Online Supplemental Information for Nadtochiy *et al*.

"Lysine Deacetylation in Ischemic Preconditioning: The Role SIRT1"

SUPPLEMENTAL METHODS INFORMATION

Materials: Z-Leu-Leu-Leu-CHO (MG-132) was purchased from BIOMOL (Plymouth Meeting, PA). All other chemicals (including splitomicin and FK-866) were analytical grade from Sigma (St. Louis, MO). Antibodies were obtained from sources as listed in the main manuscript.

Langendorff perfused heart: Mouse hearts were subjected to retrograde (Langendorff) perfusion as previously described [1]. Briefly, following anesthesia with Avertin (0.5mg/kg IP), the aorta was cannulated in situ with a 22½G needle filled with 37°C Krebs-Henseleit buffer (KH) comprising 118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 10 mM glucose, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$. Hearts were rapidly transferred (<10 s.) to a perfusion apparatus, and Langendorff perfusion begun with 37°C KH gassed with 95% O_2, 5% CO_2, in constant flow mode (4 ml/min). Coronary perfusion pressure and left-ventricular balloon pressures were monitored by transducers. Delivery of pharmacologic reagents was accomplished via a syringe pump linked to a port located just above the perfusion cannula, with the volume of added solutions never comprising more than 0.1% of total perfusion solution volume.

Measurement of Myocardial Infarct Size: Following perfusion, hearts were cut into 5 transverse slices, and the slices placed in a solution of 1% 2,3,5-triphenyltetrazolium chloride (TTC) in PBS, for 20 min. at 37 ºC. Slices were then fixed for 24 hrs. in 4% formalin. Slices were placed on a black velvet background (to eliminate reflections), and lit from the side using a fiber-optic microscopy light source, with a light diffuser (a clear plastic drum wrapped in white paper). Images were collected by digital photography using a Canon Powershot SD990-IS (14.7 mega-pixels) in digital macro mode, optical zoom 1.3x, spot-focus, superfine compression (JPEG), highest resolution, ISO-80, tungsten lighting mode. The camera was mounted/stabilized on top of the light-diffusing drum.

Images were analyzed in Photoshop™ software (Adobe). The background color was set to black (R:0 G:0 B:0) using the paint-bucket tool. A 20 x 20 pixel square of pure white (R:255 G:255

B:255) was copied from a reference file, and pasted into the top right corner of the image. Green (R:0 G:255 B:0) was selected as the foreground color, and the paint-bucket tool (with "tolerance" set to 140) used to fill the white square and all similar pixels on the image – i.e. the infarct. This resulted in a green infarct. The white reference square was then returned to black. Setting red as the foreground color (R:255 G:0 B:0), the paint bucket tool (Tolerance: 100) was then used to fill the remaining pink color of the heart slice, to turn the live tissue red. The histogram tool was then used to quantify the # of pixels of each color (black = background, green = infarct, red = live). The same parameters (paint bucket tolerance) and reference squares were used for all samples. Representative images and image masks generated using this method, are shown in Figure S10.

In-vivo IPC: Procedures were essentially as previously described [2]. Mice were anesthetized with Avertin (0.5mg/kg IP), and placed in dorsal recumbency on a heated (37ºC) surgical platform. The entire surgical suite was sterilized by UV light prior to use, and all instruments were autoclaved. Following intubation and ventilation on 100% oxygen (200 µl tidal volume, 100 breaths/min.), a vertical incision was performed and the left anterior descending coronary artery (LAD) visualized. EKG was monitored throughout the procedure. A 9-0 ethilon suture was passed under the LAD, and a small length of PE tubing overlaying the LAD. Occlusion was accomplished by tightening the suture around both the artery and the PE tube, and was monitored by both pallor in the area immediately downstream of the LAD, and an elevation in ST segment on the EKG. Mice were divided into 2 groups: (i) Ctrl., in which the suture was passed under the LAD and left in place without occlusion. (ii) IPC, in which the LAD was occluded for 3 x 5 min. cycles, interspersed with 5 min. of reperfusion. At the end of surgical protocols, tissue was either harvested immediately (acute IPC), or mice were allowed to recover for 24 hrs. (with suture still in place but not occluded) and then tissue was harvested. Cardiac tissue fractionation was performed by differential centrifugation using established methods [3]. The area-at-risk (AAR) was delineated from the area-not-at-risk (ANAR) by visualization of pallor upon transient LAD occlusion, immediately prior to tissue dissection. We have previously shown that this protocol of IPC is cardioprotective against IR injury [2].

Cardiomyocyte model of IPC: Male Sprague-Dawley rats (200-250g), 6-8 weeks of age were purchased from Harlan and handled in accordance with the a protocol approved by the University Committee on Animal Research (UCAR), and in accordance with the NIH Guide for the Care and Use of Laboratory animals (NIH Publication #85-23, 1996). Rats were housed under a 12 hr. light/dark cycle with food and water available ad libitum. Adult rat cardiomyocytes were isolated as described previously [4]. Briefly, the isolated rat heart was initially perfused with Krebs-Henseleit (KH) buffer (118mM NaCl, 4.7mM KCl, 25mM $NaHCO_3$, 10mM glucose, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, pH 7.4 @ 37ºC). Perfusion was switched to KH buffer supplemented with type II collagenase (Worthington Biochemical Lakewood, NJ) which was stripped of endotoxin using AffinityPak™ Detoxi-Gel™ columns (Pierce, Rockford, IL). Ventricular tissue was cut and digested in fresh collagenase buffer, and cells were passed through several wash steps as described [4]. Preparations with cell viability <80% (rod-shape excluding Trypan blue) were discarded. After preparation cells (5×10^5 cells in 5 ml of KH) were transferred into several round-bottom tubes for incubations in a shaking water bath (120 cycles/min. 37C). Cells were subjected to the following conditions: (i) “normoxic” incubation with 10 mM glucose, buffered to pH 7.4, gassed with O_2; (ii) “ischemic” incubation was accomplished by transferring cells to glucose-free KH media, buffered to pH 6.5, gassed with N_2 (zero oxygen). Cell transfer occurred by rapid centrifugation (30 x g, 2 min.), and resuspension of cell pellet in the new media; (iii) “reperfusion” was accomplished by transferring cells back into normoxic KH; (iv) “IPC” comprised 2 cycles of 10 min. ischemia and 10 min. reperfusion.

After 20 min. of initial normoxic incubation, the following groups were assigned: (a) Ctrl. – normoxic conditions during the entire period of incubation; (b) IR – 1 Hr. of ischemia followed by 30 min. of reperfusion; (c) IPC + IR – IPC followed by IR; (d) Split + IPC + IR – Splitomicin (10 μM) was added directly into the incubation media 10 min. prior to IPC and each time after transferring cells into new media. Splitomicin was omitted from the media during the IR protocol; (e) Sp + Ctrl. – splitomicin was incubated for 50 min. (time for drug incubation was exactly as for IPC + IR protocol). At the end of each protocol cell viability was measured using Trypan blue. Note: incubation buffers were changed in all groups simultaneously.

For protein deacetylation assay, 3 x 10^6 cells were pelleted simultaneously from the groups (a), (c), (d) and (e) at the time point which corresponded to the end of IPC protocol (before the onset of index ischemia). Cell pellets were resuspended in buffer containing sucrose (440 mM), MOPS (20 mM) and EGTA (1 mM), pH 7.2 at 4°C, followed by mechanical homogenization with a micro glass Dounce homogenizer. Cell fractionation was performed using differential centrifugation as previously described [4].

2D Gels and Protein Identification: Since single bands from 1D gels contain numerous proteins, identification of single proteins cannot be performed on bands excised from 1D gels. Thus, for protein identification purposes, 2D gels were utilized. Samples were separated by 2D electrophoresis essentially as previously described [2] with several modifications. Samples (0.4 mg of protein) were resuspended in 300 µl rehydration buffer (RB: 7M urea, 2M thiourea, 30 mM DTT, 2% CHAPS, 0.5% lauryl-maltoside, 0.2% pH 3-10 ampholytes). Duplicate samples (150 µl ea.) were loaded into Zoom-Runner cassettes (Invitrogen) with immobilized pH gradient (IPG) strips (Bio-Rad, 7 cm, pH 3-10) and rehydrated overnight at 25°C. Samples underwent IEF, and then SDS-PAGE on 10 or 12.5% gels as described [2].

For protein identification, gels were run in duplicate. One gel was western blotted for K-Ac. Corresponding spots were cut from a parallel coomassie stained gel, with alignment performed in open-source Flicker software. Criteria for spot cutting included: (i) spot position was reproducible in independent experiments; (ii) spot was identifiable by eye in coomassie stained gel; (iii) spot corresponded to different signal intensity in control and experimental samples (e.g. IPC vs. control). Excised spots were trypsinized and identified by MALDI-TOF-TOF in the University of Rochester Proteomics Core Facility (www.urmc.edu/proteomics).

LITERATURE CITED

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3. Cox B, Emili A. Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics. *Nat Protoc* 2006;**1**:1872-1878.

4. Nadtochiy SM, Burwell LS, Brookes PS. Cardioprotection and mitochondrial S-nitrosation: effects of S-nitroso-2-mercaptopropionyl glycine (SNO-MPG) in cardiac ischemia-reperfusion injury. *J Mol Cell Cardiol* 2007;**42**:812-825.

SUPPLEMENTAL RESULTS

Figure S1 shows that while acetylation was decreased in IPC in the cytosol (see main manuscript Figure 1), this was not the case in other cell fractions, including the homogenate. This result highlights the requirement for performing both cell fractionation and 2D gels to identify and visualize changes in acetylation.

Figure S2 shows 2D gels of cytosol and homogenate in control and IPC. Changes in acetylation in these fractions are discussed in the Figure legend. Notably, several proteins which change their acetylation in the cytosol were not detected in the mitochondrial fraction, and vice versa, indicating no translocation of proteins between these fractions. In further support of this, Figure S3 shows that changes in acetylation were observed at the whole homogenate level, on 2D gels. These results highlight the necessity to perform 2D gels to visualize changes in acetylation in the whole homogenate. Notably, all protein acetylation events seen in the cell sub-fractions, are not visible on the homogenate gel – this is due to restrictions in protein loading content of IPG strips, and highlights the need to pre-fractionate cells before loading on 2D gels, to improve resolution of specific proteins in each fraction.

Figure S4 shows that SIRT1 levels do not change in nuclear or cytosolic fractions in IPC. Figures S5, S6, and S7 show Ponceau S stained membranes for Figures 2, 3, and 4 of the main manuscript, respectively.

Figure S8 shows results of the IPC model in cardiomyocytes (see methods). Similar to the result obtained in intact perfused hearts (Figures 4 and 5 of the main manuscript), IPC in cells led to deacetylation of cytosolic proteins that was inhibited by splitomicin. Furthermore, splitomicin partially inhibited the protective effects of IPC in this model system.

Figure S9 shows the effects of administration of SRT1720 (a claimed SIRT1 activator) on IR injury in perfused hearts. Figure S10 shows representative infarct size images from the groups studied, with image masks generated as described in the methods. Quantitation of infarct was calculated from pixel counts on these image masks.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Protein acetylation in perfused heart during Control and IPC. (A): Mouse perfused hearts were subjected to either control perfusion (Ctrl.) or IPC without subsequent IR injury, followed by immediate tissue fractionation. Homogenate (Hmg.), nuclear (Nuc.) and mitochondrial (Mito.) protein fractions were separated by SDS-PAGE, and K-Ac visualized by western blot. Representative blot for Hmg, Nuc and Mito fractions is shown. Numbers to the left of blots are molecular weight markers (kDa). Densitometry was performed in the range 25 – 100 kDa, normalized to protein across the same molecular weight range from representative Ponceau S stained membranes shown in panel **B**. The ratio of K-Ac/Protein was calculated and is shown below the blot in panel A (mean ± SEM, N=3). **(C):** Densitometry control data for Figure 1A of the main manuscript. Ponceau S stained membrane (left panel) and WB (center panel) are shown, together with a sample densitometry profile of the two WB lanes (Ctrl. and IPC) in the range 25 – 100 kDa. **(D):** Densitometry control data for Figure 1B of the main manuscript. Ponceau S stained membrane (left panel) and WB (center panel) are shown, together with a sample densitometry profile of the two WB lanes (Ctrl. and IPC) in the range 25 – 100 kDa. For panels C and D, total area under the curve was used to calculate overall densitometry data points used in lower panels of Figure 1 of the main manuscript.

Figure S2: 2D gel and Western blots of cytosolic and mitochondrial proteins. K-Ac western blots from cytosolic (Cyto) and mitochondrial (Mito) samples isolated from perfused hearts subject to Control perfusion and IPC (as marked in the figure). Representative K-Ac western blots are on the left, while on the right are representative coomassie blue (CB) stained gels. Spot with a red circle (A) indicates a protein that exhibited increased acetylation in IPC in mitochondria. This spot was absent in all cytosolic samples examined. Spots with blue (B) and green (C) circles indicate proteins that were deacetylated in IPC in cytosolic samples. These spots were absent in all mitochondrial samples examined. Spots with a black circle (1,2,3,4) indicate proteins that were deacetylated in IPC in mitochondria. These spots were absent in all cytosolic samples examined. The identity of these proteins A-C and 1-4 (determined by MALDI-TOF-TOF of excised gel spots) is shown below the gel/blot images.

Figure S3: 2D gel and Western blots of heart homogenates. K-Ac western blots from whole heart homogenate samples isolated from perfused hearts subject to Control perfusion and IPC (as marked in the figure). Representative K-Ac western blots are on the left, while on the right are representative coomassie blue (CB) stained gels. Spots with a black circle (4,5) indicate identified proteins that were deacetylated in IPC. Arrows indicate non-identified proteins that were deacetylated in IPC. The identity of targets 4 & 5 is shown below the gel/blot images.

Figure S4: SIRT1 levels in cell fractions from hearts exposed to IPC in-vivo. (A): Mice were exposed to IPC in-vivo as described in the methods, and heart tissue fractionated into nuclear and cytosolic fractions, followed by western blotting. The blot was cut in half, with the upper half probed for SIRT1, and the lower half probed with an antibody to histones 1-4 as a nuclear marker. Both halves of the blot were re-united for secondary antibody and ECL development. Blot is representative of at least 3 independent experiments. Densitometry was performed on the upper Sirt1 band (see main manuscript) and the corresponding Ponceau S stained membrane (panel **B**), in the range 37 – 250 kDa. The ratio of Sirt1/Protein was then calculated, and is shown below the blot (mean ± SEM).

Figure S5: Ponceau S staining of western blot membranes for Figure 2. (A): Whole heart homogenates from perfused hearts subjected to control perfusion (Ctrl.) or IPC, were western blotted for p53 lysine 379 acetylation and total p53 (see Figure **2A** in main manuscript). Ponceau S staining of western blot membrane is shown herein in the left panel. Homogenates from AAR and ANAR of hearts exposed to IPC in-vivo were western blotted for acetylated vs. total p53. Ponceau S staining of western blot is shown herein in the right panel. **(B/C):** Samples from perfused or in vivo hearts were obtained as described in the legend to **Figure 2** (main manuscript), and western blotted for Sirt1 and actin. Ponceau S stained membranes of corresponding blots are shown herein.

Figure S6: Ponceau S staining of western blot membranes for Figure 3: SIRT1 was immunoprecipitated from perfused or in vivo hearts as described in the methods and in the legend for Figure 3 (main manuscript). Ponceau S stained membranes of corresponding western blots are shown herein. **(A):** Perfused hearts (c.f., Fig. 3A). **(B)**: In-vivo (c.f., Fig. 3B).

Figure S7: Ponceau S staining of western blot membranes for Figure 4: Cytosolic fractions from hearts subjected to control perfusion (Ctrl.) or IPC, in the absence or presence of splitomicin (Sp), or FK-866 were western blotted for K-Ac. Ponceau S staining of representative western blot membranes is shown herein.

Figure S8: Effect of splitomicin and IPC on Lys acetylation and cell viability, in isolated cardiomyocytes. Isolated adult rat cardiomyocytes were subjected to control (Ctrl.) incubation with or without splitomicin (Sp), IR injury, IPC + IR, or Sp + IPC + IR (see methods). **(A):** Cytosolic fractions from cells subjected to Ctrl. or IPC, in the absence or presence of splitomicin (Sp, 10 μM), were western blotted for K-Ac. Representative blot is shown, numbers to the left are molecular weight markers (kDa). Densitometry was performed in the range 25 – 100 kDa, normalized to protein across the same molecular weight range (representative Ponceau S stained membrane is shown in panel **B**). The ratio of K-Ac/Protein was calculated and is shown below the blots (mean ± SEM, N≥3). *p<0.05 vs. ctrl). **(C):** Cell viability at the end of each protocol, analyzed by Trypan blue staining. *p<0.05 (ANOVA) between the indicated group and all other groups.

Figure S9: Effect of SRT1720 on IR injury. (A): Perfused hearts were subjected to IR injury, with or without infusion of SRT1720 (1 μM, see methods). Left-ventricular contractile function (rate pressure product, RPP) was monitored throughout the procedure. **(B):** Following IR protocols, hearts were stained with TTC and infarct size measured as % of LV area. In the graph, infarct is quantified, with individual data points for each condition shown on the left. N.B.: IR alone was the same as in Figure 5 (main manuscript), thus, statistical analysis was performed by ANOVA. Data are means ± SEM, N≥5.

Figure S10: Representative images of heart slices and image masks used to delineate infarct areas. Perfused hearts were subjected to conditions as indicated in Figures 5 & S9. Upper images show representative TTC stained hearts from each experimental group, with red indicating live tissue, and pale/white indicating dead tissue (infarct). Lower images show image masks generated as described, using Adobe Photoshop software, for delineation of infarct area (see suppl. Methods). Red = live tissue, green = infarct.

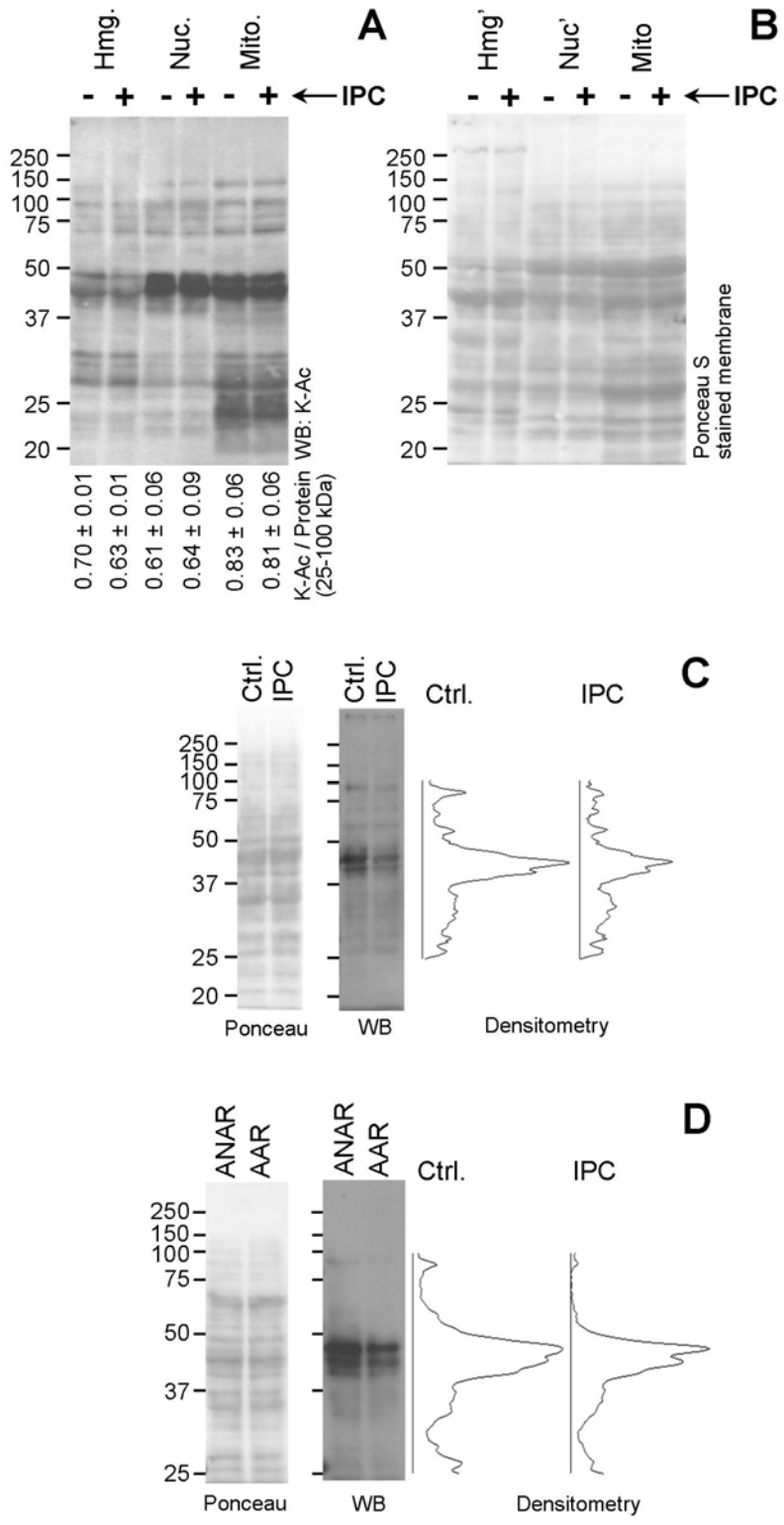
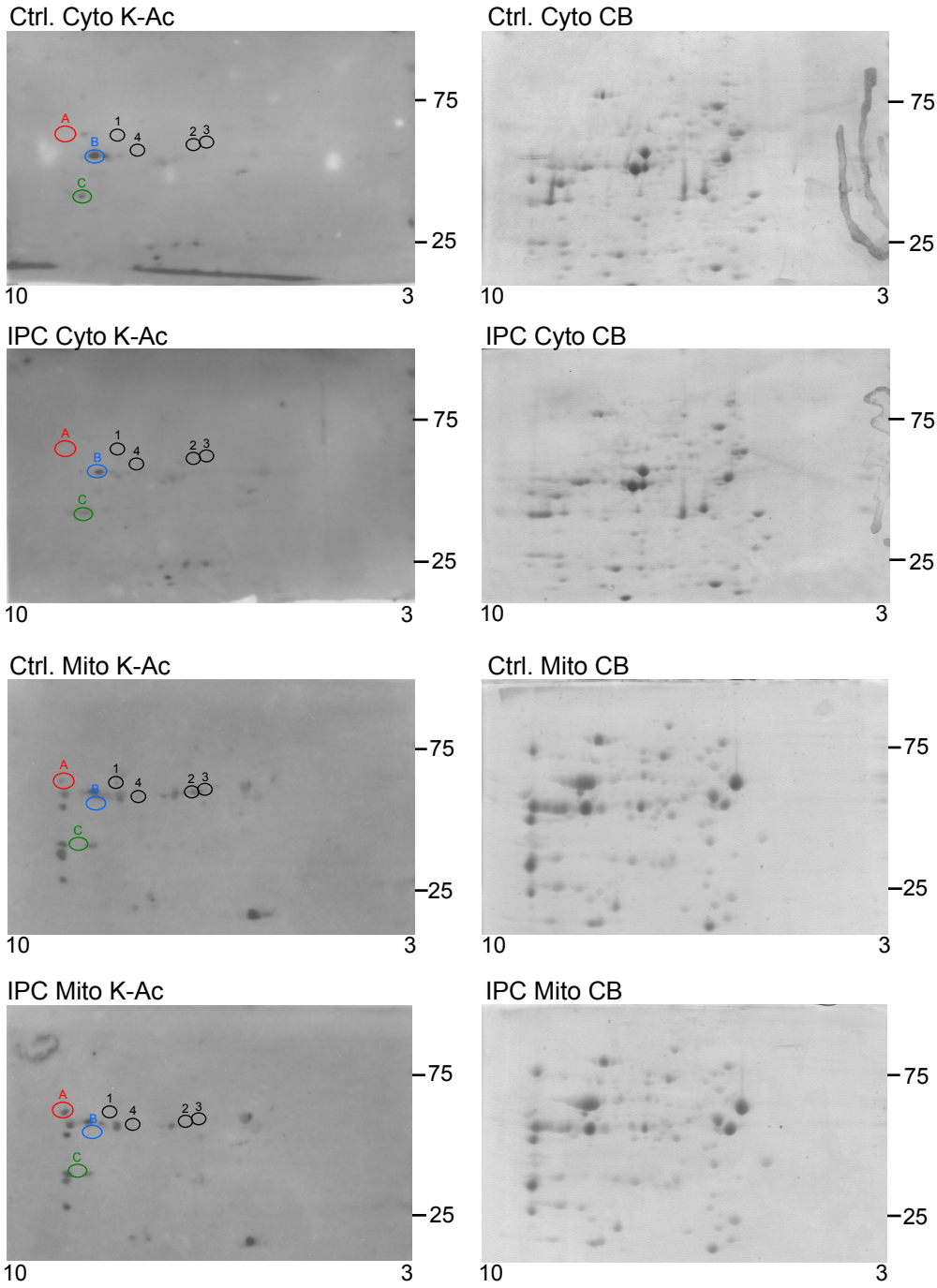

A
Hmg.
Nuc.
Mito.
- + - + - + ← IPC
250
150
100
75
50
37
25
20
WB: K-Ac
0.70 ± 0.01
0.63 ± 0.01
0.61 ± 0.06
0.64 ± 0.09
0.83 ± 0.06
0.81 ± 0.06
K-Ac / Protein (25-100 kDa)
B
Hmg'
Nuc'
Mito
- + - + - + ← IPC
Ponceau S stained membrane
C
Ctrl.
IPC
Ponceau
WB
Densitometry
D
ANAR
AAR
Ponceau
WB
Densitometry

Figure S1



A. gi21704100 Trifunctional protein, β subunit
B. gi158518416 Isocitrate dehydrogenase, mitochondrial precursor
C. gi149266431 Glyceraldehyde 3 phosphate dehydrogenase
1. gi19526814 NADH dehydrogenase, flavoprotein 1
2. gi148685430 mCG22399, isoform CRA_e
3. gi6679261pyruvate dehydrogenase E1 alpha 1
4. gi38259206 creatine kinase, mitochondrial 2

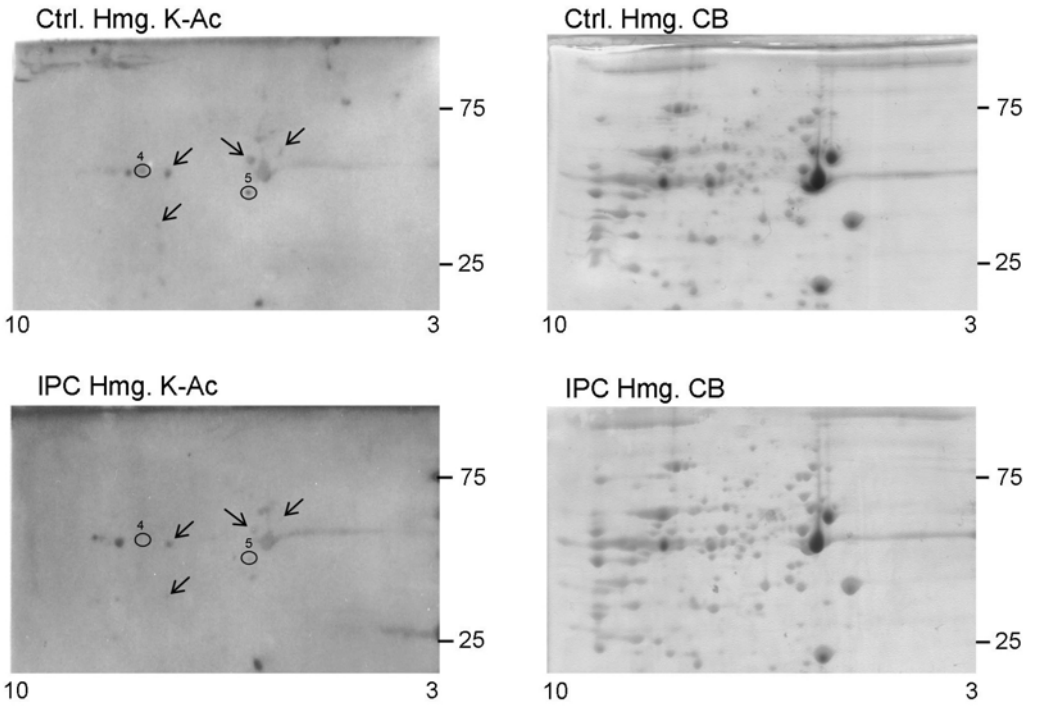


Figure S3

4. gi38259206 creatine kinase, mitochondrial 2
5. gi148693875 isocitrate dehydrogenase 3 (NAD^+) alpha, isoform CRA_e

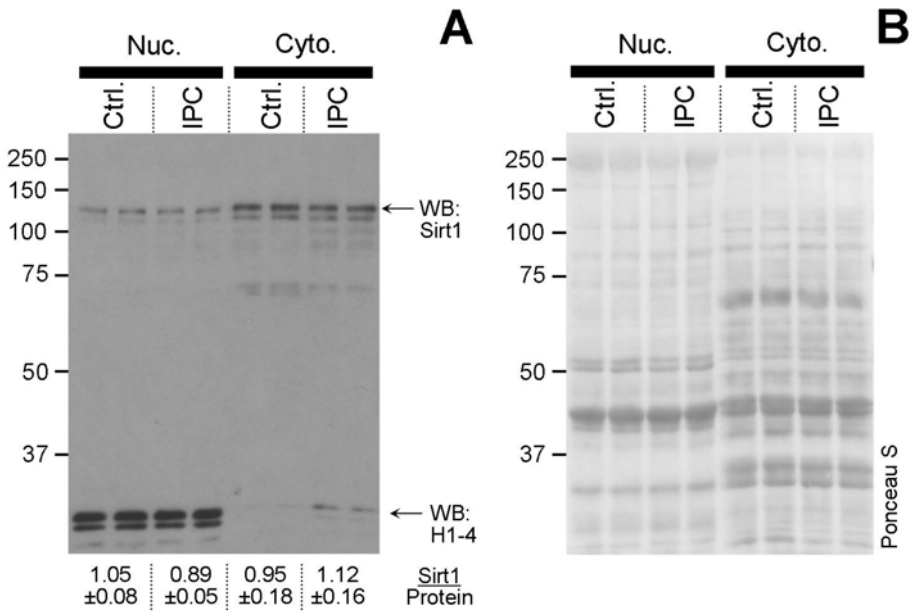
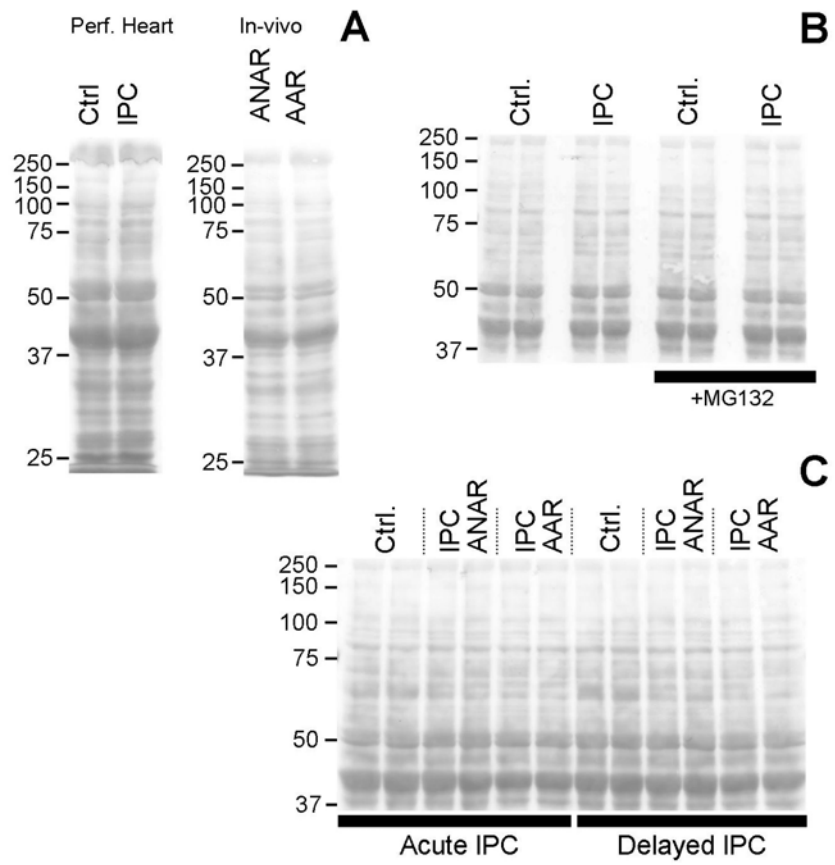

A
Nuc.
Cyto.
Ctrl.
IPC
Ctrl.
IPC
250
150
100
75
50
37
WB:
Sirt1
WB:
H1-4
1.05
±0.08
0.89
±0.05
0.95
±0.18
1.12
±0.16
Sirt1
Protein
B
Nuc.
Cyto.
Ctrl.
IPC
Ctrl.
IPC
250
150
100
75
50
37
Ponceau S

Figure S4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Perf. Heart
In-vivo
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IPC
ANAR
AAR
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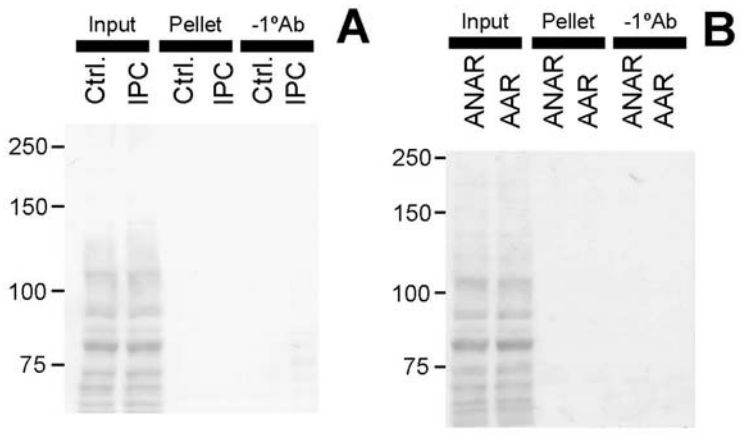
150
100
75
50
37
25
B
Ctrl.
IPC
Ctrl.
IPC
+MG132
C
Ctrl.
IPC ANAR
IPC AAR
Ctrl.
IPC ANAR
IPC AAR
Acute IPC
Delayed IPC

Figure S5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Ctrl.
IPC
Ctrl.
IPC
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100
75
B
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ANAR
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ANAR
AAR
250
150
100
75

Figure S6

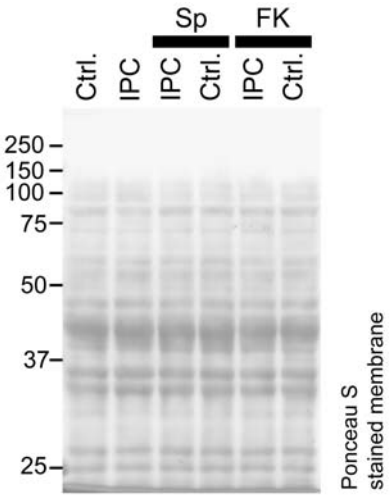
Sp
FK
Ctrl.
IPC
IPC
Ctrl.
IPC
Ctrl.
250
150
100
75
50
37
25
Ponceau S
stained membrane

Figure S7

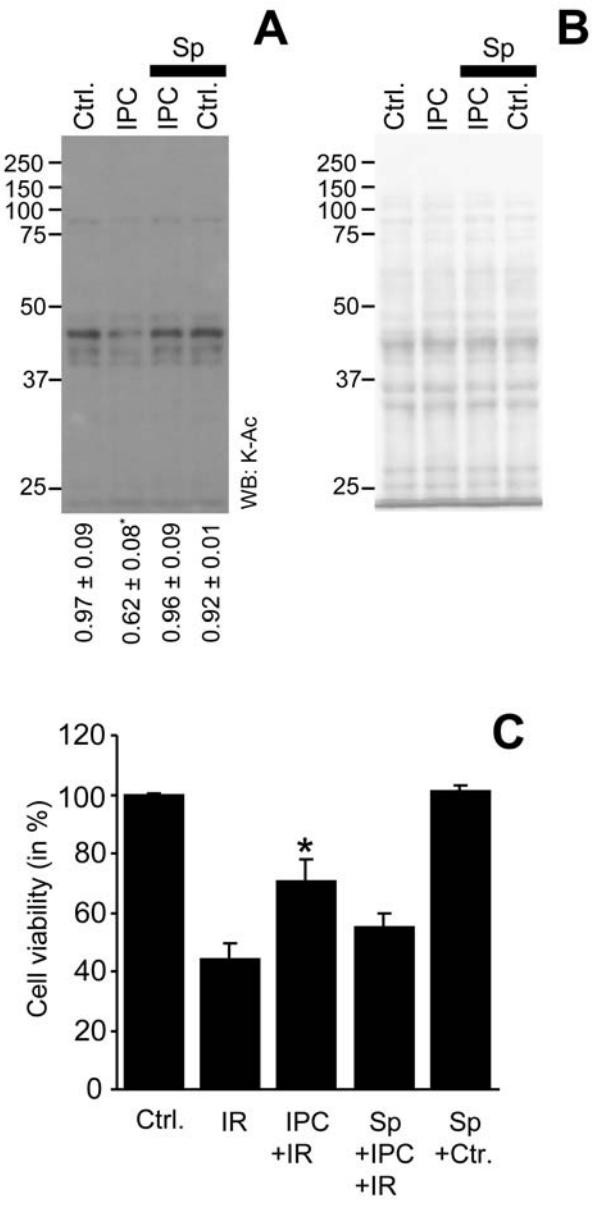
A
B
Sp
Ctrl.
IPC
IPC
Ctrl.
250
150
100
75
50
37
25
WB: K-Ac
0.97 ± 0.09
0.62 ± 0.08*
0.96 ± 0.09
0.92 ± 0.01
C
Cell viability (in %)
120
100
80
60
40
20
0
*
Ctrl.
IR
IPC +IR
Sp +IPC +IR
Sp +Ctr.

Figure S8

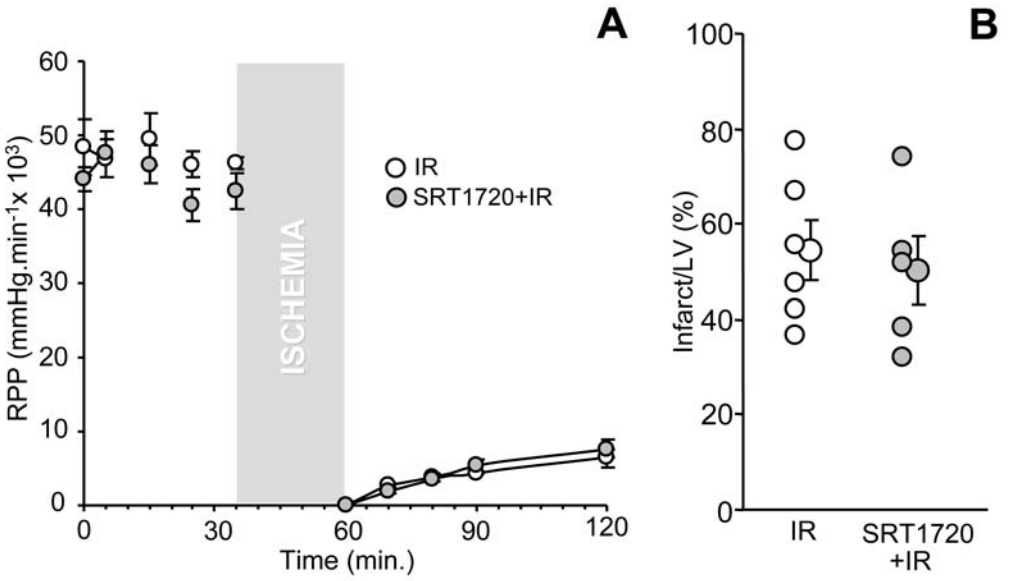
A
B
RPP (mmHg.min-1x 10³)
ISCHEMIA
IR
SRT1720+IR
Time (min.)
Infarct/LV (%)
IR
SRT1720 +IR

Figure S9

Figure S10