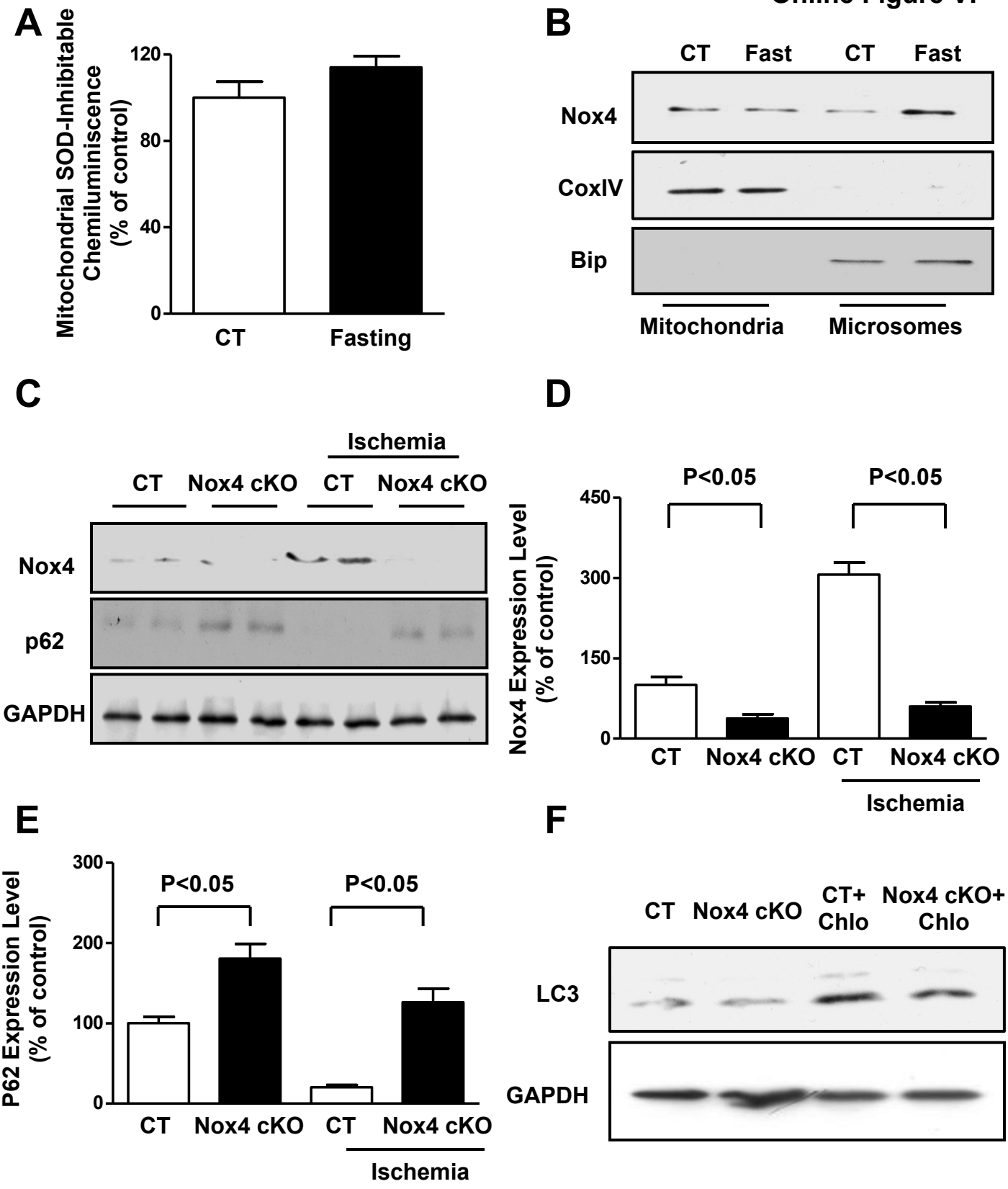



**Online Figure IV. A-B.** Cardiomyocytes were transduced with Ad-LacZ or Ad-ER-Catalase, together with Ad-ER-HyPer for 48 hours, and then cultured with glucose-free medium for 4 hours. CMs were stained with ER-tracker red. Representative images of ER-HyPer and ER-tracker fluorescence are shown (A) together with quantification (B). Bar=50 μm. **C.** Cardiomyocytes were transduced with Ad-LacZ or Ad-PRX-3 for 48 hours, and then cultured with normal or glucose-free medium for 4 hours. PRX-3 and LC3 levels were evaluated. **D.** Cardiomyocytes were transduced with Sh-CT or Sh-PHD4. PHD4 level was evaluated. **E.** Phospho-ERK and ERK levels were evaluated in cardiomyocytes with or without Nox4 deletion cultured with either regular medium or glucose-free medium. **F-K.** Cardiomyocytes with or without Nox4 knockdown were subjected to GD for 4 hours. As a positive control cardiomyocytes were treated with thapsigargin (TG, 1 μM) for 4 hours. ATF6 expression levels (full length and active) were evaluated (F-G). ATF6 reporter luciferase activity was evaluated (H). N=5-6. mRNA levels of GRP78 and GRP94 (downstream targets of ATF6) were measured in order to further assess ATF6 activity (I). N=4. Abundance of unspliced and spliced forms of XBP-1 was evaluated (J-K). N=3. **L.** Cardiomyocytes with or without Nox4 knockdown were subjected to GD for 24 hours. ATF4, ATF6 and XBP-1 isoform levels were evaluated. **M.** Cardiomyocytes were treated with thapsigargin for 4 hours with or without PERK inhibitor (GSK2606414, 1 μM), ATF6 inhibitor (AEBSF, 100 μM) or IRE-1α inhibitor

(4μ8C, 32 μM). Phospho-EIF2α, EIF2α, active ATF6 and XBP-1 isoforms were evaluated. **N.** Cardiomyocytes were subjected to 4 hours of GD with or without PERK, ATF6 or IRE-1α inhibitor. P62 and LC3 levels were evaluated. **O-P.** Cardiomyocytes were transduced with Ad-LacZ or Ad-Nox4, with or without Ad-ER-Catalase or Ad-PRX-3 for 48 hours. Phospho-PERK (T980) and LC3 expression levels were evaluated.

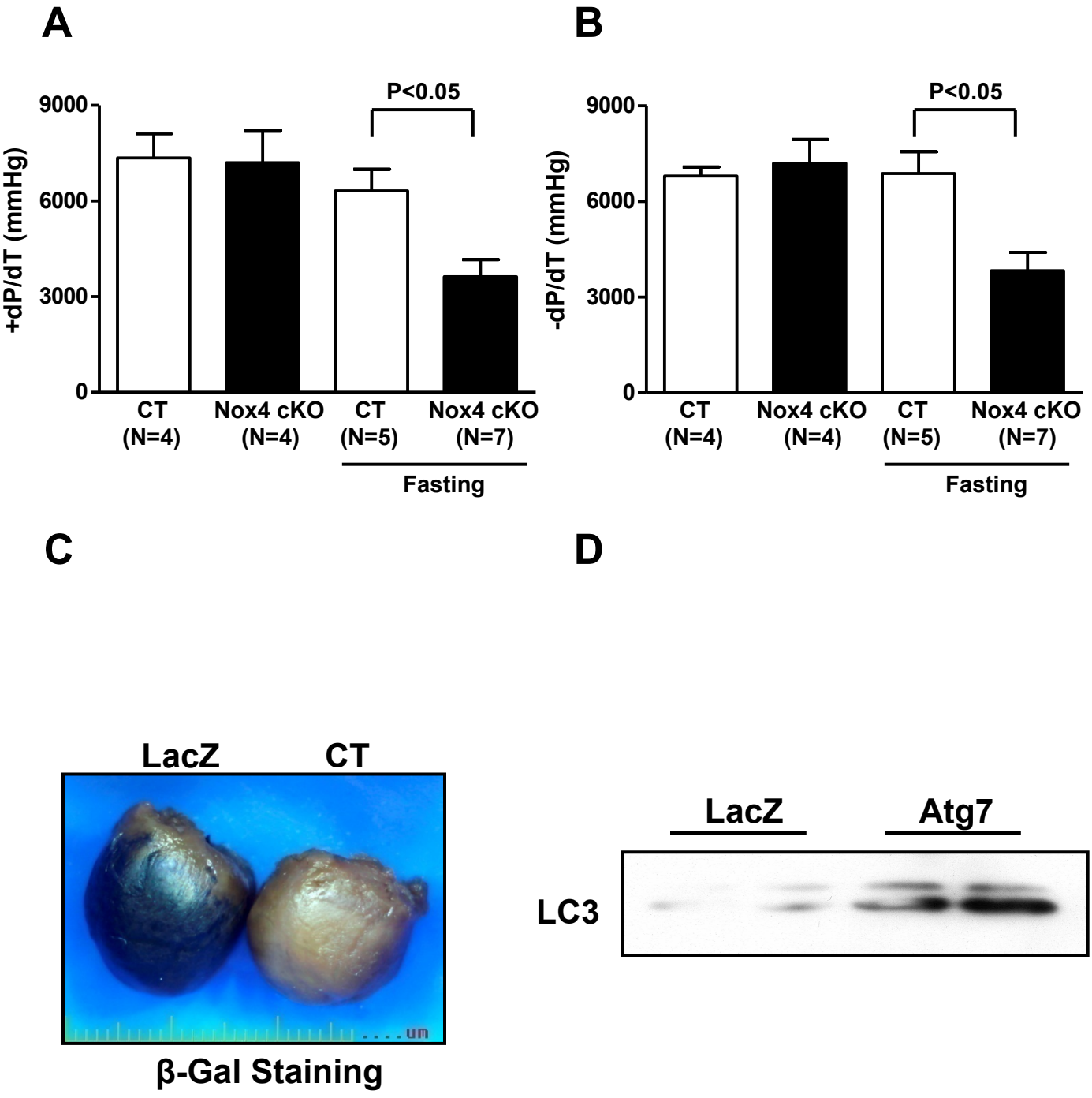


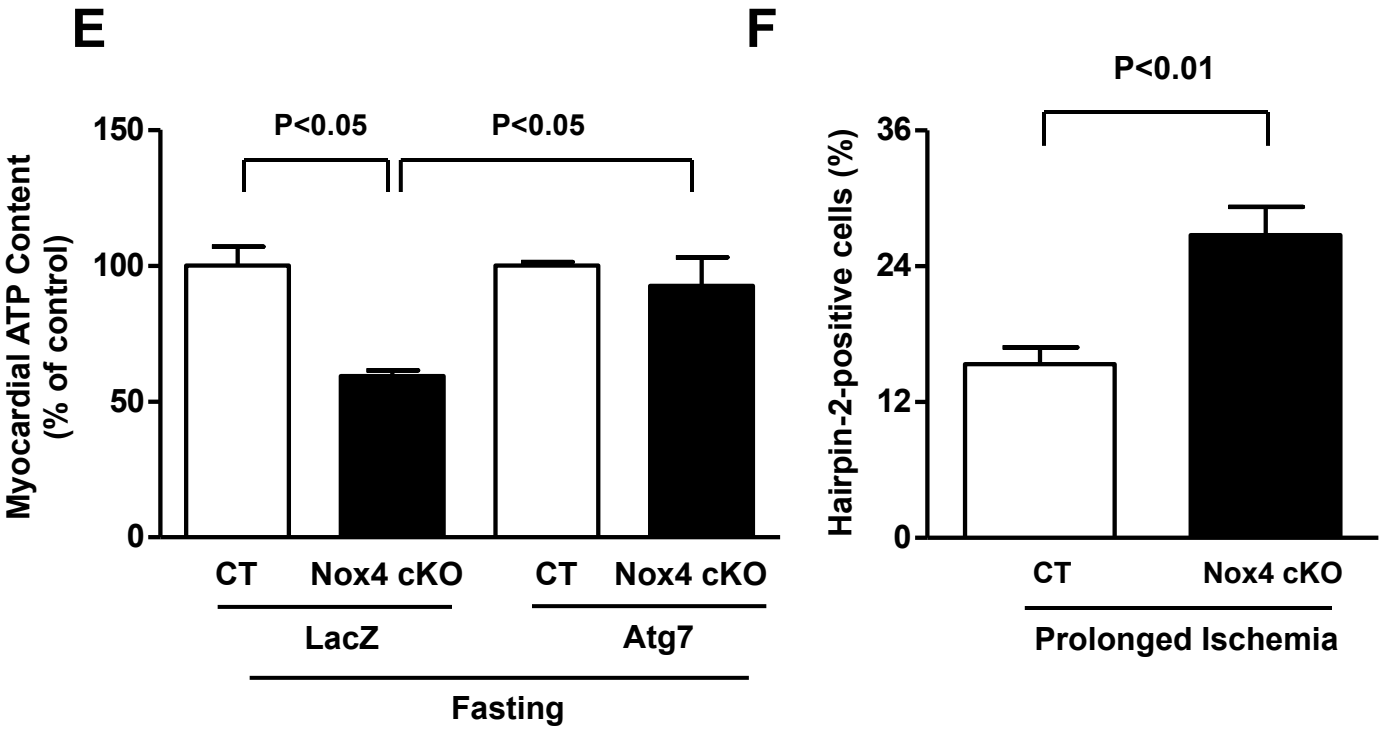

**Online Figure V. A-B.** Cardiomyocytes were transduced with Sh-Nox4 for 96 hours, and with Ad-LacZ or Ad-Atg7, together with Ad-mRFP-GFP-LC3 for the last 48 hours. Representative images of mRFP and GFP dots are shown (A), together with quantification of autophagosomes and autolysosomes (B). N=3. Bar=10 μm

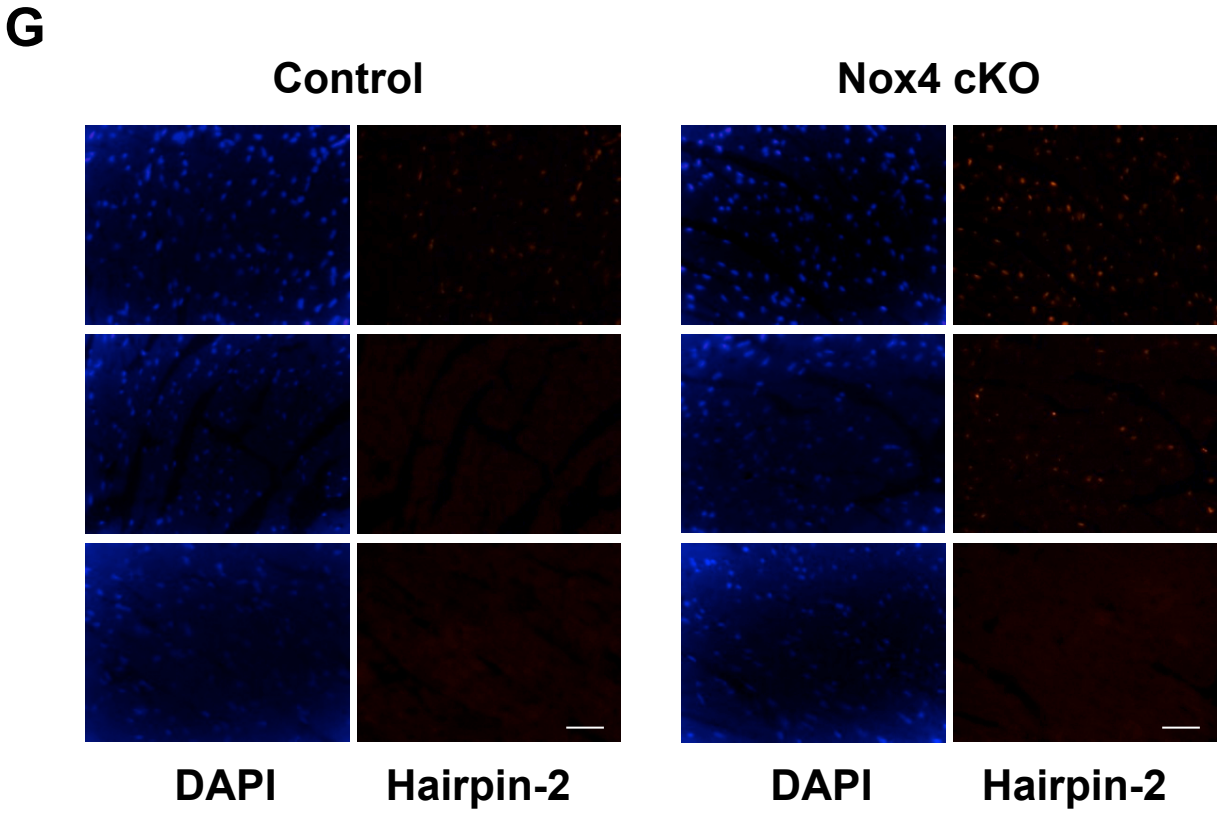



**Online Figure VI. A.** Control mice were subjected to 48 hours of fasting. Cardiac mitochondria were isolated and NADPH-dependent $O_2^-$ production was assessed by lucigenin assay. N=3. **B.** Nox4 levels were evaluated in the mitochondria and microsomes from mouse hearts at baseline and after fasting. **C-E.** Control and Nox4 cKO mice were subjected to 3 hours of ischemia. Nox4 and p62 protein levels were evaluated. Representative immunoblot (C) and densitometric analyses (D-E) are shown. N=4.

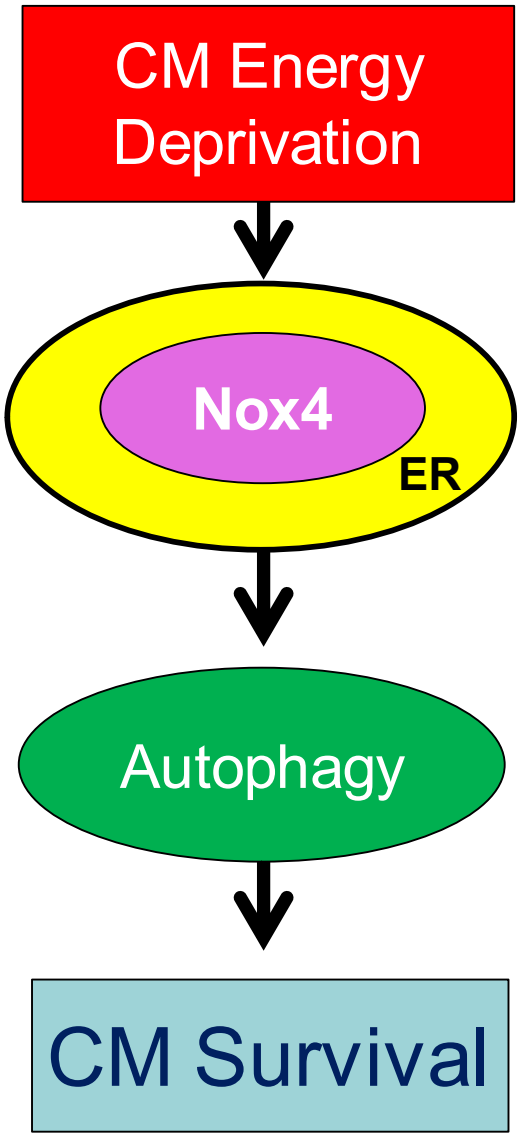
**F.** Control and Nox4 cKO mice received chloroquine (10 mg/kg i.p) or vehicle 4 hours before they were sacrificed. Representative immunoblot of cardiac LC3 is shown.


A
+dP/dT (mmHg)
9000
6000
3000
0
P<0.05
CT (N=4)
Nox4 cKO (N=4)
CT (N=5)
Nox4 cKO (N=7)
Fasting
B
-dP/dT (mmHg)
9000
6000
3000
0
P<0.05
CT (N=4)
Nox4 cKO (N=4)
CT (N=5)
Nox4 cKO (N=7)
Fasting
C
LacZ
CT
β-Gal Staining
D
LacZ
Atg7
LC3


E
Myocardial ATP Content
(% of control)
150
100
50
0
P<0.05
P<0.05
CT
Nox4 cKO
CT
Nox4 cKO
LacZ
Atg7
Fasting
F
P<0.01
Hairpin-2-positive cells (%)
36
24
12
0
CT
Nox4 cKO
Prolonged Ischemia


G
Control
Nox4 cKO
DAPI
Hairpin-2
DAPI
Hairpin-2

**H**




**Online Figure VII. A-B.** Hemodynamic analysis was performed in control and Nox4 cKO mice at baseline and after fasting. dP/dT was measured. **C-D.** Control and Nox4 cKO mice received cardiac injection of Ad-LacZ or Ad-Atg7. Beta-galactosidase staining (C) and LC3 protein levels (D) were evaluated. **E.** Control and Nox4 cKO mice received cardiac injection of Ad-LacZ or Ad-Atg7 and were then subjected to 48 hours of fasting. Myocardial ATP content was evaluated. N=4-7. **F-G.** Control and Nox4 cKO mice were subjected to prolonged ischemia (3 hours). Hairpin-2 staining was performed on myocardial sections. Quantification of the percentage of Hairpin-2-positive nuclei is presented (F) together with representative pictures of ischemic and non-ischemic areas in the two groups (G). N=4. Bar=50 μm. **H.** Representative schema of the hypothesis and findings of the study. In energy-depleted cardiomyocytes, Nox4 is activated in the ER, thereby promoting autophagy and survival.