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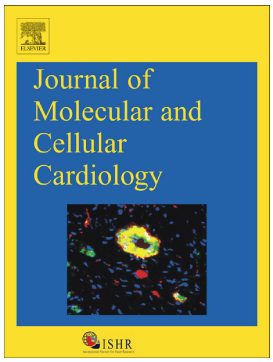

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Mitochondrial LonP1 protects cardiomyocytes from ischemia/reperfusion injury in vivo

Sundararajan Venkatesh [a,*] sundarve@njms.rutgers.edu, Min Li [a], Toshiro Saito [b], Mingming Tong [b], Eman Rashed [a], Satvik Mareedu [a], Peiyong Zhai [b], Clea Bárcena [c], Carlos López-Otín [c], Ghassan Yehia [d],
Junichi Sadoshima [b], Carolyn K. Suzuki [a,*] suzukick@njms.rutgers.edu

[a]Department of Microbiology, Biochemistry & Molecular Genetics, New Jersey Medical School-Rutgers, The State University of New Jersey, Newark, NJ, USA,
[b]Department of Cell Biology & Molecular Medicine, New Jersey Medical School- Rutgers, The State University of New Jersey, Newark, NJ, USA,
[c]Dpto. de Bioquímica y Biología Molecular, Instituto Universitario de Oncología (IUOPA), Universidad de Oviedo, Oviedo, Spain,
[d]Genome Editing Core Facility, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901.

[*]Corresponding authors at: Department of Microbiology, Biochemistry and Molecular Genetics, New Jersey Medical School- Rutgers, The State University of New Jersey, 225 Warren Street Newark, New Jersey 07103, USA.

ABSTRACT

Rationale: LonP1 is an essential mitochondrial protease, which is crucial for maintaining mitochondrial proteostasis and mitigating cell stress. However, the importance of LonP1 during cardiac stress is largely unknown.

Objective: To determine the functions of LonP1 during ischemia/reperfusion (I/R) injury *in vivo,* and hypoxia-reoxygenation (H/R) stress *in vitro*.

Methods and Results: LonP1 was induced 2-fold in wild-type mice during cardiac ischemic preconditioning (IPC), which protected the heart against ischemia-reperfusion (I/R) injury. In contrast, haploinsufficiency of LonP1 (*LONP1*$^{+/-}$) abrogated IPC-mediated cardioprotection. Furthermore, *LONP1*$^{+/-}$ mice showed significantly increased infarct size after I/R injury, whereas mice with 3-4 fold cardiac-specific overexpression of LonP1 (LonTg) had substantially smaller infarct size and reduced apoptosis compared to wild-type controls. To investigate the mechanisms underlying cardioprotection, LonTg mice were subjected to ischemia (45 min) followed by short intervals of reperfusion (10, 30, 120 min). During early reperfusion, the left ventricles of LonTg mice showed substantially reduced oxidative protein damage, maintained mitochondrial redox homeostasis, and showed a marked downregulation of both Complex I protein level and activity in contrast to NTg mice. Conversely, when LonP1 was knocked down in isolated neonatal rat ventricular myocytes (NRVMs), an up-regulation of Complex I subunits and electron transport chain (ETC) activities was observed, which was associated with increased superoxide production and reduced respiratory efficiency. The knockdown of LonP1 in NRVMs caused a striking dysmorphology of the mitochondrial inner membrane, mitochondrial hyperpolarization and increased hypoxia-reoxygenation (H/R)-activated apoptosis. Whereas, LonP1 overexpression blocked H/R-induced cell death.

Conclusions: LonP1 is an endogenous mediator of cardioprotection. Our findings show that upregulation of LonP1 mitigates cardiac injury by preventing oxidative damage of proteins and

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lipids, preserving mitochondrial redox balance and reprogramming bioenergetics by reducing Complex I content and activity. Mechanisms that promote the upregulation of LonP1 could be beneficial in protecting the myocardium from cardiac stress and limiting I/R injury.

Key Words: LonP1 protease, mitochondria, cardioprotection, oxidative stress, ischemia and reperfusion,

1. Introduction

Myocardial infarction and heart failure are leading causes of morbidity worldwide. Ischemic and reperfusion (I/R) injury are major determinants of myocardial death [1]. Intracellular mechanisms mediating I/R injury include oxidative stress, calcium overload, metabolic dysfunction and inflammation [2]. In addition, microvascular damage and endothelial cell dysfunction make up the complex sequelae that provoke myocardial injury and death. Mitochondrial reactive oxygen species (ROS) have been shown to drive ischemia-reperfusion injury [3] leading to extensive oxidative damage resulting in cardiac myocyte apoptosis [1]. Effective therapies to ameliorate myocardial I/R injury remain unavailable [4]. Hence, identifying novel endogenous cardioprotectants and specific mechanisms by which to mitigate I/R injury can provide insights into new strategies for limiting myocardial injury in individuals with ischemic heart disease and/or heart failure.

The mitochondrial LonP1 is a highly conserved ATP-powered protease, which ensures mitochondrial proteostasis and regulates adaptive responses to cell stress [5-8]. *In vitro* studies have shown that LonP1 is up-regulated by acute cell stressors such as hypoxia, oxidative stress, nutrient deprivation and the unfolded protein response at the endoplasmic reticulum [9-12]. Several *in vitro* studies have shown that LonP1 up-regulation adapts and preserves normal cell viability in response to acute cell stress [11, 13-15], whereas another study reported that LonP1 up-regulation induces cell death [16]. The overexpression of LonP1 has also been observed in several cancers, and is proposed to overcome the hypoxic, metabolic and proteotoxic stress associated with the oncogenic transformation of tumor cells [13-15, 17]. However, the function of LonP1 during cardiac stress *in vivo* is not known.

The roles of LonP1 in disease and aging are only beginning to emerge. In mice, homozygous deletion of the *LONP1* gene causes early embryonic lethality, whereas

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heterozygous *LONP1*$^{+/-}$ mice are phenotypically normal under baseline conditions [14]. In humans, we and others have reported that homozygous and compound heterozygous mutations in *LONP1* are associated with CODAS syndrome, a rare multi-system developmental disorder affecting the cerebral, ocular, dental, auricular and skeletal systems of children from diverse ethnic backgrounds [18, 19]. CODAS syndrome is the first human disease linked to pathogenic mutations in *LONP1*. Some affected individuals present with atrioventricular defects and incomplete cardiac septation [18]. However, all CODAS individuals are still young children, thus the impact of these mutations on stress or age-related disorders (e.g. heart disease), has not been studied. In mice selected for lifelong voluntary exercise, LonP1 expression in the heart is upregulated 2.3 fold and these mice have a longer median lifespan than sedentary mice [20]. In addition, LonP1 expression levels are highest in heart, brain, liver and skeletal muscle [21], highlighting its importance in maintaining mitochondrial function in these energy demanding tissues. During aging, LonP1 expression has been shown to decline [22], whereas its overexpression in model systems has been found to extend lifespan [23].

In this study, we employed both gain- and loss-of-function mouse models and isolated neonatal rat ventricular myocytes (NRVMs) to investigate the role of LonP1 in cardioprotection. We show that LonP1 selectively regulates the levels of Complex I subunits, reduces Complex I activity, preserves mitochondrial redox status and reduces both oxidative protein damage and cardiomyocyte apoptosis during I/R.

2. Methods

2.1. Mouse models

Heterozygous knockout *LONP1*$^{+/-}$ mice in the C57BL/6 background were obtained from Dr. Carlos López-Otín (Universidad de Oviedo, Spain), and have been previously described [14]. Transgenic mice with cardiac-specific LonP1 overexpression were generated in FVB/NJ background (LonTg). The *LONP1* cDNA was expressed under the control of the murine alpha-Myosin Heavy Chain (αMHC) promoter and injected into ES cells. Non-transgenic (NTg) mice were used as controls. Cardiac-specific overexpression of LonP1 was confirmed by immunoblotting (Suppl. Figure 1 A). Three different mouse lines were established ranging from low (1.5 fold), medium (3-4 fold), and high (5-6 fold) levels of LonP1 expression (Suppl. Figure 1B). In this study, we employed the mouse line with medium LonP1 expression level (3-4 fold) as these levels are close to the fold expression of LonP1 observed during ischemic preconditioning (IPC) (Fig.1D), which was cardioprotective against I/R. Male animals were used throughout the study. All animal protocols were carried out as described and approved by the Institutional Animal Care and Use Committee (IACUC) of New Jersey Medical School- Rutgers, The State University of New Jersey.

2.2. Ischemic preconditioning and ischemia-reperfusion surgeries

Mice used in the study were housed as per the standard temperature-controlled environment with 12-hour light/dark cycles where they received food and water ad libitum. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60µg/g). A rodent ventilator (model 683; Harvard Apparatus Inc., Holliston, Massachusetts, USA) was used with 65% oxygen during the surgical procedure. Rectal temperature was monitored and maintained between 36˚C and 37˚C. The chest was opened by a horizontal incision through the muscle

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between the ribs (third intercostals 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 (1mm 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, silicon tubing was removed to achieve reperfusion. The chest wall was then closed by with *5-0* silk. The animal was removed from the ventilator and kept warm in the cage maintained at 37°C until normal activity was observed. For ischemic preconditioning, 3 month old C57BL/6 mice were subjected to a series of 6 cycles of brief ischemia (3 min) and reperfusion (3 min) and the chest wall was then closed by with *5-0* silk. The hearts were harvested at specified time points. All the I/R experiments were performed at the same time period of the day to control for circadian processes. In this study, we used two different mouse strains, FVB/NJ and C57BL/6. The FVB strain was employed for cardiac-specific overexpression of LonP1 (LonTg), whereas C57BL/6 strain was employed in the heterozygous LonP1 knock out (*LONP1*$^{+/-}$) model. In our laboratory settings, we have standardized the I/R experiments for each strain and found that ischemia of 30 min for C57BL/6 and 45 min for FVB strains was optimal for minimizing mortality rate. We have analyzed these strains separately and have not compared any outcome between these strains. In IPC-IR experiments, we used younger C57BL/6 mice (3.5-4.5 months) to minimize the mortality rate, whereas in I/R we employed 6-8 months old mice.

2.3. Cellular bioenergetics

Mitochondrial bioenergetics and glycolytic flux were measured in NRVMs, plated at 90,000 cells/well in Seahorse XF24 well plates in Dulbecco's Modified Essential Medium/Ham F12 (1:1) (Invitrogen, 12500-062) supplemented with 5% Horse Serum (Invitrogen, 16050-122) and 1% penicillin/streptomycin (Invitrogen, 10378-016). The next day, for LonP1 knockdown, the cells were treated with control or LonP1 shRNA adenovirus particles, and for overexpression studies, cells were transduced with control LacZ or LonP1 adenoviral particles. Adenoviral particles were added in DMEM medium containing reduced FBS (3%) for 24 h, after which complete medium was added and refreshed every 24 h for 4-6 days for knockdown and 2 days for overexpression. On the day of analysis, the medium was replaced with unbuffered DMEM supplemented with 17.5 mM glucose, 2 mM pyruvate, and 1 mM glutamine for 1 hour in non-CO_2 incubator. The Mito Stress test was performed as per the manufacturer's recommendations (Seahorse, Agilent Technologies). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured at baseline and after the sequential addition of: (1) oligomycin (1 µM final)- a Complex V ATP synthase inhibitor used to measure ATP-dependent oxygen consumption; (2) FCCP (3 µM final)- an uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone used to measure maximum respiratory capacity; and (3) antimycin A and rotenone (each 1µM final), which are Complex I and III inhibitors, respectively, used to measure non-mitochondrial OCR. The calculation of basal OCR, % spare respiratory capacity, proton leak, coupling efficiency, non-mitochondrial respiration and extracellular acidification rate (ECAR) were carried out using Excel/Mito Stress Report generator (Seahorse, Agilent technologies).

2.4. Cellular OXPHOS complex activities

To measure *in situ* respiratory complex activities we employed the Seahorse XF24 analyzer, using adenoviral transduced NRVMs as described above. On the day of the assay, cells were washed with 1X MAS buffer [mannitol 220 mM, sucrose 70 mM, KH_2PO_4 10 mM, $MgCl_2$ 5 mM, HEPES 2 mM, EGTA 1 mM, fatty acid-free (FAF) BSA 0.2% (*w*/*v*)]. Assay medium was added consisting of MAS buffer supplemented with pyruvate, malate, and ADP (10 mM, 1 mM, 4 mM, respectively). Cells were treated with plasma membrane permeabilizer (PMP) (1 nM) (Seahorse Biosciences, Agilent), to initiate the experiment and the plate was immediately transferred to the

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XF24 analyzer. ETC complex inhibitors and substrates prepared in MAS buffer were added sequentially to the wells as follows: (1) rotenone (2 µM)- a Complex I inhibitor; (2) succinate (10 mM), a substrate of Complex II; (3) antimycin A (2 µM)- a Complex III inhibitor, (4) ascorbate (10 mM) and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) (100 µM)- a Complex IV substrate and electron donor, respectively. Initial readings were used to determine the basal OCR of mitochondria. Complex I activity was determined as the difference between OCR at baseline and after Complex I inhibition by rotenone. Complex II and III activity was determined as the difference in OCR after Complex II substrate addition and Complex III inhibition. Complex IV activity was determined by directly stimulating cytochrome c oxidase with ascorbate (10 mM) TMPD (100 µM). The assay used mix/wait/measure times of 0.5 min/0.5 min/2 min with no equilibration step, and 3 measurements per step were taken as per the manufacturer's (Seahorse XF analyzer, Agilent) instruction.

2.5. In-gel aconitase assay

Aconitase activity was measured by in-gel assay as described by Tong and Rouault [24]. Heart tissues were ground in dry ice, then dry ice-evaporated and washed one time with cold PBS. The tissue powder was suspended in aconitase lysis buffer (50 mM Tris-HCl, pH 8, 50 mM NaCl, 1% TX-100, 10% v/v glycerol, 2 mM Na-citrate and 15 U catalase) on ice for 20 min with occasional mixing. Protein samples were centrifuged at 14,000 rpm for 20 min at 4°C. Proteins were quantified by bicinchoninic acid method (BCA, Pierce) and 20 - 40 µg samples were loaded on a native acrylamide gel (6% separating and 4% stacking gel) and electrophoresed in running buffer (132 mM Tris base, 132 mM boric acid, 3.6 mM sodium citrate). In-gel activity was assayed by incubation in developing buffer (100 mM Tris-HCl, pH 8, 1 mM NADP, 2.5 mM cis-aconitic acid, 5 mM $MgCl_2$, 1.5 mM methylthiazolyldiphenyl-tetrazolium bromide, 0.3 mM phenazine methosulfate, and 5 U/ml isocitrate dehydrogenase) in the dark at 37˚C.

2.6. In vitro Complex I activity assay

Complex I activity in the protein extracts from the left ventricle of the heart was determined using a microplate assay (Abcam, ab109721). In this kinetic assay, Complex I activity was measured as the change in absorbance of the reaction product following the procedure recommended by the manufacturer. Briefly, 20 µg protein extract was incubated in a 96 well plate pre-coated with antibody capturing the Complex I holoenzyme, followed by washing to remove non-specific proteins. Enzyme activity was determined by measuring the oxidation of NADH to NAD+ and the simultaneous reduction of a dye, leading to increased absorbance at 450 nm. The background absorbance was determined using assay buffer alone. The activity was determined by calculating the slope, and is expressed as the change in absorbance per minute per amount of sample present in the well.

All other methods employed in this study are available in the Supplemental Materials.

3. Results

3.1. LonP1 mitigates ischemic cardiac injury and promotes cardioprotection

To address whether LonP1 is involved in the endogenous signaling pathway of cardioprotection, we investigated its importance during IPC, which is a potent cardioprotective experimental procedure [25, 26]. IPC attenuates the severity of reperfusion-induced damage, thus reducing

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infarct size. C57BL/6 male mice were subjected to short cycles of ischemia followed by reperfusion, and the left ventricles of sham control and IPC-treated mice were analyzed for both LonP1 transcript and protein levels at selected timepoints post-IPC (Figure 1A). Both LonP1 transcript (Figure 1B) and protein levels (Figure 1C) were significantly upregulated 2-fold within 4 h after IPC compared to controls ($p<0.05$) (Figure 1D). This suggests that LonP1 may participate in ameliorating cardiac injury.

To determine whether LonP1 plays a crucial role in cardioprotection, we employed heterozygous *LONP1* knockout mice (*LONP1*$^{+/-}$) in a C57BL/6 background as described previously [14], which have approximately 50-60% reduced LonP1 protein levels in the heart (Suppl. Figure 2A). Under baseline conditions, *LONP1*$^{+/-}$ mice developed normally and did not display any obvious pathological alterations [14]. Our further analysis showed that heart weight, cardiac fibrosis, left ventricular thickness and intraventricular septum thickness of *LONP1*$^{+/-}$ mice were similar to wild-type *LONP1*$^{+/+}$ littermates (Suppl. Figure 2B and 2C) [14]. Both *LONP1*$^{+/+}$ and *LONP1*$^{+/-}$ mice were subjected to ischemia for 30 min followed by reperfusion for 24 h. Cardiac morphometric analyses showed that heterozygous *LONP1*$^{+/-}$ hearts had 37% larger infarct size after I/R as compared to wild-type *LONP1*$^{+/+}$ littermates ($p<0.01$), and there was no significant change in the percent area at risk relative to the left ventricle size (AAR/LV) (Figure 2A). Furthermore, haploinsufficiency of *LONP1* (*LONP1*$^{+/-}$) abrogated IPC-mediated cardioprotection. *LONP1*$^{+/-}$ mice subjected to IPC prior to I/R were not protected and showed a significantly larger infarct size as compared to IPC-protected wild type *LONP1*$^{+/+}$ mice ($p<0.0001$) (Figure 2B). Taken together, these data demonstrate that reduced LonP1 protein levels exacerbate I/R-induced cardiac injury and block the cardioprotective effects of IPC.

As the deficiency of LonP1 aggravates I/R injury, we examined whether its overexpression could confer cardioprotection using transgenic mice with cardiac-specific expression of the human *LONP1* cDNA (LonTg). These mice showed no significant change in basal hemodynamic parameters as compared to their littermate non-transgenic (NTg) controls (Suppl. Figure 1C). LonTg mice with 3-4 fold overexpression of LonP1 subjected to ischemia for 45 min followed by reperfusion for 24 h showed significantly ($p<0.005$) smaller infarct size compared to NTg controls, with no significant change in the AAR/LV ratio (Figure 2C). The average infarct size of the LonTg group was found to be ~30% less than NTg controls, thus showing that LonP1 overexpression was cardioprotective against I/R injury. *In situ* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of heart sections from both LonTg and NTg mice showed that left ventricular infarct area of LonTg mice had approximately 50% fewer apoptotic cells than NTg controls ($p<0.005$) (Figure 2D). Consistent with these *in situ* data, immunoblot analysis (Figure 2E) of left ventricular extracts showed that LonTg mice subjected to I/R (LonTg-I/R) had significantly lower levels of cleaved caspases -3 ($p<0.05$) and -9 ($p<0.05$) as compared to NTg-I/R mice (Figure 2F), suggesting that LonP1 ameliorates apoptotic cell death during I/R. Taken together, these findings suggest that LonP1 mitigates I/R-induced cardiac injury by reducing apoptotic cell death and promoting cardioprotection.

3.2. LonP1 preserves cardiac redox status and reduces oxidative stress

Oxidative stress-induced damage to lipids and proteins are hallmarks of I/R injury *in vivo* [27, 28]. Increased lipid peroxidation induced by oxidative stress elevates the production of 4-hydroxynonenal (4-HNE), which modifies proteins. Mt-aconitase is a tricarboxylic acid (TCA) cycle enzyme containing a $[Fe_4S_4]^{2+}$ cluster, which is vulnerable to oxidative inactivation [29]. To identify whether LonP1 prevents oxidative stress and damage, both of these markers were analyzed in left ventricular protein extracts from LonTg and NTg mice, which were subjected to ischemia (45 min), followed by a time course of reperfusion (0, 10, 30 and 120 min). NTg hearts

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showed significantly ($p<0.05$) elevated 4-HNE modified proteins after 30 min reperfusion compared to sham control in contrast to LonTg extracts in which 4-HNE modified proteins were reduced significantly at reperfusion time point 30 min ($p<0.01$) compared to corresponding NTg reperfusion time point (NTg-30 min) (Figure 3A and B). Mt-aconitase activity was also analyzed at the same time points using an in-gel activity assay (Figure 3C) [24]. Interestingly, in LonTg mice, mt-aconitase activity was significantly ($p<0.05$) increased 2.5-fold after ischemia and reperfusion (10 min) and remained above the basal level throughout reperfusion (30 and 120 min) as compared to the LonTg sham control (Figure 3D). By contrast, NTg mice subjected to I/R did not show increased mt-aconitase activity, which instead declined albeit non-significantly at 120 min reperfusion (Figure 3C and D). The corresponding protein levels of mt-aconitase in NTg and LonTg were comparable and unchanged throughout the time course (Figure 3C and D), demonstrating that increased activity of mt-aconitase in LonTg hearts, was not caused by its increased protein levels (Figure 3D). Collectively, these results show that LonP1 overexpression reduces oxidative protein-lipid damage and maintains mitochondrial redox balance during cardiac I/R.

3.3. LonP1 downregulates Complex I levels and decrease Complex I activity

The reduced oxidative stress in LonTg versus NTg mice during I/R prompted us to examine the respiratory chain complexes, which are the major source of mitochondrial reactive oxygen species (ROS), and a critical driver of I/R injury. Complexes -I and -III are the major sites generating ROS, which can form highly reactive and damaging hydroxyl radicals [3, 30, 31]. Recent evidence shows that Complex I is the principal site of superoxide production during I/R promoting cardiac injury [3, 31]. The protein levels of LonP1 and subunits belonging to Complexes I through V were examined during an I/R time course (Figure 3E and F). LonP1 levels were not changed during acute ischemia (45 min) or reperfusion times (Suppl. Fig. 3). However, in LonTg mouse hearts, we found that both peripheral (Ndufs1 and Ndufa9) and membrane (Ndufb8) arm subunits of Complex I were significantly ($p<0.05$) reduced during an early reperfusion phase time point, 10-30 min (Figure 3E and F), which was consistent with the reduction in 4-HNE and increased mt-aconitase activity (Figure 3A-D). No changes were observed in the other Complexes II-IV subunits that were examined (Figure 3E and F). Notably, even at a baseline, the Complex I subunit Ndufs1 was present at significantly ($p<0.05$) lower levels in LonTg versus NTg hearts (Figure 3E and F). Collectively, these results raise the possibility that LonP1 downregulates Complex I activity thereby limiting superoxide generation during I/R.

We examined Complex I activity in protein extracts from left ventricular tissue of the hearts from NTg and LonTg mice with or without I/R. Interestingly, LonTg hearts showed significantly slower Complex I kinetics as compared to NTg hearts (Figure 3G). Under baseline conditions, Complex I activity was significantly less ($p<0.05$) in LonTg hearts as compared to NTg (Figure 3H). After 30 min reperfusion, Complex I activity in LonTg hearts was also significantly less ($p<0.05$) than in NTg hearts (Figure 3H). Interestingly, the activity of Complex I in LonTg hearts after I/R was comparable to the basal activity of NTg hearts without I/R (Figure 3H). These observations are consistent with reduced Complex I subunits, oxidative stress and damage observed in LonTg hearts during I/R (Figure 3A and C). We also examined Complex I activity in protein extracts from left ventricular tissue of the hearts from heterozygous knockdown *LONP1*$^{+/-}$ and normal *LONP1*$^{+/+}$ mice under basal conditions, but found no significant difference in enzyme kinetics (Suppl. Figure 4).

3.4. LonP1 overexpression ameliorates H/R induced stress in primary neonatal rat ventricular myocytes (NRVMs)

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To complement our *in vivo* findings, we also employed NRVMs, which were cultured with reduced nutrients (10 mM glucose, 0.3% FBS) and hypoxia (0.5% oxygen) to mimic ischemic-like stress. The purity of NRVMs was ~85% (Suppl. Figure 5A). Under these conditions, LonP1 transcripts and protein levels were upregulated by 2-3-fold (Figure 4A and B), however, hypoxia-reoxygenation (H/R) reduced the LonP1 expression to normal levels (Figure 4B). H/R was performed to mimic I/R wherein NRVMs were nutrient-deprived (10 mM glucose, 0.3% FBS) and cultured at low O_2 tension (0.5-2%) for 20 h, and then reoxygenated at normoxia for 2 h. Under hypoxia, NRVMs viability was reduced to 55% by 24 h (Suppl. Figure 5B). Also, H/R significantly ($p<0.01$) reduced cell viability (Figure 4D). However, overexpressing human LonP1 for 2 days (Ad-LonP1) (Figure 4C) significantly ($p<0.05$) increased cell viability to 73% upon hypoxia (20 h) - reoxygenation (2 h) (Figure 4D). By contrast, the Ad-LacZ control group showed only 51% viability ($p<0.05$) (Figure 4D). Also, overexpressing LonP1 in NRVMs for 2 days significantly upregulated anti-apoptotic Bcl2 expression under normoxia as well as H/R conditions (Figure 4E). Altogether, LonP1 overexpression reduces cell death in NRVMs during H/R stress, which is in line with our *in vivo* findings demonstrating reduced apoptosis during I/R in cardiac tissue of LonTg mice (Figure 2D and 2E). We also examined whether LonP1 overexpression affected cellular energetics under basal conditions (Suppl. Figure 6). Overexpressing LonP1 in NRVMs significantly ($p<0.01$) reduced spare respiratory capacity (Suppl. Figures 6A and 6B), but did not affect basal respiration and glycolytic capacity (Suppl. Figures 6C-E).

3.5. LonP1 knockdown dysregulates mitochondrial respiratory efficiency

To further investigate the mechanism underlying our *in vivo* findings, NRVMs were transduced with a LonP1 shRNA using an adenovirus (LonP1-KD) or a control shRNA (Control) delivery system. Four days after the knockdown, LonP1 protein levels were ~30% of control (Figure 5A). LonP1 depletion did not alter cell viability compared to control (Suppl. Figure 7). Strikingly, the cell culture medium of the LonP1-KD NRVMs was more acidic 4 days after transduction compared to controls (Suppl. Figure 8A). This implied that LonP1-KD increased acidification of the culture medium by increasing glucose utilization leading to increased lactate production and secretion. Spectrophotometric measurement of the culture medium containing phenol red [32] from LonP1-KD NRVMs showed significantly decreased pH compared to controls ($p<0.05$) (Suppl. Figure 8B). Further, LonP1-KD NRVMs showed increased levels of Complex I subunits Ndufb8, Ndufs1, and Ndufa9 (Figure 5A), reiterating the findings from Figure 3E that these subunits are regulated by LonP1. In addition, when these NRVMs were subjected to H/R, LonP1-KD NRVMs were more susceptible to apoptosis as shown by increased cleavage of caspase-3 (Figure 5B).

To determine whether LonP1-KD in NRVMs affects mitochondrial bioenergetics, we measured the mitochondrial oxygen consumption rate (OCR) and extracellular acidification (ECAR), which are readouts of oxidative phosphorylation (OXPHOS) and glycolysis, respectively. LonP1-KD significantly increased both basal OCR ($p<0.05$) (Figure 5C) and ECAR ($p<0.05$) (Suppl. Figure 8C). This was accompanied by significantly decreased percent spare respiratory capacity (% SRC) ($p<0.05$) (Suppl. Figure 8D). SRC is defined as the difference between basal OCR and maximal OCR induced by dissipating the mitochondrial membrane potential using an uncoupler such as FCCP. SRC is a measure of the cell's ability to respond to increased energy demand [33]. Specific analysis of ETC complex activities in LonP1-KD NRVMs showed that Complex I, Complex II+III and Complex IV activities were significantly increased compared to the controls (Figure 5D and 5E). In addition, LonP1-KD NRVMs showed

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significantly reduced coupling efficiency ($p<0.005$), as well as increased proton leak ($p<0.05$) and increased non-mitochondrial oxygen consumption ($p<0.05$) (Suppl. Figure 8E) compared to controls. Cell energy phenotype analysis suggested that LonP1-KD promoted a metabolic shift, resulting in increased glucose utilization (Suppl. Figure 8F).

3.6. LonP1 knockdown increases mitochondrial superoxide production and membrane potential.

To investigate further the effect of LonP1 knockdown, NRVMs were subjected to ROS and mitochondrial membrane potential estimation. LonP1-KD NRVMs increased superoxide generation as shown by increased MitoSOX fluorescence staining, which colocalized with MitoTracker Green labeled mitochondria (Figure 6A). Analysis of mean fluorescence intensities showed a significantly increased ratio of MitoSOX (red) to MitoTracker Green (green) fluorescence ($p<0.0001$), in LonP1-KD versus controls, demonstrating increased superoxide production (Figure 6B), which is consistent with the observed increased ETC activity. Further, flow cytometry analysis using MitoSOX red indicator showed increased superoxide generation in LonP1-KD cells demonstrated by a rightward shift in the MitoSOX signal as shown in the histogram (Figure 6C). The corresponding quantification showed LonP1-KD cells produce significantly ($p< 0.01$) more superoxide ions (Figure 6D), which is consistent with our microscopy findings (Figure 6A). In addition, LonP1-KD NRVMs showed significantly increased mitochondrial membrane potential ($\Delta\psi$) as compared to control cells ($p <0.0001$) (Figure 6E and 6F), as determined by TMRE staining. As a positive control, FCCP was used to dissipate the $\Delta\psi$ (Figure 6E), which reduced TMRE fluorescence in both control and LonP1-KD cells. Mitochondrial hyperpolarization was also demonstrated by flow cytometry using JC-1, which showed that LonP1-KD cells accumulated more aggregated JC-1 red fluorescence in mitochondria compared to control, indicating a higher mitochondrial membrane potential (Figure 6G). Upon uncoupling $\Delta\psi$ with FCCP, JC-1 red fluorescence was substantially reduced in both control and LonP1-KD cells with a coordinate increase in monomeric JC-1 green fluorescence in the cytosol (Figure 6G). Previous work has shown that hyperpolarization of $\Delta\psi$ causes a burst of ROS production, oxidative stress and cell injury [34-36]. Taken together, these findings support the notion that LonP1 plays a key role in regulating the flux of electrons through the ETC and ROS generation.

3.7. LonP1 knockdown in cardiomyocytes leads to mitochondrial dysmorphology

As LonP1-KD disrupts the stability of ETC complex subunits, oxidative metabolism, and redox homeostasis, we examined the structural integrity of mitochondria by electron microscopy (Figure 7). Strikingly, approximately 78% of LonP1-KD NRVMs showed single mitochondrial outer membranes surrounding the organelles, rather than clearly distinct outer- and inner-membranes separated by the intermembrane space as observed in control cells (Figure 7A). Moreover, ~75% of the LonP1-KD CMs showed densely packed and compressed cristae (CC), with what appear to be small, vesiculated membranous structures, which were not present in control CMs (Figure 7A). Additionally, 54% of LonP1-KD CMs showed fragmented mitochondria compared to 14% in the control cells (Figure 7B). These cells did not show the presence of electron-dense inclusions or aggregates, which have been shown in other cell types knocked down for LonP1 [13, 37].

4. Discussion

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In this study, we demonstrate that the mitochondrial LonP1 protease is an endogenous cardiac stress response protein that mitigates I/R induced cardiac injury *in vivo*. LonP1 combats oxidative stress-induced damage and prevents cardiomyocyte apoptosis by reprogramming bioenergetics during I/R, thus reducing myocardial infarct size (Figure 8). LonP1 is transcriptionally upregulated by ischemic preconditioning (IPC) (Figure 1), a procedure shown to protect the heart from subsequent I/R insult [38, 39]. Furthermore, IPC-mediated cardioprotection in wild-type mice is abrogated by LonP1 haploinsufficiency (Figure 2B). Previous work has shown that IPC-dependent cardioprotection is abolished in heterozygous knockout mice partially deficient for the hypoxia-inducible factor 1 α (HIF-1 α) [40]. In that study, HIF-1α was required to trigger levels of ROS in mitochondria that are cardioprotective [40], needed to activate signal transduction pathways mediating IPC [41]. Interestingly, HIF-1 α has also been shown to up-regulate LonP1 transcription, promoting adaptation to oxygen deprivation in a variety of cells lines cultured *in vitro* [11]. This prompted us to hypothesize that the upregulation of myocardial LonP1 *in vivo* may be a direct endogenous cardioprotectant. Consistent with this notion, the overexpression of LonP1 in LonTg mice reduces myocardial death and infarct size, and the partial expression of LonP1 in heterozygous knockout *LONP1*$^{+/-}$ mice exacerbates left ventricular injury.

We propose that LonP1 mediates cardioprotective mechanisms during acute ischemia and early reperfusion thus limiting myocardial death, by abrogating oxidative stress, maintaining mitochondrial redox balance, and reprogramming bioenergetics. In LonTg hearts during acute phases of ischemia and reperfusion, we observed a striking reduction in oxidative damage and the preservation of redox status (Figure 3A and 3C). In LonTg hearts, lipid peroxidation and oxidative protein modification were substantially reduced during ischemia and early reperfusion, as compared to NTg mice (Figure 3A and 3B). A protein quality control function of LonP1 is the elimination of oxidatively damaged proteins, thus preventing their accumulation and aggregation. In addition, LonP1 may participate in a stress response network, which regulates metabolic function, permitting mitochondria to withstand or adapt to acute oxidative stress. Intriguingly, we observed in LonTg hearts that mt-aconitase activity was increased 2.5 fold during ischemia (45 min) and early reperfusion time points (10 and 30 min) (Figure 3C and 3D). This is in contrast to NTg controls hearts, which showed no substantial increase but a decline in mt-aconitase activity at 120 min reperfusion. Published *in vitro* findings suggested that LonP1 selectively degrades oxidized mt-aconitase [42]. However, our *in vivo* findings and those of others [27] have not observed LonP1-dependent degradation of mt-aconitase during an I/R time course (Figure 3C and 3D). This difference may be because mt-aconitase is not oxidatively modified *in vivo* during early I/R, even though other proteins are carbonylated [27] and lipids undergo peroxidation within mitochondria (Figure 3A). Mt-aconitase activity is a sensitive redox sensor of oxidative stress caused by elevated levels of reactive oxygen and nitrogen species in cells [43]. Previous work has demonstrated the oxidative inactivation of mt-aconitase by superoxide and hydrogen peroxide causes the release of free iron(II) from its $[4Fe\text{-}4S]^{2+}$ cluster [44]. Free iron catalyzes the formation of hydroxyl radicals, which are highly reactive and damaging [29]. The increased activity of mt-aconitase in LonTg may prevent its oxidation and the release of free iron, thereby thwarting the surge of hydroxyl radical formation during reperfusion. Further work is needed to determine the mechanism(s) by which LonP1 regulates cardiac mt-aconitase activity, which may promote the metabolic reprogramming of cardiomyocytes. Increased mt-aconitase activity is expected to influence the levels of citrate, which is either fed into the TCA cycle or transported to the cytosol where it can activate lipid synthesis and inhibit glycolysis during I/R [45].

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Complex I and III have been shown to produce large amounts of ROS during I/R [3, 46]. Superoxide generation by Complex I during the early phases of reperfusion contributes substantially to myocardial I/R injury [3]. The accumulation of succinate during ischemia is rapidly re-oxidized during reperfusion, resulting in extensive superoxide generation by reverse electron transport at Complex I [3]. Our findings show that in LonTg hearts, there is a significant reduction of Complex I subunits at baseline (Ndufs1), and also immediately after ischemia and throughout reperfusion (Ndufa9 and Ndufb8) (Figure 3E and F). LonP1 is one of the major mitochondrial proteases, which plays an important role in metabolic programming by remodeling OXPHOS complexes and modulating OXPHOS activity [11, 14, 47]. For example, hypoxia transcriptionally upregulates LonP1 leading to the selective degradation of the Cox4-1 subunit of Complex IV cytochrome c oxidase (COX), permitting the assembly of an alternate subunit, Cox4-2, into the COX complex [11]. Such subunit switching within the COX complex is thought to be an adaptive mechanism to maximize the efficiency of respiration during reduced oxygen availability. In addition, LonP1 overexpression in melanoma cells significantly decreases Complex I, II and IV levels leading to decreased OXPHOS and increased glycolysis at normal oxygen tension [14]. This is accompanied by a decrease in all ETC complex activities as well as their content [14]. The specific overexpression of LonP1 in melanoma cells reduces Complex I activity by degrading catalytic subunits of Complex I such as NDUFV1, NDUFV2, NDUFS3, and NDUFS7. However, in other cancer cells such as cervical carcinoma (HeLa) and neuroblastoma (SH-SY5Y), LonP1 degrades certain peripheral arm subunits of Complex I such as NDUFS1, NDUFV1, NDUFA9, and NDUFV2 only upon mitochondrial depolarization but not at basal or normal conditions [47]. Interestingly, our data in the heart shows that overexpression of LonP1 reduces Complex I level and activity even at the basal conditions. Even at baseline, the Complex I subunit Ndufs1 was present at a significantly ($p<0.05$) lower levels in LonTg hearts compared to NTg (Figure 3E and F), which is accompanied by reduced Complex I activity (Figure 3G and H). Such reduction in Complex I activity in LonTg was not apparently deleterious as we did not observe any abnormal phenotypes or cardiovascular events. We hypothesize that this reduction in Complex I activity during reperfusion may reduce the generation of superoxide ions and thus limit cardiac stress and injury. Our observation is consistent with a recent report by Zhang et. al, showing that the cardiac-specific knockout of Complex I in mice, have ~70% less Complex I activity than control mice, but do not show any abnormal phenotype [48]. Furthermore, these mice are protected from I/R-induced injury, which is attributed to reduced levels of ROS [48].

Reduction in both the membrane and peripheral arm subunits of Complex I may reduce Complex I activity, as Complex I degradation is shown to abolish mitochondrial ROS generation during stress conditions [47]. The reduction in Complex I levels in LonTg hearts is coincident with reduced levels of oxidatively modified proteins and increased mt-aconitase activity (Figures 3A and 3C). We speculate that the down-regulation of Complex I by LonP1 during reperfusion may provide a mechanism for limiting electron flow between Complexes I and II, thus attenuating superoxide generation [3, 49]. This possibility is supported by our *in vitro* findings showing that LonP1 depletion in NRVMs up-regulates Complex I subunits, and increases the activities of Complex I and succinate-driven Complex II, while also augmenting superoxide anion levels [31, 50]. Further support for this proposition, is provided by studies demonstrating that direct pharmacologic inhibition of Complex I by rotenone [51], metformin [52], or amobarbital [53] blocks ROS production and prevents reperfusion injury. Taken together, these data suggest that LonP1 may remodel Complex I and its activity to modulate superoxide production, minimizing excessive ROS generation and ROS-induced damage to the myocardium.

Changes in OXPHOS complex assembly and complex interactions may underlie the striking

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dysmorphology of mitochondrial inner membranes in LonP1 knockdown NRVMs (Figure 7). Notably, LonP1-KD in NRVMs did not lead to depolarization of mitochondria but rather a hyperpolarization, which is consistent with the increased ETC activities. Previous studies have reported that hyperpolarization of the mitochondrial membrane potential is associated with the burst of ROS production during reperfusion, leading to apoptosis and/or necrosis [34-36]. Findings obtained from NRVMs cannot be directly translated to adult cardiomyocytes because of differences in mitochondrial energetics and cell death mechanisms, which occur during cardiac development. Thus, future global experiments conducted in adult cardiomyocytes are warranted to determine whether LonP1 is directly responsible for specifically regulating the assembly state, activity and interactions of OXPHOS complexes by directly degrading specific Complex subunits during I/R.

In conclusion, this study shows that LonP1 ensures mitochondrial integrity during acute ischemia and early reperfusion *in vivo,* thereby mediating cardioprotection against I/R injury. LonP1 is induced by acute stress and disease states as observed in various experimental systems and disease models [54, 55]. However, during chronic stress and aging, the inducibility of LonP1 appears to decline [55]. By contrast, exercise up-regulates LonP1 [20], and caloric restriction is found to prevent the age-associated decrease in LonP1 expression [22]. We speculate that developing specific strategies to boost LonP1 activity (e.g. exercise, diet, small molecule activation), may play a protective role by mitigating myocardial injury in cardiovascular diseases such as chronic ischemia, myocardial infarction, dilated cardiomyopathy and congestive heart failure.

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Disclosures

None.

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Figure 1

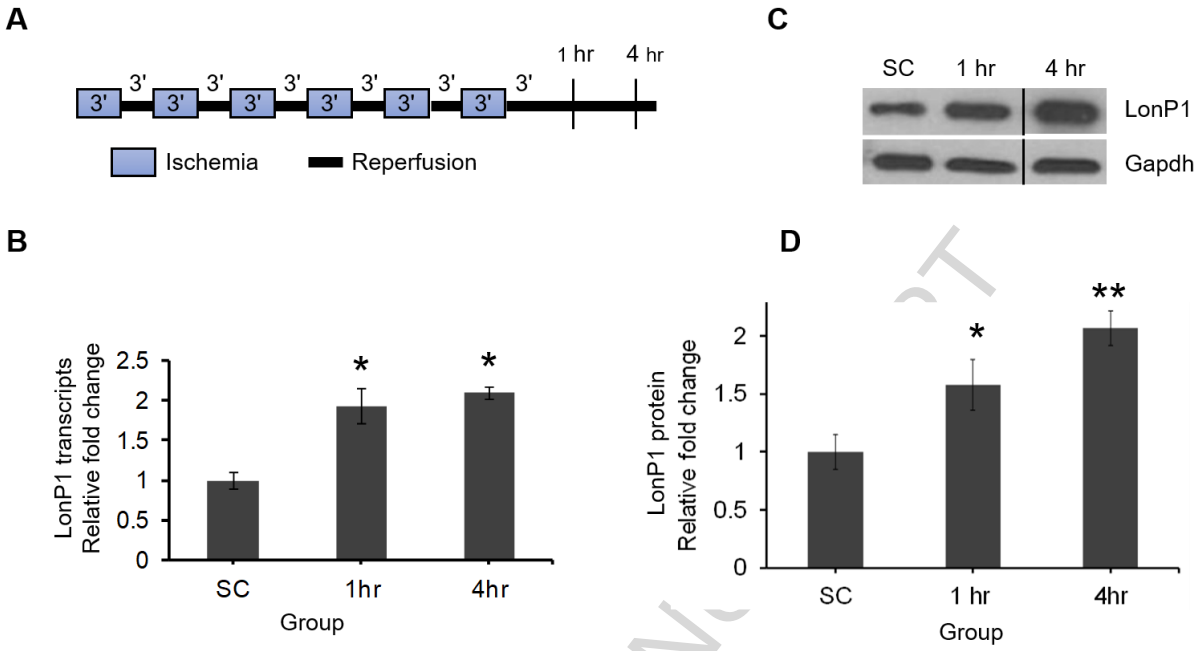

Figure 1. LonP1 is upregulated by ischemic preconditioning (IPC). **(A)** Schematic representation of the IPC procedure using 3 months old C57BL/6 male mice, which entailed 6 cycles of surgically-induced cardiac ischemia (3 min), followed by reperfusion (3 min). Sham control (SC) mice were subject to the same surgical procedures without cycles of ischemia and reperfusion. At 1 and 4 h post-IPC, the left ventricles were harvested for protein and transcript analysis. **(B)** Relative fold change in LonP1 transcript levels after 1 and 4 h post-IPC surgery (n=3, each time point). Gapdh was used as an endogenous control **(C)** Representative immunoblot of LonP1 and Gapdh after 1 and 4 h post-IPC surgery. The lanes were run on the same gel but were noncontiguous. **(D)** Relative fold change of LonP1 protein levels normalized to Gapdh, quantified by ImageJ (n=3, each time point). All values are mean ± S.E.M. *P <0.05, **P <0.01 is significant compared to sham control. Statistical significance was calculated by one-way ANOVA followed by post test.

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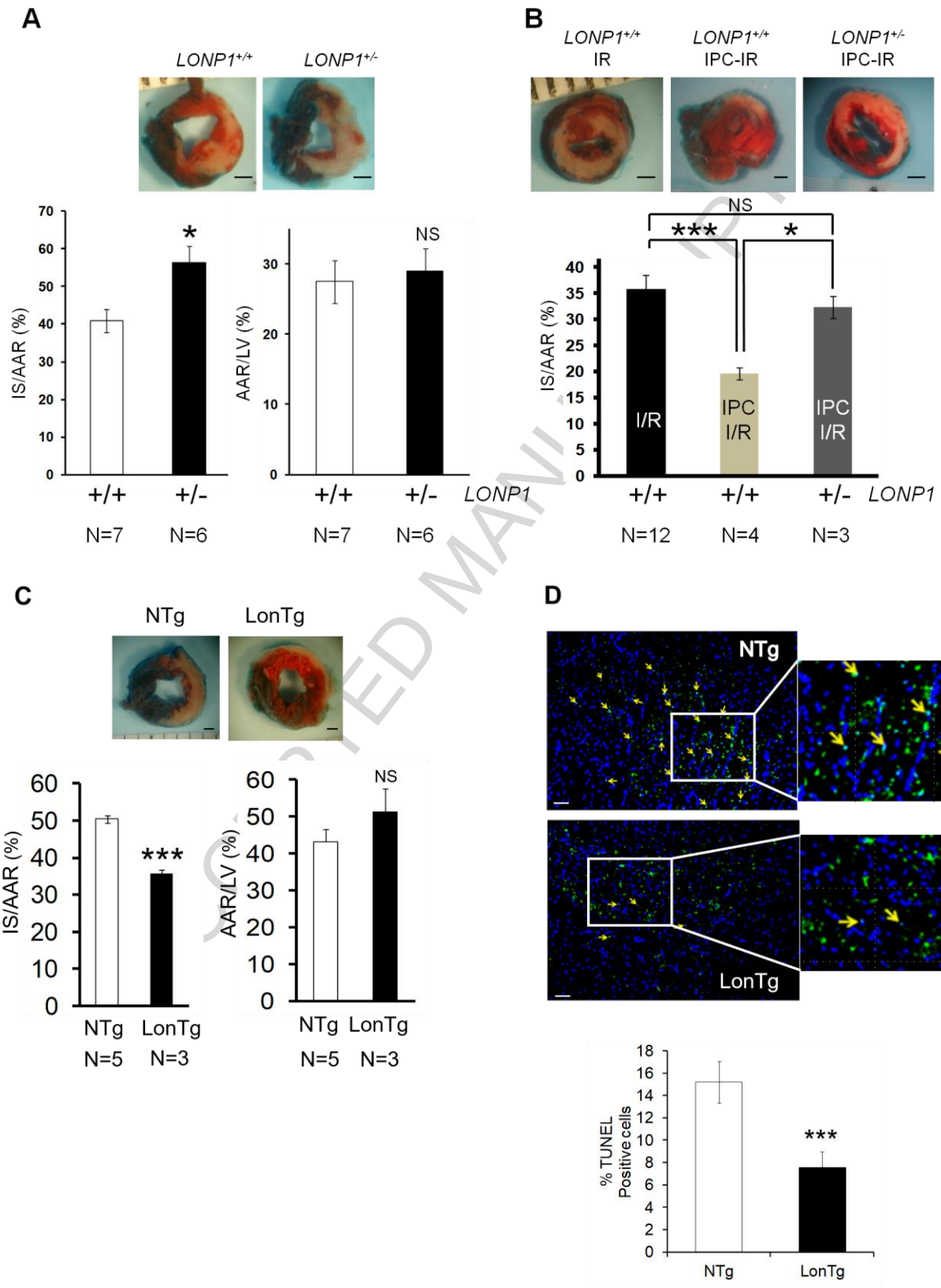
Figure 2



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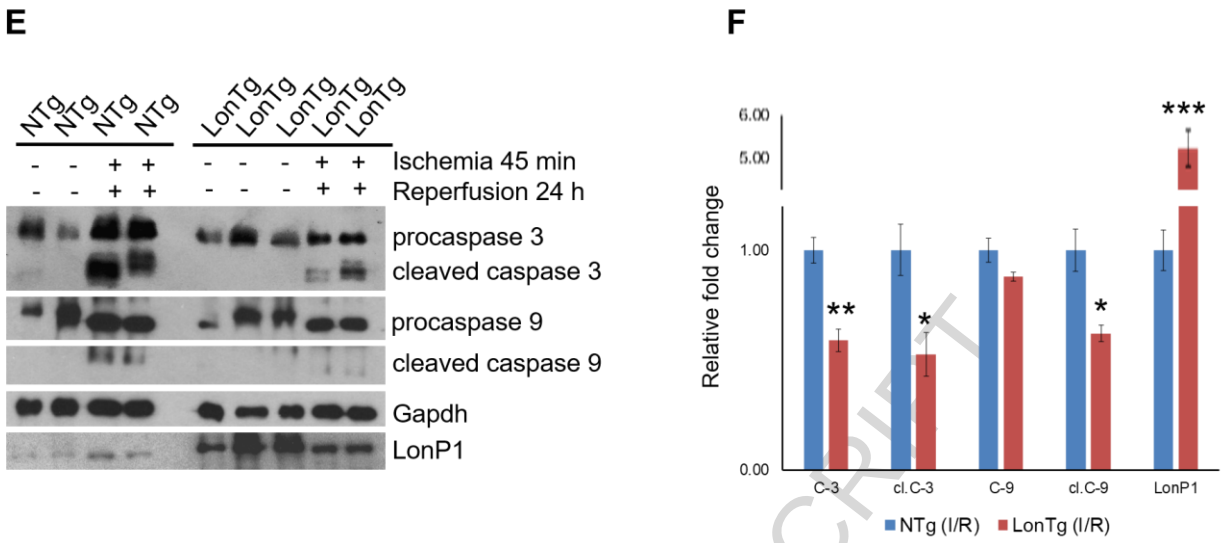

Figure 2. LonP1 mitigates cardiac injury and promotes cardioprotection in vivo. (A) Top panels- representative images of alcian blue and triphenyl tetrazolium chloride (TTC) stained heart sections of LONP1$^{+/+}$ and LONP1$^{+/-}$ male mice after ischemia (30 min) and reperfusion (24 h). Scale bar: 1mm. Bottom panel, left- quantitative measurements of percent myocardial infarct size (IS)/area at risk (AAR) (% IS/AAR) from the heart sections of LONP1$^{+/+}$ and LONP1$^{+/-}$ male mice, (age 6 – 7.5 months old). Bottom panel right- quantitative measurements of percent AAR/left ventricle area (% AAR/LV), of heart sections from LONP1$^{+/+}$ and LONP$^{+/-}$, male mice (age, 6 – 7.5 months). **(B)** Top panels- representative images of alcian blue and TTC stained heart sections of LONP1$^{+/+}$ male mice subjected to I/R (age 6 - 7.5 months old), IPC-I/R (age 3.5 - 4.5 months), and LONP1$^{+/-}$ male mice subjected to IPC-I/R (3.5 - 4.5 months). Scale bar: 1mm. Bottom panel - quantitative measurements of percent AAR/left ventricle area (% AAR/LV) of corresponding groups. **(C)** Top panels - representative images of alcian blue and TTC stained heart slices from NTg and LonTg mice subjected to ischemia 45 min and reperfusion 24 h (age 7.7-8.5 months old). Scale bars: 1mm. Bottom left- Quantitative measurements of percent % IS/AAR. Bottom right- % AAR/LV. **(D)** Representative images of TUNEL analysis from NTg and LonTg hearts subjected to I/R showing positive cells colocalized with nuclei (DAPI) (yellow arrow). Bottom- Quantification of percentage TUNEL positive cells of NTg and LonTg group (n=3). Scale bar is 50 µm **(E)** Immunoblot analysis of apoptotic markers, cleaved caspase-9 and -3 probed from the left ventricular protein extracts of NTg (n=4) and LonTg mice (n=5) subjected to no I/R or I/R. **(F)** Densitometry quantification of apoptotic markers comparing NTg vs. LonTg after I/R. All values are the mean ± S.E.M. * P<0.01, **P<0.001, *** P<0.0001 by student's 't' test.

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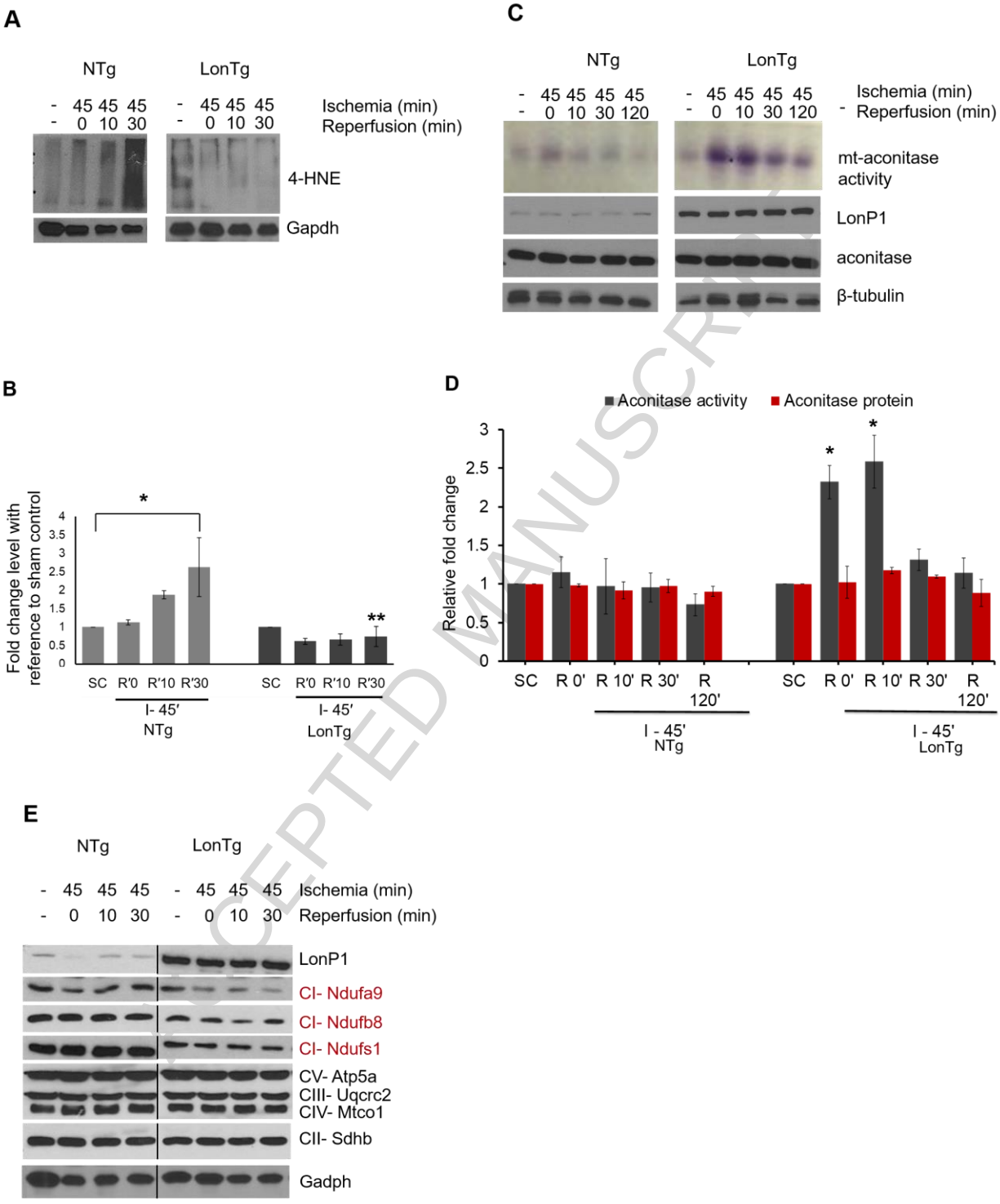
Figure 3



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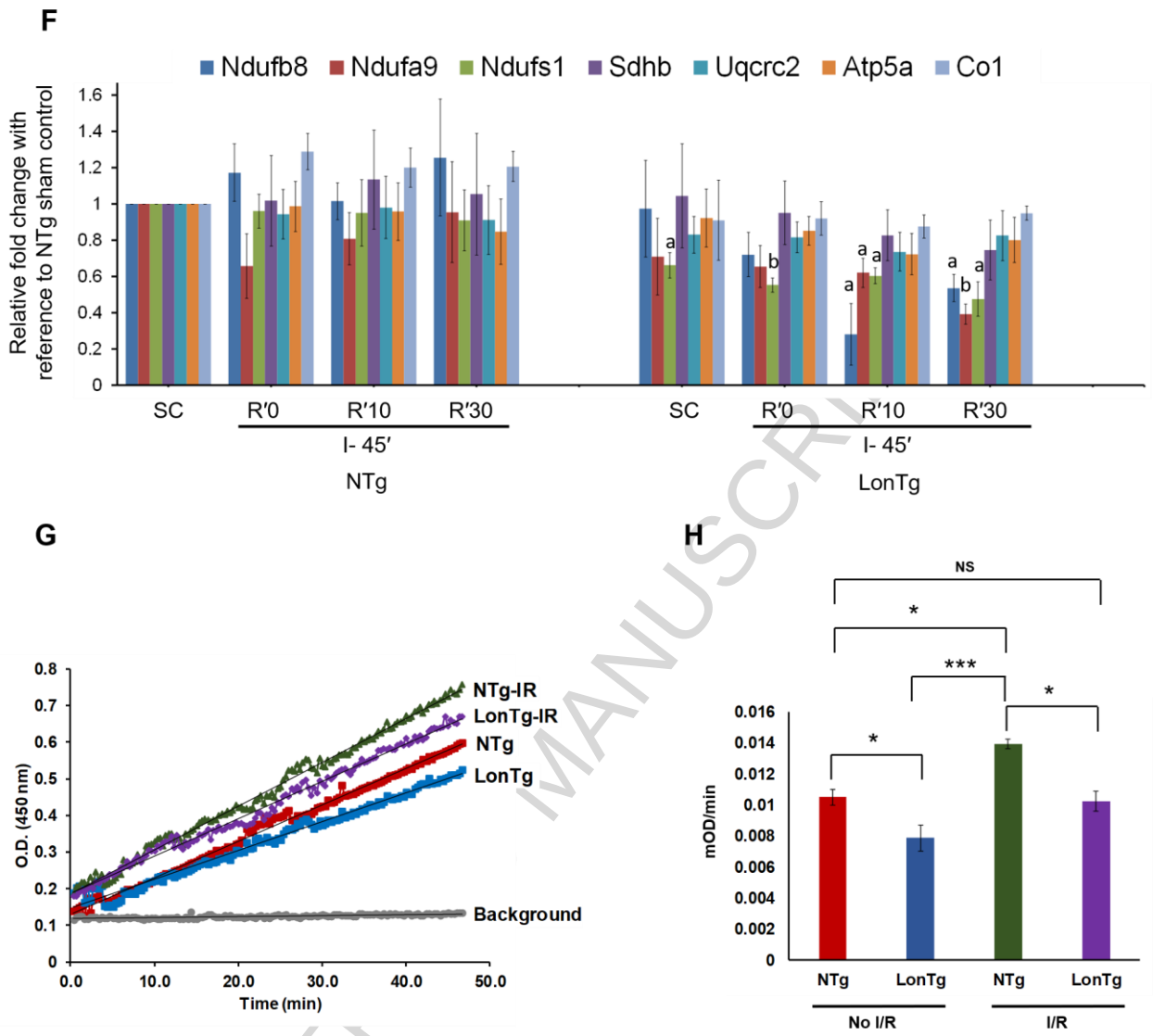

Figure 3. LonP1 preserves cardiac redox status by attenuating Complex I activity and I/R induced oxidative stress in vivo. **(A)** Representative immunoblot of protein extracts from the left ventricles of NTg and LonTg male mice (age, 6.5 months) subjected to ischemia (45 min), followed by a time course of reperfusion (0, 10, 30 min), probed with antibodies recognizing 4-HNE and Gapdh (n=3). **(B)** Relative fold change of 4-HNE level with reference to the respective sham control. *p<0.05 is significant compared to NTg sham control and **p<0.01 is significant compared to corresponding NTg time point (NTg-30 min). **(C)** In-gel mt-aconitase activity from the left ventricle of NTg and LonTg mice subjected to an I/R time course and representative immunoblots of the corresponding protein extracts were probed for LonP1, mt-aconitase, and β-tubulin (n=3). **(D)** Relative fold change of aconitase activity and protein level with reference to their respective sham control. *p<0.05 is considered as significant compared to SC **(E)** Representative immunoblots of protein extracts from the left ventricles of NTg and LonTg mice subjected to I/R time course as shown in panels A and B were probed for LonP1 and OXPHOS complex subunits. The lanes were run on the same gel but were noncontiguous. **(F)** Relative fold change of Complex subunits compared to the NTg sham control (n=3). The complex I subunit Ndufs1 was significantly (p<0.05) reduced in LonTg sham control at basal condition compared to NTg SC. This significant reduction was maintained during all reperfusion time

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course (10 and 30 min) in LonTg hearts. However, other Complex I subunits ndufb8 and ndufa9 levels in LonTg hearts were significantly reduced ($p<0.05$) during 10- and 30-mins reperfusion phase, where ndufa9 is further reduced very significantly at 30 min reperfusion time ($p<0.01$). All values are Mean ± S.E.M. [a] $p<0.05$, [b] $p<0.01$ is significant compared to their respective NTg sham control groups. Statistical significance was calculated by oneway ANOVA followed by post tests. **(G)** Complex I activity in cardiac tissue extract from NTg (n=5), LonTg (n=4), NTg-I/R (n=3) and LonTg-I/R (n=5) mice. **(H)** Complex I activity was calculated and represented as a change in optical density per min. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by one way ANOVA followed by post test.

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Figure 4

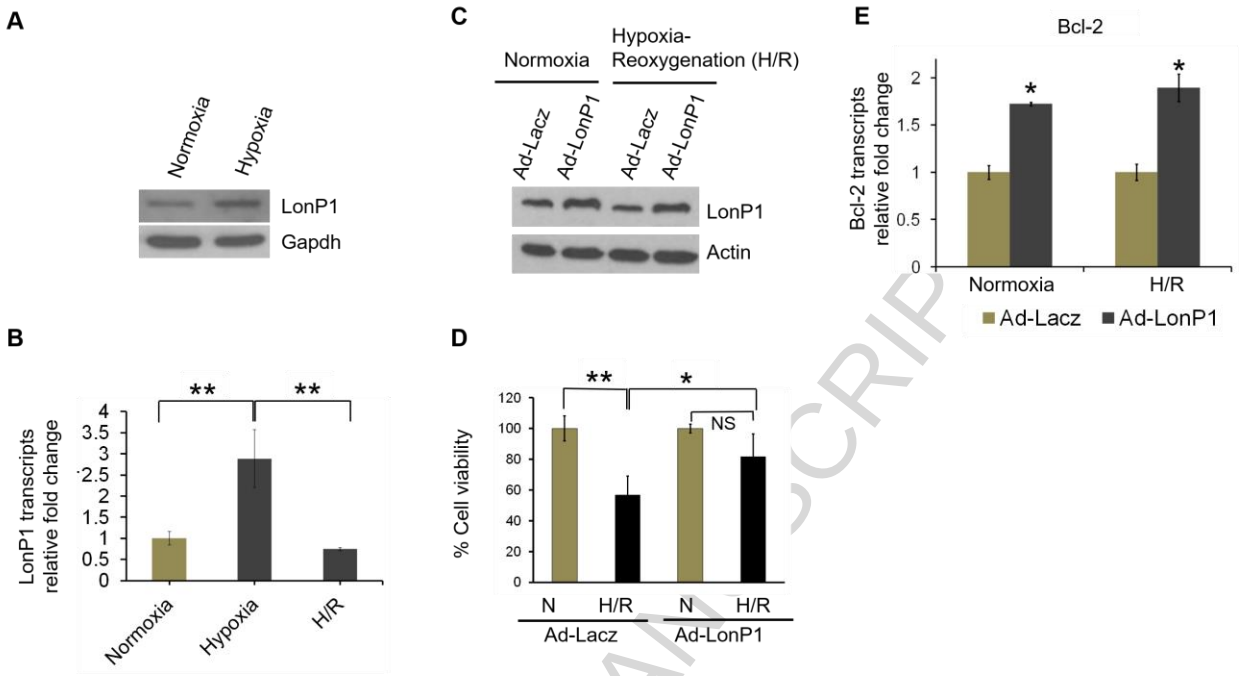

Figure 4. LonP1 ameliorates hypoxia/reoxygenation-induced cell death in primary neonatal rat ventricular myocytes (NRVMs). (A) Representative immunoblot of the protein extracts from NRVMs subjected to normoxia or hypoxia for 20 h. **(B)** Relative fold LonP1 transcript levels in NRVMs subjected to hypoxia (26 h), and hypoxia (20 h)-reoxygenation (6 h) versus normoxic conditions for 26 h (n=3). Gapdh/18S transcripts were used as an endogenous reference (n=3). **(C)** Representative immunoblot of protein extracts from NRVMs transduced with Ad-LacZ or Ad-LonP1 for 2 days and then subjected to hypoxia for 20 h followed by reoxygenation for 2 h (H/R) or normoxia for 22 h. **(D)** Quantification of percent viability of NRVMs after H/R, with reference to normoxic controls (n=3). **(E)** Relative fold change in Bcl-2 transcript levels in NRVMs transduced with Ad-Lacz or Ad-LonP1 for 2 days (n=3). All values are mean ± S.E.M. * P <0.05, ** P <0.01 is significant by one-way ANOVA followed by post test. For Bcl-2 comparison, student's t test was employed. * P <0.05 is significant compared to Ad-LacZ at normoxia or H/R.

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Figure 5

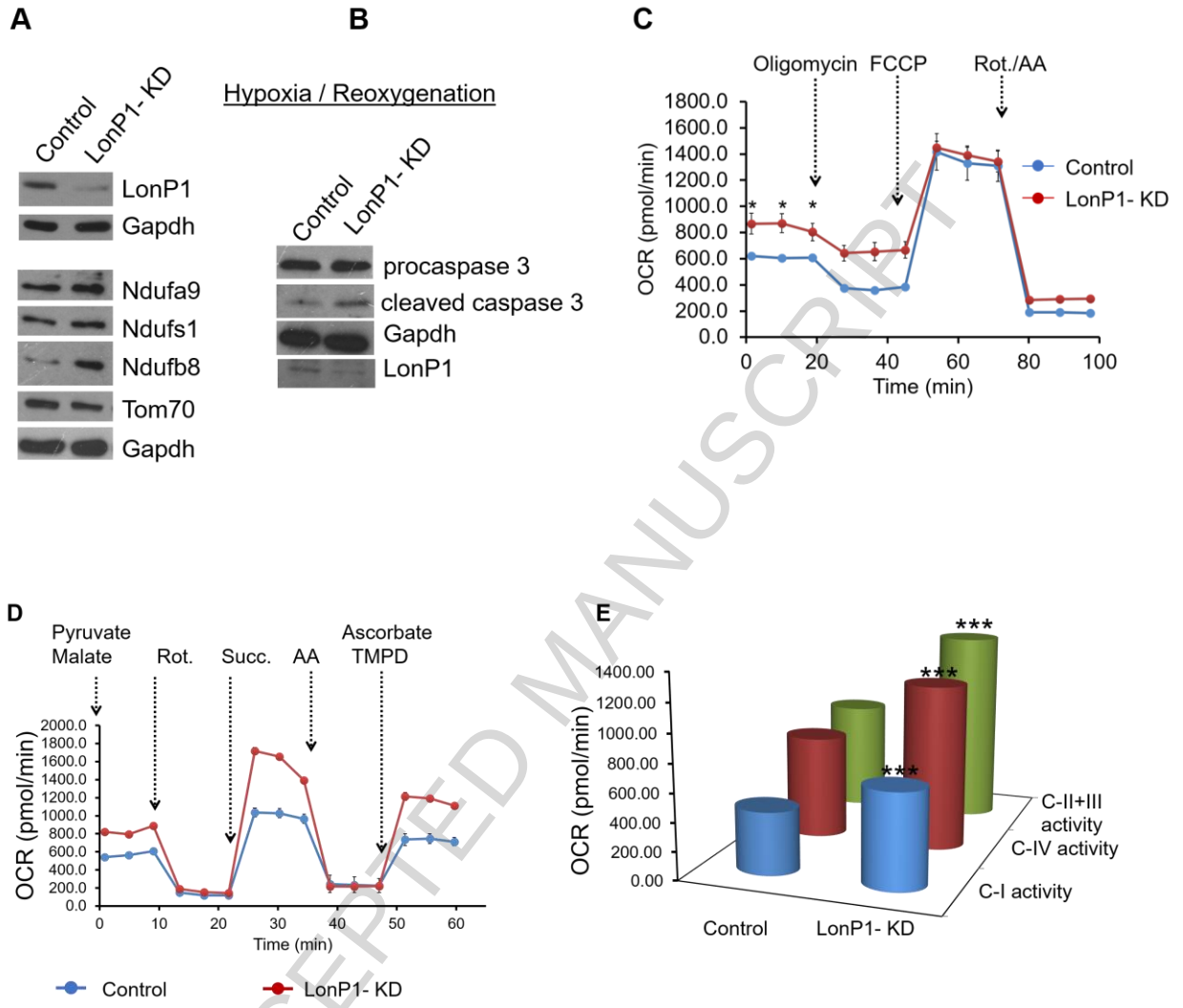

Figure 5. LonP1 is required for mitochondrial coupling and respiratory efficiency. (A) Representative immunoblot of protein extracts from Ad-control shRNA (Control) or Ad-LonP1 shRNA (LonP1-KD) transduced NRVMs (4-5 days) probed for LonP1, Gapdh, Tom70 and complex I subunits. **(B)** Immunoblot of protein extracts from control and LonP1-KD NRVMs subjected to H/R probed for procaspase 3, cleaved caspase 3, Gapdh and LonP1. **(C)** Oxygen consumption rate was monitored in live cells from both control and LonP1-KD NRVMs at baseline, and then after sequential injection of oligomycin (1 µM), FCCP (3 µM), and rotenone/antimycin (1 µM each) (n=3). **(D)** Representative measurements of OCR from *in situ* analysis of Complex I, II+III and IV activities in the isolated NRVMs transduced with Ad-control or Ad-LonP1 shRNA for 4-5 days by supplying complex specific substrates and inhibitors as shown in the figure. **(E)** Complex specific activities were quantified and shown as a measurement of OCR.

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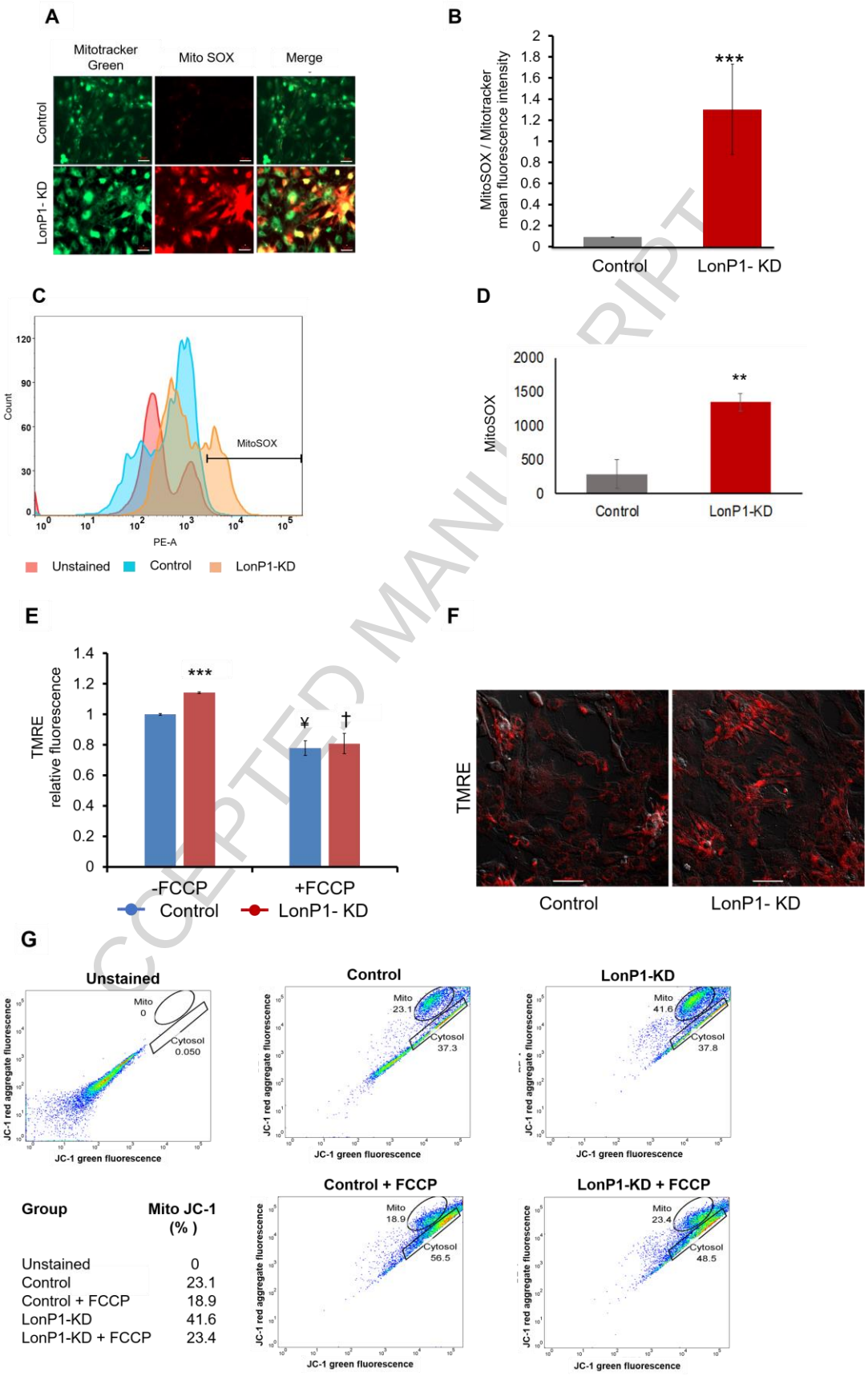
Figure 6



Group	Mito JC-1 (%)
Unstained	0
Control	23.1
Control + FCCP	18.9
LonP1-KD	41.6
LonP1-KD + FCCP	23.4

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Figure 6. LonP1 downregulation increase mitochondrial superoxide level and membrane potential. (A) Live cell staining of control and LonP1-KD NRVMs for MitoSOX Red (superoxide) and Mito Tracker Green (mitochondria). Co-localization of MitoTracker Green with MitoSox Red is shown in the merged images. Scale bar is 50 μm. **(B)** Quantification of the ratio of mean MitoSOX fluorescence intensity to mean MitoTracker green fluorescence intensity of 100 cells in each group analyzed by using Zen lite software (ZEISS). **(C)** Representative histogram of MitoSOX analysis by flow cytometry. LonP1-KD NRVMs show a right shift of MitoSOX fluorescence. **(D)** Quantification of MitoSOX positive cells from control and LonP1-KD NRVMs. **E)** Relative TMRE fluorescence of LonP1-KD compared to control. **(F)** Representative Images of control and LonP1-KD NRVMs (4 days) stained with TMRE. Scale bar is 50 μm. **(G)** Flow cytometry analysis of mitochondrial membrane potential by JC-1 dye. LonP1-KD NRVMs show higher percent JC-1 mitochondrial aggregates (41.6%) compared to control (23.1%). Aggregated JC-1 red fluorescence emission at 590 nm was recorded in the PE-A channel and monomeric JC-1 green fluorescence emission at 529 nm was recorded in FITC channel. Upon FCCP treatment (positive control), JC-1 fluorescence substantially shifted to the cytosol in both control and LonP1-KD NRVMs. Data values are mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control, †$p<0.05$ compared to control with no FCCP treatment, ¥ $p<0.05$ compared to LonP1-KD with no FCCP treatment. Significance was determined by student's 't' test.

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Figure 7

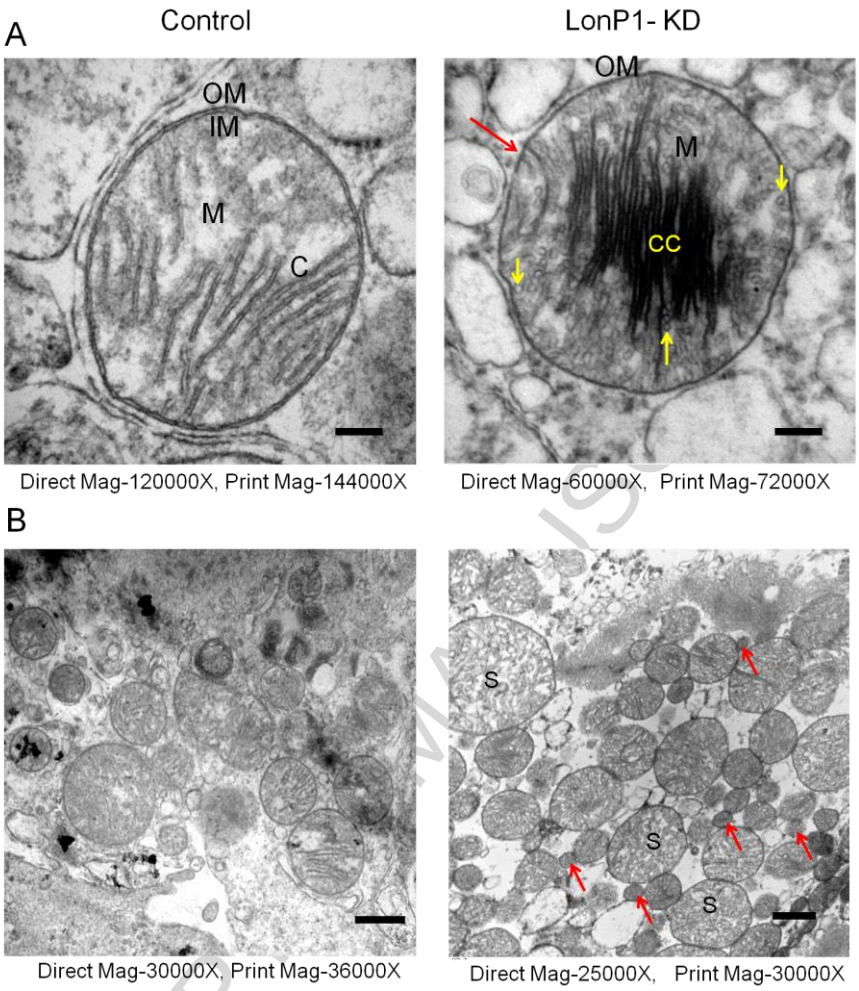

Figure 7. LonP1 is required for mitochondrial structural integrity. Transmission electron microscope (TEM) images. **(A)** Higher magnification. Left- control NRVMs, scale bar: 100nm, right- LonP1-KD NRVMs, scale bar is 200 nm. OM- outer membrane, IM- inner membrane, C- cristae, M-mitochondrial matrix. Red arrow- compressed OM, compressed cristae (CC), and yellow arrow- vesiculated membranous structures. **(B)** Lower magnification. Left- control NRVMs with uniformly sized mitochondria, scale bar: 600 nm. Right- LonP1-KD NRVMs show numerous fragmented small (red arrow) and swollen (S) mitochondria. Scale bar: 600nm. Direct and print magnifications are shown below in the respective images.

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Figure 8

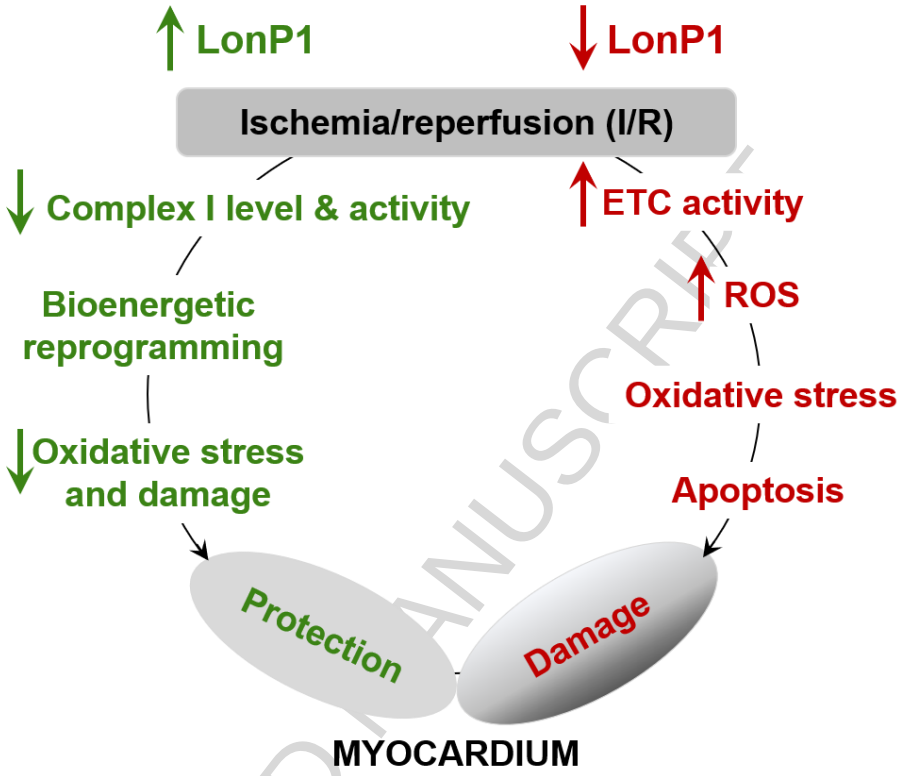

Figure 8. The functional role of LonP1 in the heart during ischemia-reperfusion injury

Highlights

- LonP1 haploinsufficiency ablates IPC-mediated cardioprotection
- Cardiac-specific LonP1 overexpression decreases I/R injury
- LonP1 downregulates Complex I subunits and activity during I/R
- LonP1 maintains mitochondrial redox status during I/R
- LonP1 reduces oxidative stress during I/R

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