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DJ-1 Protects the Heart Against Ischemia-Reperfusion Injury by Regulating Mitochondrial Fission

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Abstract

Recent data indicates that DJ-1 plays a role in the cellular response to stress. Here, we aimed to examine the underlying molecular mechanisms mediating the actions of DJ-1 in the heart following myocardial ischemia-reperfusion (I/R) injury. In response to I/R injury, DJ-1 KO mice displayed increased areas of infarction and worsened left ventricular function when compared to WT mice, confirming a protective role for DJ-1 in the heart. In an effort to evaluate the potential mechanism(s) responsible for the increased injury in DJ-1 KO mice, we focused on SUMOylation, a post-translational modification process that regulates various aspects of protein function. DJ-1 KO hearts after I/R injury were found to display enhanced accumulation of SUMO-1 modified proteins and reduced SUMO-2/3 modified proteins. Further analysis, revealed that the protein expression of the de-SUMOylation enzyme SENP1 was reduced, whereas the expression of SENP5 was enhanced in DJ-1 KO hearts after I/R injury. Finally, DJ-1 KO hearts were found to

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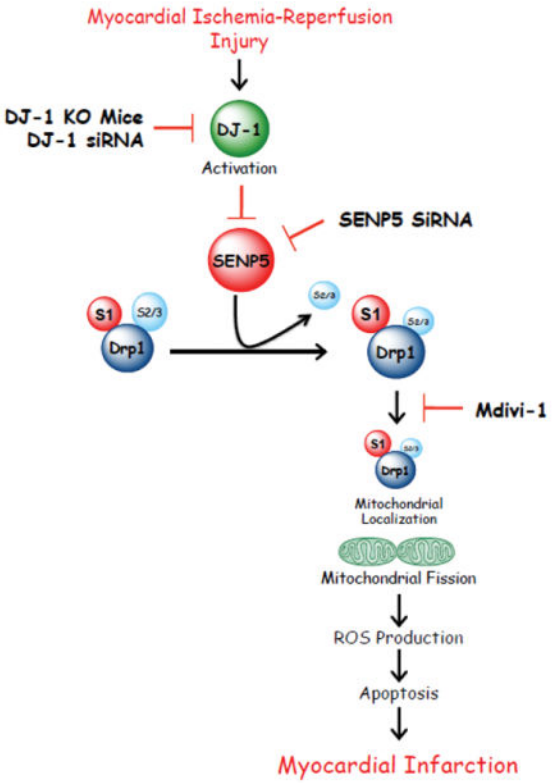
display enhanced SUMO-1 modification of dynamin-related protein 1, excessive mitochondrial fission, and dysfunctional mitochondria. Our data demonstrates that the activation of DJ-1 in response to myocardial I/R injury protects the heart by regulating the SUMOylation status of Drp1 and attenuating excessive mitochondrial fission.

Graphical Abstract



Keywords

DJ-1; myocardial ischemia; mitochondrial fission; SUMOylation

1. Introduction

DJ-1, also knows as Park7 (Parkinson's Disease autosomal recessive, early onset 7), is an evolutionarily conserved 189-amino acid protein [1]. Although originally identified as on oncogene [2], it is now recognized that DJ-1 promotes cytoprotection in response to various pathological stimuli. For instance, in the brain, the deletion or loss of function of DJ-1 is associated with autosomal recessive, early-onset Parkinson's Disease [3]. Additionally, *in vitro* and *in vivo* models show that over-expression of DJ-1 protects cells against oxidative stress-induced injury, whereas knockdown or knockout of DJ-1 increases susceptibility to oxidative injury in models of cerebral ischemia and neuronal cell death [4, 5]. In regards to mechanisms of action, these studies indicate that DJ-1 plays an important role in multiple cellular processes, including oxidative stress response, protein quality control, anti-apoptotic

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signaling, and transcriptional regulation [1, 5, 6]. Due in large part to the association of DJ-1 with Parkinson's Disease, most studies aimed at investigating its role in response to pathological stimuli have been confined to the brain or neuronal cells. However, DJ-1 is expressed in many other tissues, including the heart [7]. Two recent studies report that mice deficient in DJ-1 develop more severe heart failure in response to aortic banding [8] and display exaggerated myocardial injury in response to ischemia [9]. While these studies provide evidence that DJ-1 plays a protective role in the heart, its mechanism of action remains unclear.

Post-translational modifications (PTMs) are essential for controlling the function and stability or proteins. As such, PTMs regulate cell fate under physiological and pathological conditions. SUMOylation is a PTM process in which small ubiquitin-like modifier (SUMO, also called Sentrin) proteins are covalently and reversibly conjugated to target proteins [10]. In recent years, SUMOylation has been shown to regulate and influence a number of cellular processes, including cell cycle regulation, apoptosis, epigenetic regulation, and transcription [11]. It has also been reported to play a role in several disease states, such as cancer and cerebral ischemia [12]. In the heart, SUMOylation contributes to normal cardiac development and function [13]. It also plays a role in the adaptation of the heart to pathological stress [14, 15]. Mammalian cells express three isoforms of SUMO that can be covalently conjugated to proteins: SUMO-1, SUMO-2, and SUMO-3 [16]. Because SUMO-2 and SUMO-3 are nearly homologous (~97% identical) and cannot be distinguished from each other under most contexts, they are collectively referred to as SUMO-2/3. In contrast, SUMO-1 shares very little homology to SUMO-2/3 (~47% identical) [13]. Whereas SUMO-1 and SUMO-2/3 share some overlap in the modifications of certain proteins, each has a distinct pool of targets, suggesting that they may play different role in cellular processes [13, 17]. A key feature of protein SUMOylation is its reversibility by a family of Sentrin/SUMO-specific proteases (SENPs), whose activity is relatively specific for distinct SUMO proteins [18]. For instance, SENP1 targets all SUMO isoforms for deconjugation, whereas SENP5 preferentially target SUMO-2/3 isoforms [19]. Importantly, cellular homeostasis is dependent on balancing SUMOylation with de-SUMOylation as evidenced by studies demonstrating that tipping the balance either way results in aberrant signaling and pathological conditions [14, 15]. Therefore, understanding the cellular mechanisms by which the SUMO machinery is regulated under physiological and pathological conditions has become an important area of research.

DJ-1 has been implicated as a cellular inhibitor of SUMO-1 modifications in human dopaminergic cell lines [10]. However, the mechanism(s) responsible for this inhibition are not known and it is not known if DJ-1 alters SUMO modifications *in vivo*. Moreover, the cellular mechanisms that underlie the reported cardioprotective actions of DJ-1 remain largely unknown. To clarify some of these issues, we examined the actions of DJ-1 in the heart using an established *in vivo* murine myocardial I/R model.

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2. Materials and Methods

2.1. Animals

Male mice with a global deficiency in DJ-1 (B6.Cg-*Park7*^{tm1Shn}/J; DJ-1 KO) maintained on a C57BL/6J background, were purchased from Jackson Labs (Bar Harbor, ME). In all experiments, Wild-Type (WT) littermates were used as controls. All experimental protocol were approved by the Institute for Animal Care and Use Committee at Emory University School of Medicine and conformed to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996), and with federal and state regulations.

2.2. Materials

Mitochondrial division inhibitor, Mdivi-1 (Sigma Aldrich; St. Louis, MO) was dissolved in di-methyl sulfoxide (DMSO) and given as intraperitoneal injection at a dose of 50 mg/kg (final volume 50 μL) 15 minutes before the onset of myocardial ischemia. DMSO was administered in the same manner for the vehicle group.

2.3. Myocardial I/R Protocol and Myocardial Injury Assessment

Surgical ligation of the left coronary artery, myocardial infarct size determination, Troponin-I measurements, echocardiography and invasive hemodynamics were performed similar to methods described previously [20, 21].

2.4. Subcellular Fractionation, Western Blot Analysis

Whole cell and mitochondrial fractions were obtained from hearts excised from separate groups of mice. Whole cell homogenates were obtained as previously described [20]. Mitochondrial fractions were obtained using the Mitochondria Isolation Kit (MITOISO1, Sigma). Western blot analysis was performed as described previously [20].

2.5. Immunoprecipitation

Heart homogenates were immunoprecipitated with antibodies to either DJ-1 or dynamin-related protein 1 (Drp1) using the Dynabeads® Protein G Immunoprecipitation Kit according to manufacturer's instructions. The samples were then subjected to standard Western blot techniques and the membranes probed with antibodies to SENP1, SENP5, SUMO-1, or SUMO-2/3.

2.6. Isolation of mRNA and Taqman qPCR

RNA was isolated using the RiboPure kit according to manufacturer's instructions (Ambion). Reverse transcription was performed in a standard fashion with QuantiTect Reverse Transcription Kit (QIAGEN) supplemented with DNase treatment. Taqman qPCR was carried out according to the manufacturer's instructions using probe sets for senp1 and senp5. Analysis was carried out using the ΔΔ-CT method with 18S correction and reported as relative fold change versus WT Sham.

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2.7. Electron Microscopy

Heart tissue was dissected along the muscle fiber while immersed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2). Samples were stored in the same fixative overnight at 4°C. Samples were washed with the same buffer and post-fixed in 1% buffered osmium tetroxide, dehydrated through a graded ethanol series to 100%, and embedded in Eponate 12 resin. Ultrathin sections were cut on a Leica UC6rt ultraJMCC9711-R1 microtome at 70–80 nm and counter-stained with 4% aqueous uranyl acetate and 2% lead citrate. Sections were examined using a Hitachi H-7500 transmission electron microscope equipped with a Gatan BioScan CCD camera.

2.8. Myocyte Isolation and Immunohistochemistry

Adult cardiomyocytes were isolated and fixed as previously described [22]. Cardiomyocytes were stained with primary antibodies to α-MHC and DJ-1 followed by incubation with secondary antibodies. Coverslips were mounted using Vectashield H-1500--4′,6-Diamidino-2-phenylindole (DAPI)-containing medium (Vector Laboratories). Images were acquired on a Leica DM6000.

2.9. Neonatal Cardiomyocyte Isolation and siRNA transfection

Neonatal rat ventricular cardiomyocytes (NRVMs) were isolated from 1- to 2-day-old neonatal rat pups and cultured as a monolayer as described previously [23]. The entire ventricles were excised without contaminating atrial myocytes. After 24 hours of culture, monolayers of NRVMs were transfected with siRNA (10 μM) against: DJ-1 (Ambion, ID:s128737), SENP5 (Ambion, ID:s192872), both DJ-1 and SENP5, or scrambled sequence (negative control) (Ambion, cat#4390843). Transfections were carried out with Lipofectamine 2000 (Life Technologies, Carlsbad, CA) by incubating the NRVMs with the respective siRNA for one day. Afterwards, media containing the transfecting siRNA agent was removed from the cell monolayers, washed twice with cold PBS, and replenished with fresh 2% FBS-containing NRVM media. For all in vitro experiments, NRVMs were plated at a density of 210,000 cells per each cm^2 of surface area. Unless indicated otherwise, the NRVMs were plated and transduced in 96-well plates. Transfections were performed in a routine NRVM culture media, based on M199 with the following components: 10 mM HEPES, 0.1 mM non-essential amino acids, 3.5 mg/mL glucose, 2 mM L-glutamine, 4 μg/ml vitamin B12, 100 U/ml penicillin and heat-inactivated FBS at 10% (first two days of culture) or 2% (after two days of culture) final concentration.

2.10. Hypoxia/Reoxygenation

One cohort of cells were transferred to a hypoxic conditioned incubator with 1% O_2, while the other cohort was kept under normoxic conditions (20% O_2). After 12 hours of hypoxia, the cohort was then transferred to a normoxic incubator for 24 hours, to simulate reperfusion. Cell viability was determined with the Cell-Titer Blue Assay (Promega) according to the manufacture's instructions.

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2.11. Intracellular ROS Assay

ROS was measured using 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) as a fluorescent probe (Sigma-Aldrich). DCFH-DA is a non-fluorescent analog of fluorescein which will emit fluorescence after being oxidized by intracellular ROS. Following H/R, cells were loaded with DCFH-DA (10 μM) for 30 min, followed by washing with PBS. DCF fluorescence was detected using a fluorescence spectrophotometer with an excitation of 485 nm and an emission of 520 nm. A cohort of cells was incubated with MitoTempo. MitoTemp (10μM; Sigma-Aldrich) was added immediately after hypoxia was ended. The cells were allowed to reperfuse in normoxia for 1 day.

2.12. Analysis of mitochondrial interconnectivity

Cells were plated in four well-chambered slides. After conclusions of experiments, cells were washed once in PBS, fixed in 1% paraformaldehyde (30 minutes), washed in PBS, followed by two washes with 0.1% Triton X-100/PBS and blocked in 5% normal donkey serum (1 hour). Fixed cells were then incubated with rabbit anti-TOM20 (SantaCruz, clone sc-11415, 1:1000) overnight at 4°C. Following primary antibody incubation, cells were washed in PBS then incubated with either Alexa 488- conjugated donkey anti-rabbit secondary antibodies (Molecular Probes, Eugene, Ca). Cells were imaged for mitochondrial morphology using a Leica DM6000 with a 100X oil objective lens. 25–30 fields were imaged per well. To quantify mitochondrial morphology, custom-written macros were developed for the NIH Image J software (1.47). In brief, indices of mitochondrial interconnectivity (area/perimeter ratios) were quantified using NIH Image J macros that allow for semi-automated image analysis of individual mitochondrial particles and measure the cytosolic area per cell as previously characterized [24].

2.13. Statistics

All the data in this study are expressed as mean ± standard error (SEM). Differences in data between groups were compared using Prism 5 (GraphPad Software, Inc) with Student's paired 2-tailed t-test, one-way analysis of variance (ANOVA), or two-way ANOVA. For the one-way ANOVA, if a significant variance was found, the Dunnett test was used as the post hoc analysis. For the echocardiography data, a 2-way repeated measures ANOVA with a Bonferroni test as the posthoc analysis was used. When we compared data between the WT and DJ-1 KO mice under sham and myocardial I/R settings, we used a 2-way non-repeated measures ANOVA with a Bonferroni test as the posthoc analysis. In all cases, a p value less than 0.05 was considered statistically significant and p-values were two-sided.

3. Results

3.1. Myocardial I/R Induces the Cleavage of DJ-1

Our initial experiments confirmed the expression of DJ-1 in the heart and in isolated cardiomyocytes from adult Wild-Type mice (Fig. 1A). Further analysis revealed the uniform expression of DJ-1 throughout the adult cardiomyocyte (Fig. 1B). Exposure to oxidative stress induces the cleavage of DJ-1 at its C-terminal end, and this cleavage enhances the cytoprotective function of DJ-1 against oxidative damage [1]. Since, it was not known if the

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cleavage of DJ-1 occurs in the heart following the onset of I/R, we evaluated the expression of the full-length and cleaved forms of DJ-1 using a custom antibody that recognizes both forms [1]. Heart homogenates were obtained from mice subjected to various periods of reperfusion following ischemia (Fig. 1C–F). The expression of the full-length form of DJ-1 was elevated at the end of ischemia, but returned to near baseline levels during reperfusion. The cleaved form of DJ-1 was evident at the end of ischemia and persisted for up to 24 hours of reperfusion. A previous report indicated that DJ-1 localizes to the cytosol and nucleus, but not the mitochondria in isolated neonatal cardiomyocytes.[8] Since this study only evaluated the localization under basal conditions, we performed a series of experiments to determine if the localization of DJ-1 changed in response to myocardial I/R injury. Our analysis confirmed the previous report, as evidenced by the findings that DJ-1 was localized in the cytosolic and nuclear fractions, but not the mitochondrial fractions collected from Sham hearts (Supplemental Figure 1A–B). Early after the onset of myocardial I/R, the expression of DJ-1 redistributed from the cytosol to the nucleus and mitochondria. Additional experiments revealed that following 4 hours of reperfusion the cleaved form of DJ-1 was also localized to the mitochondria (Fig. 1G–I).

3.2. DJ-1 Deficiency Exacerbates Acute Myocardial I/R Injury

To determine if the myocardial I/R-induced activation of DJ-1 plays a protective role, DJ-1 KO and WT mice were subjected to 45 minutes of ischemia followed by 24 hours of reperfusion. Representative mid-ventricular photomicrographs of hearts from both groups are shown in Fig. 2A. DJ-1 KO mice displayed significant increases in both infarct size relative to the area-at-risk (INF/AAR) and INF relative to the left ventricle (INF/LV) when compared to WT mice (Fig. 2B). DJ-1 KO mice also displayed higher circulating levels of troponin-I when compared to WT mice (Fig. 2C). The effects of DJ-1 deficiency on LV structure and function following myocardial I/R were next evaluated in separate groups of mice using *in vivo* transthoracic echocardiography and invasive hemodynamics following 7 days of reperfusion. When compared to WT mice, DJ-1 KO mice displayed exacerbated systolic dysfunction, as evidenced by significant increases in LV end-systolic diameter (LVESD; Supplemental Figure 1) and significant lower ejection fraction and fractional shortening (Fig. 2D–E). Likewise, LV dP/dt, an index of contractility, was significantly reduced in DJ-1 KO mice, whereas an elevation of Tau provided evidence of impaired relaxation (Supplemental Fig. 1D–E).

3.3. DJ-1 Regulates Protein SUMOylation following Myocardial I/R Injury

Next, we turned our attention to evaluate the signaling mechanisms responsible for these actions. Here, we focused on SUMOylation for two main reasons. First, recent evidence implicates it as a regulator of proteins involved in apoptosis [25]. Second, DJ-1 has been shown to regulate protein SUMOylation *in vitro* [10]. Experiments were, therefore, conducted to determine if the increased injury observed in the DJ-1 KO hearts was associated with alterations in protein SUMOylation.

Using two separate antibodies specific to SUMO-1 and SUMO-2/3, respectively, we observed distinct changes in the expression of SUMO-modified proteins in the hearts of WT and DJ-1 KO mice following myocardial I/R (Fig. 3A–D). Specifically, myocardial I/R

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injury induced a significant increase in both SUMO-1 modified proteins and SUMO-2/3 modified proteins in the hearts of WT mice. In contrast, myocardial I/R injury induced a significant increase in only SUMO-1 modified proteins in the hearts of DJ-1 KO mice. In both cases, these changes were significantly different between the two strains. Protein SUMOylation is regulated through changes in the expression of the various components of the SUMO pathway [26]. Experiments were, therefore, conducted to determine if the ischemic-induced alterations in protein SUMOylation observed in the DJ-1 KO hearts were associated with changes in the expression of proteins in the SUMO pathway. First, we evaluated the expression of the SUMO proteins (Fig. 3E–F).

Myocardial I/R did not alter the protein expression of SUMO-1 in hearts from either strain. However, a significant difference in SUMO-1 expression was observed between the WT and KO hearts under basal and myocardial I/R conditions. A decrease in SUMO-2/3 protein expression was observed in WT hearts following myocardial I/R. No significant difference was observed in the KO hearts. These changes are consistent with the observed changes in protein conjugation of each SUMO protein. Next, we evaluated the expression of ubiquitin-like conjugating enzyme 9 (Ubc9), the only E2 enzyme identified in mammalians [11]. Under basal conditions, the expression of Ubc9 was found to be elevated in the hearts of DJ-1 KO mice compared to the hearts of WT mice (Fig. 4A–B). In response to myocardial I/R injury Ubc9 levels increased in the hearts of WT mice but remained unaltered in the hearts of DJ-1 KO mice. However, the levels of Ubc9 were similar in the hearts of WT and DJ-1 KO mice following myocardial I/R injury, suggesting that the alterations in protein SUMOylation were not caused by changes in the levels of Ubc9. Next, we evaluated the expression of two SUMO E3 ligases; protein inhibitor of activated STAT 2 (PIAS2) and PIAS4. Under basal conditions, the protein expression of PIAS2 and PIAS4 were the same in the hearts of WT and DJ-1 KO mice (Fig. 4A&C). In response to myocardial I/R, both proteins increased in a similar manner over basal levels in the hearts of WT and DJ-1 KO mice, suggesting that the alterations in protein SUMOylation were likely not caused by changes in the levels of PIAS2 nor PIAS4. Finally, we evaluated the protein expression of two deconjugating enzymes; sentrin-specific protease 1 (SENP1) and SENP5 (Fig. 4D–F). Under basal conditions, no significant differences in the protein expression of each SENP were observed in the hearts of WT and DJ-1 KO mice. In response to myocardial I/R injury, the expression of SENP1 increased significantly in the hearts of WT mice, but remained unaltered in the hearts of DJ-1 KO mice. In contrast, the expression of SENP5 increased in both WT and DJ-1 KO hearts following myocardial I/R, with more of an increase observed in the DJ-1 KO hearts. Together, this data suggests that the distinct protein SUMOylation patterns observed in the DJ-1 KO heart may be caused by changes in the expression of SENPs.

DJ-1 plays an important role in multiple cellular processes including transcriptional regulation. No significant differences in the gene expression of SENP1 and SENP5 were observed between either strain under basal conditions nor in response to myocardial I/R (Supplemental Fig. 2). This indicates that the changes in the protein expression of the SENPs observed in the DJ-1 KO hearts was not likely caused by changes in gene expression. There is evidence in the literature that DJ-1 stabilizes proteins in response to oxidative stress [27]. Therefore, we evaluated the interaction of DJ-1 with SENP1 and SENP5 in heart

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samples collected from WT mice (Fig. 4G–I). Under basal conditions, DJ-1 was found to interact with both SENPs. In response to myocardial I/R injury, the interaction between DJ-1 and SENP1 was significantly increased when compared to basal conditions. In contrast, the interaction between DJ-1 and SENP5 was decreased in response to myocardial I/R injury.

3.4. DJ-1 Regulates the SUMOylation Status of Drp1 following Myocardial I/R Injury

The findings above provide evidence that DJ-1 regulates global SUMOylation levels. It is important to note that although generalities can be made regarding the global SUMO status of a cell or organ, the SUMO status of individual proteins is equally important in determining the fate of said cell/organ following a stressful stimuli. SUMOylation of a specific protein can be influenced by its cellular localization, the presence of specific stimuli, or the availability and activity of SUMO conjugation/de-conjugating enzymes [26]. In this sense, different stresses increase or decrease the SUMOylation of proteins. We, therefore, turned our attention to evaluate the ability of DJ-1 to regulate the SUMOylation of a specific protein, Drp1. We focused on Drp1 for two main reasons. First, mitochondrial fission requires the activity of Drp1, a GTPase that causes scission of the mitochondrial outer membrane, resulting in fission of mitochondrial tubules into fragments [28]. Second, Drp1 is regulated predominantly by post-translational modifications. For instance, the SUMOylation status of Drp1 regulates its cellular localization, with modifications by SUMO-1 inducing mitochondrial localization [25, 29] and modifications by SUMO-2/3 preventing localization to the mitochondria [30]. Initial experiments found that the whole cell expression of Drp1 was similar in the hearts of WT and DJ-1 KO mice under both basal and I/R conditions (Supplemental Figure 3A–B). Under basal conditions, a significant increase in SUMO-1 modified Drp1 was observed in the hearts of DJ-1 KO mice (Fig. 5A–B). No difference in SUMO-2/3 modification was observed between the two strains under basal conditions (Fig. 5A&C). In response to myocardial I/R, we observed a significant increase in SUMO-1 modified Drp1 in both WT and KO hearts with an enhanced increase observed in the absence of DJ-1. A significant increase in SUMO-2/3 modified Drp1 was observed only in the hearts of WT mice. Additional analysis comparing the ratio of SUMO-2/3 to SUMO-1 modified Drp1 revealed a propensity for an increase in SUMO-2/3 modifications in the hearts of WT mice and a decrease in SUMO-2/3 modifications in the hearts of DJ-1 KO mice following myocardial I/R injury (Fig. 5D). In agreement with the changes in its SUMOylation status, the mitochondrial expression of Drp1 increased in response to myocardial I/R in both strains with an enhanced increase observed in the absence of DJ-1 (Fig. 5E–F). Interestingly, DJ-1 KO mice exhibited a higher basal mitochondrial expression of Drp1 compared to the WT mice; likely due to the higher degree of SUMO-1 modification.

3.5. DJ-1 Regulates Mitochondrial Fission following Myocardial I/R Injury

Next, we examined the mitochondrial network in each strain following I/R injury to determine if the alterations in the cellular localization of Drp1 were associated with changes in mitochondrial dynamics. By electron microscopy, we observed more mitochondria per field of view in the hearts of DJ-1 KO mice under basal conditions when compared to WT hearts (Fig. 6A–B). The mitochondria in the hearts of DJ-1 KO mice were also smaller under basal conditions when compared to WT hearts, as evidenced by reduced areas and

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perimeters (Fig. 6C–D). This is likely due to the higher basal mitochondrial expression of Drp1, as the whole cell and mitochondrial expression of the fusion proteins (mitofusion-1, Mfn1; mitofusion-2, Mfn2; and Opa-1, and the fission protein, fission-1 (Fis1), were not altered in the hearts of DJ-1 KO mice under basal conditions (Supplemental Figures 3C–F). I/R injury led to an increase in mitochondria number and a decrease in size in both strains with more of change in each parameter observed in the absence of DJ-1, suggesting enhanced fission. Further analysis of mitochondrial fission was achieved by calculating the percentage of mitochondria in a given field that fell into three size categories based on area: <0.6 μm^2, 0.6 μm^2 – 1.0 μm^2, and >1.0 μm^2 [31]. In support of the overall area and perimeter calculations, this analysis demonstrated that DJ-1 KO hearts experienced enhanced mitochondrial fission when compared to WT hearts as evidenced by the significant increase in the number of mitochondria smaller than <0.6 μm^2 (Fig. 6E). These changes were associated with alterations in the expression of proteins involved in mitochondrial fusion and fission (Supplemental Figure 3C–F). In response to myocardial I/R, Fis1 levels were unaltered in the hearts of WT mice, but were significantly increased in the hearts of DJ-1 KO mice. Opa1 levels did not change following myocardial I/R in the WT hearts, but were significantly decreased in DJ-1 KO hearts. Mfn1 levels were not altered in either strain in response to myocardial I/R. In contrast, the mitochondrial levels of Mfn2 were increased in both strains with a more of an increase observed in the absence of DJ-1.

Excessive mitochondrial fission is associated with the production of ROS and the initiation of apoptosis [31]. Oxidative fluorescence microtopography with the fluorescent probe DHE (red staining) demonstrated that myocardial I/R-induced superoxide production was intensified in tissue slices from DJ-1 KO hearts when compared to slices from WT hearts (Fig. 6F–G). Hearts from DJ-1 KO mice also displayed a significant increase in caspase-3 activity when compared to WT (Fig. 6H).

3.6. Inhibition of Drp1 Reduces Myocardial I/R Injury in DJ-1 KO Mice

In an effort to determine if the enhanced myocardial injury observed in the DJ-1 KO hearts was dependent on Drp1, DJ-1 KO mice were treated with the mitochondrial division inhibitor-1 (Mdivi-1), a selective pharmacological inhibitor of Drp1 activity [32], and subjected to myocardial I/R. Initial experiments revealed that Mdivi-1 reduced the myocardial I/R-induced localization of Drp1 in both WT and DJ-1 KO hearts (Supplemental Figure 4A–B). DJ-1 KO mice treated with Mdivi-1 displayed reduced levels of mitochondrial fission (Fig. 6E), superoxide levels (Fig. 6F–G), caspase-3 activity (Fig. 6H), and myocardial injury compared to vehicle-treated mice (Supplemental Fig. 4C–D).

3.7. SENP5 contributes to the enhanced mitochondrial fission observed in the absence of DJ-1

Next, we delved further into the mechanism by which DJ-1 regulates the SUMOylation of Drp1 and mitochondrial fission. We specifically focused on SENP5 based on our evidence above and because it preferentially targets SUMO-2/3 modifications for deconjugation [19] and SENP5 transgenic mice display a reduced SUMO-2/3 modified Drp1 [13]. For these experiments, we employed an *in vitro* hypoxia/reoxygenation (H/R) model using isolated cardiomyocytes from neonatal rats. Initial experiments validated this approach, as exposure

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of cells to H/R induced the cleavage of DJ-1, increased the expression of SENP5, altered the SUMOylation status of cellular proteins, induced mitochondrial fission, induced ROS production and induced cell death (Fig. 7 and Supplemental Figs. 5–6). To mimic our in vivo conditions, cells were transfected with siRNA to DJ-1 (siRNA-DJ1). This reduced DJ-1 levels by 83% (Fig. 7A) and resulted in a further increase in the expression of SENP5 levels (Supplemental Fig. 5A–B), as well as enhanced mitochondrial fission, ROS production, and cell death (Fig. 7E–H). The source of the ROS was confirmed to be from the mitochondria given that MitoTempo (a mitochondrially targeted antioxidant) reduced DCFH oxidation to a similar level in both siRNA-scr and siRNA-DJ1 treated cells. A significant increase in SUMO-2/3 modified Drp1 was observed in cells treated with control siRNA (siRNA-scr) following H/R (Supplemental Fig. 6). In contrast, H/R failed to alter the SUMO-2/3 modified Drp1 in siRNA-DJ1 treated cells. Transfecting cells with siRNA to both DJ-1 and SENP5 (siRNA-DJ1/SENP5) resulted in a 22% reduction in SENP5 levels (Supplemental Fig. 5) and a significant increase in SUMO-2/3 modified Drp1 (Supplemental Fig. 6) following H/R when compared with cells treated with siRNA-DJ1. Importantly, cells treated with siRNA-DJ1/SENP5 displayed lower levels of mitochondrial fission, ROS production, and cell death following H/R when compared to cells treated with siRNA-DJ1 (Fig. 7E–H). Together this data suggest that SENP5 contributes to the enhanced mitochondrial fission observed in the absence of DJ-1.

4. Discussion

While the physiological function of DJ-1 has not been fully established, there is evidence suggesting several different functions, including the characterization of DJ-1 as a protease, a molecular chaperone and a transcriptional co-activator [33]. The ability of DJ-1 to perform these cellular actions is dependent on a conformational change. Specifically, the C-terminal cleavage of DJ-1 converts it into an active conformation that is more effective in providing cytoprotection against oxidative injury [1]. Importantly, oxidative stress induces the cleavage of DJ-1, suggesting that its physiological actions are redox sensitive [34]. Two recent studies report that mice deficient in DJ-1 developed more severe heart failure in response to aortic banding [8] and displayed exaggerated myocardial injury in response to ischemia [9]. While these studies suggest DJ-1 plays a protective role in the heart, neither study investigated if stress induced its activation. Therefore, our current findings are the first to demonstrate the activation of myocardial DJ-1 in response to stress. Specifically, we observed the cleavage of DJ-1 early after the onset of myocardial I/R injury. Consistent with previous findings that this cleavage is associated with cytoprotection, we also found that a deficiency in DJ-1 resulted in exacerbated injury following myocardial I/R. Additionally, we found that in response to myocardial I/R injury DJ-1 translocates to the nucleus and mitochondria. Our findings related to the mitochondrial localization of DJ-1 are in contrast to those recently reported by Billia and colleagues who indicated that DJ-1 does not localize to the mitochondria in the heart [8]. However, it is important to note that the study by Billia et al[8] only evaluated the distribution of DJ-1 in isolated neonatal cardiomyocytes under non-stressed conditions, whereas our current study evaluated the distribution of DJ-1 in the adult heart at different time points following the onset of myocardial I/R.

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SUMOylation influences several cell signaling pathways [11]. It is also involved in the development of a number of human diseases, including heart failure [12]. For example, mutations in laminin A which render the protein less susceptible to SUMO-2 modifications were found in patients presenting with familial cardiomyopathy [35]. Likewise, congenital heart defects are observed in mice with a global knockdown of SUMO-1 and in mice overexpressing SENP2 [15, 36]. Furthermore, SENP1 deficient mice display larger infarct sizes compared to wild-type mice after I/R injury and the overexpression of SENP2 or SENP5 induces fibrosis and cardiac dysfunction. Together, these studies suggest that a balanced SUMOylation-deSUMOylation pathway is critical for normal heart development and proper cardiac function. However, very little is known about how SUMOylation and de-SUMOylation are regulated in the heart under physiological or pathological conditions. Furthermore, very few studies have investigated the dynamics of SUMO-1 and SUMO-2/3 modifications in response to myocardial injury. Although SUMO-1 and SUMO-2/3 are conjugated via the same set of enzymes and share some overlap in the modifications of certain proteins, they can preferentially target specific proteins and show different dynamics [13, 17, 37]. For example, virtually all of SUMO-1 is engaged in protein conjugation at a given time [19]. In contrast, SUMO-2/3 is more abundant in a large free pool than SUMO-1 and is also much more dynamically conjugated and removed [19, 37] consistent with a role for SUMO-2/3 in stress responses [38]. In the brain, SUMO-2/3 modifications are associated with neuroprotection [38, 39]. Here, we report that early after the onset of myocardial I/R injury there is an increase in both SUMO-1 and SUMO-2/3 protein modifications. Much like the observations from the studies focusing on the brain, we found that the elevation in SUMO-2/3 modifications was higher than the elevation in SUMO-1 modifications. Together these findings support a role for SUMO modifications in the heart's response to ischemic stress.

Consistent with the reported role of DJ-1 in the inhibition of SUMO-1 conjugation *in vitro* [10], the current study also indicates that DJ-1 negatively influences SUMO-1 conjugation following myocardial I/R injury, as evidenced by the findings that global SUMO-1 modifications are elevated more in the hearts of DJ-1 KO mice. In contrast, the findings of the current study suggest that DJ-1 influences SUMO-2/3 conjugation in a positive manner following myocardial I/R injury as evidenced by the dampened elevation of SUMO-2/3 modifications observed in the hearts of DJ-1 KO mice. Our findings also support a beneficial role for SUMO-2/3 conjugation in response to myocardial ischemia based on the evidence that DJ-1 KO hearts display more extensive injury than WT hearts. The exact mechanism by which DJ-1 influences SUMOylation is currently not known. There are some reports that DJ-1 interacts with several components of the SUMOylation-deSUMOylation machinery, but the consequences of these interactions are unclear. After determining that DJ-1 influenced SUMOylation in the heart following the onset of myocardial I/R injury, we set out to investigate potential mechanisms. Our results suggest that DJ-1 in part influences SUMOylation via interactions with the deSUMOyation machinery. This is based on the observations of distinct changes in the expression of SENP1 and SENP5 in WT and DJ-1 KO hearts after myocardial I/R injury. Specifically, the expression of SENP1 and SENP5 were elevated in the hearts of WT mice, whereas only the expression of SENP5 was elevated in the hearts of DJ-1 KO mice. Importantly, the expression of SENP5 was found to be higher

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in the hearts of DJ-1 KO mice when compared to WT mice. Since changes in mRNA levels did not account for the altered protein expression of SENP-1 and SENP-5, we evaluated the direct interaction of DJ-1 with both proteins in hearts of WT mice following I/R. Interestingly, we found that SENP1 binding with DJ-1 was up-regulated, whereas SENP5 binding with DJ-1 was down-regulated following myocardial I/R injury. Currently, the implications of these interactions are not known. There is evidence from the literature that DJ-1 stabilizes proteins in response to oxidative stress [27]. Therefore, we postulate that the increased interaction of DJ-1 with SENP1 in response to myocardial I/R injury could be a mechanism to stabilize SENP1. This could be a response mechanism by which DJ-1 directs SENP1 to remove SUMO modifications from certain proteins as a way of changing their function or cellular localization. For instance, the removal of SUMO-1 modifications could make way for the addition of SUMO-2/3 modification or vice versa. Based on our findings, we also postulate that the dissociation of DJ-1 and SENP5 after the onset of myocardial I/R injury possibly leads to the destabilization of SENP5. This is mainly supported by our evidence that the ischemic-induced elevation in SENP5 is enhanced by the absence of DJ-1. This could also be part of an adaptive mechanism that promotes SUMO-2/3 modifications. Further work is warranted to investigate our working hypotheses regarding the mechanisms by which DJ-1 regulates the expression of SENP1 and SENP5 in response to myocardial I/R injury.

Another main finding of the current study relates to the ability of DJ-1 to regulate mitochondrial dynamics, a highly organized process by which mitochondria change size via fusion and fission [40]. Both processes are necessary for the maintenance of organelle fidelity and cell homeostasis [31], as a disruption in either results in abnormal mitochondrial function and cell death [40]. Mitochondrial fission requires the activity of Drp1 [28]. In the heart, targeting mitochondrial fission acutely with pharmacological inhibitors of Drp1 in the setting of myocardial I/R injury results in a reduction in infarction and an improvement in LV function [32, 41]. These studies suggest that controlling fission via the inhibition of Drp1 is cardioprotective. Equally important to these studies regarding the pharmacological inhibition of Drp1 is understanding the signaling mechanisms that regulate its activation and translocation to mitochondria. Previous studies have identified Pim kinase as a negative regulator [32] and calcineurin as a positive regulator [31]. Drp1 is regulated predominately by posttranslational modifications, with phosphorylation being the most characterized [42]. However, the SUMOylation status of Drp1 also regulates its cellular localization, with modifications by SUMO-1 inducing mitochondrial localization [25, 29] and modifications by SUMO-2/3 preventing localization to the mitochondria [30]. Recently, the overexpression of SENP5 in murine cardiomyocyte was reported to induce cardiac dysfunction that was accompanied by elevated apoptosis [13]. An interesting finding in this study was that the hearts of the SENP5 transgenic mice displayed a reduction in SUMO-2/3 modified Drp1. Based on our findings that DJ-1 regulates the expression of SENP5 and the levels of SUMO-2/3 modifications, we sought to determine if DJ-1 influenced Drp1 SUMOylation and subsequent localization. Our findings provide clear evidence that in the absence of DJ-1, Drp1 is SUMOylated predominantly by SUMO-1 and localizes to the mitochondria. These changes were associated with enhanced fission, increased DHE staining, and increased caspase-3 activity, which supports the idea that abnormal mitochondrial fission induces the

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production of ROS and initiates of apoptosis [31]. Further evidence implicating excessive mitochondrial fission as a contributor to the exacerbated myocardial injury observed in DJ-1 KO mice, is that inhibiting Drp1 with mdivi-1 reduced the levels of mitochondrial fission, ROS production, caspase-3 activity, and the degree of infarction. It is important to note, that our study is not the first to suggest that DJ-1 regulates mitochondrial fission, as there is evidence for such regulation in neuronal cells [6]. Additionally, in a recent study, Dongworth and colleagues reported a greater portion of short mitochondria in DJ-1 deficient hearts under basal conditions [9]. While this study extended the observations from neuronal cells to the heart, the authors did not provide any mechanistic insights into why there is enhanced mitochondrial fission in the absence of DJ-1. Here, we provide direct evidence to fill this knowledge gap in the literature. Specifically, our data suggests that SENP5 contributes to the lower SUMO-2/3-modfied Drp1 levels and the enhanced mitochondrial fission observed in the absence of DJ-1. This evidence leads to the postulate that through its interactions with SENP5, DJ-1 acts as a negative regulator of Drp1 and mitochondrial fission in the heart. As noted above, the exact implications of the interaction between DJ-1 and SENP5 are not fully understood. Therefore further work is needed to fully evaluate this interaction and further test our postulate.

While our study focused on the ability of DJ-1 to regulate mitochondrial fission via its actions on SENP5 and Drp1, other mechanisms are likely involved given the complexity of myocardial I/R injury and mitochondrial dynamics. For example, there is evidence that Fis1 plays a role in recruiting and locking Drp1 to the mitochondria to start the fission process. [43] Mitochondrial Fis1 levels were found to be elevated in the hearts of DJ-1 KO mice. This could simply be the result of damaged mitochondria that are trying to recruit Drp1 or it is possible that DJ-1 directly influences the mitochondrial localization of Fis1 through and unexplored mechanism. The same could be proposed for observed decrease in Opa-1 and increased Mfn2 levels at the mitochondria observed in the hearts of DJ-1 KO mice. Additionally, Drp1 can be regulated by other PTMs, such as cysteine modifications [44]. Given, the increased levels of ROS observed in the absence of DJ-1, it is possible that other PTMs are playing a role in the regulation of Drp1. Whether such changes are directly dependent on DJ-1 remain to be determined. Therefore, future studies are warranted to fully investigate the actions of DJ-1 in the heart.

In response to stress stimuli, the fate of a cell lies in its ability to mount a defense against the stimuli through the upregulation and/or activation of endogenous substances. The results of the current study have identified DJ-1 as an endogenous cardioprotective protein that is activated in response to myocardial I/R injury. Furthermore, our results provide novel evidence suggesting that the activation of DJ-1 by myocardial I/R injury protects the heart by regulating the SUMO status of Drp1 and attenuating excessive mitochondrial fission. Thus, DJ-1 may represent a novel target for protecting the heart from I/R injury.

Supplementary Material

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Highlights

- Myocardial ischemia-reperfusion (I/R) injury induces the cleavage of DJ-1
- DJ-1 influences protein SUMOylation following myocardial I/R injury
- DJ-1 protects against myocardial I/R injury by attenuating mitochondrial fission
- DJ-1 alters the expression of SENP5 to influence the SUMO status of Drp1

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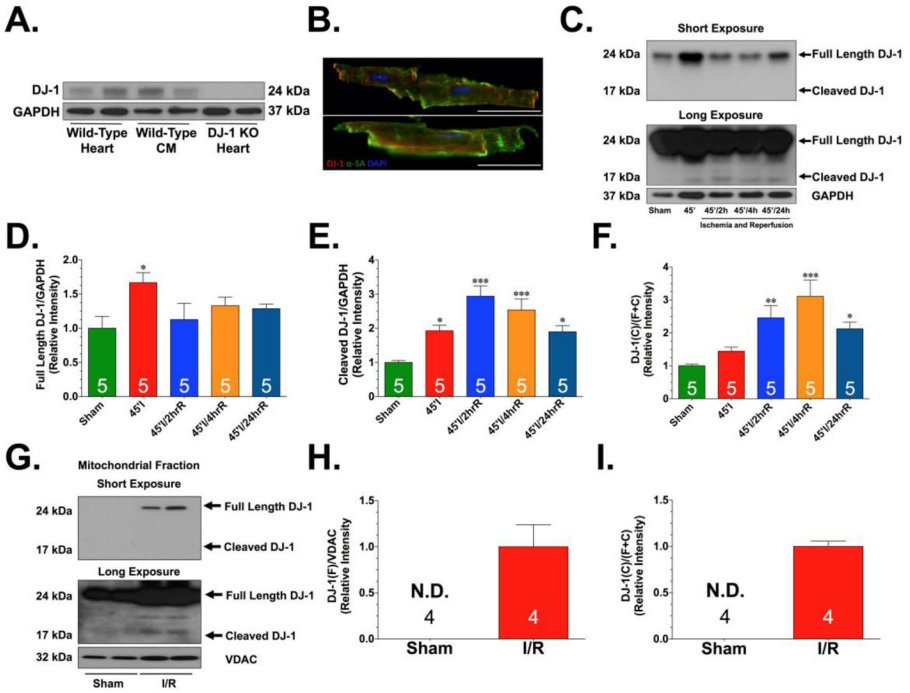

Figure 1.

Representative immunoblots of DJ-1 protein expression in **(A)** heart homogenates and **(B)** isolated cardiomyocytes from adult C57BL/6J mice and in heart homogenates from adult DJ-1 deficient (DJ-1 KO) mice. Immunohistochemical identification of DJ-1 (red), α-sarcomeric actin (green) and DAPI (blue) in isolated cardiomyocytes from adult C57BL/6J mice. Scale bar denotes 100 μm. **(C)** Representative immunoblots of the cardiac expression of the full length and cleaved form of DJ-1 following 45 minutes of myocardial ischemia and different periods of reperfusion. The cleaved form is shown in the long exposure image. Densitometric analysis of the (**D**) full-length form of DJ-1, **(E)** the cleaved form of DJ-1, and **(F)** cleaved DJ-1 protein as a percentage of the total level of DJ-1 proteins (full-length DJ-1 plus cleaved DJ-1). (**G**) Representative immunoblots of the expression of the full length and cleaved form of DJ-1 in mitochondrial fractions following 45 minutes of myocardial ischemia and 4 hours of reperfusion. Densitometric analysis of the (**H**) full-length form of DJ-1 and **(I)** cleaved DJ-1 protein as a percentage of the total level of DJ-1 proteins Numbers inside bars indicates sample size. Values are means ±SEM. N.D., not detected. *p<0.05, **p<0.01 and ***p<0.001 vs. Sham.

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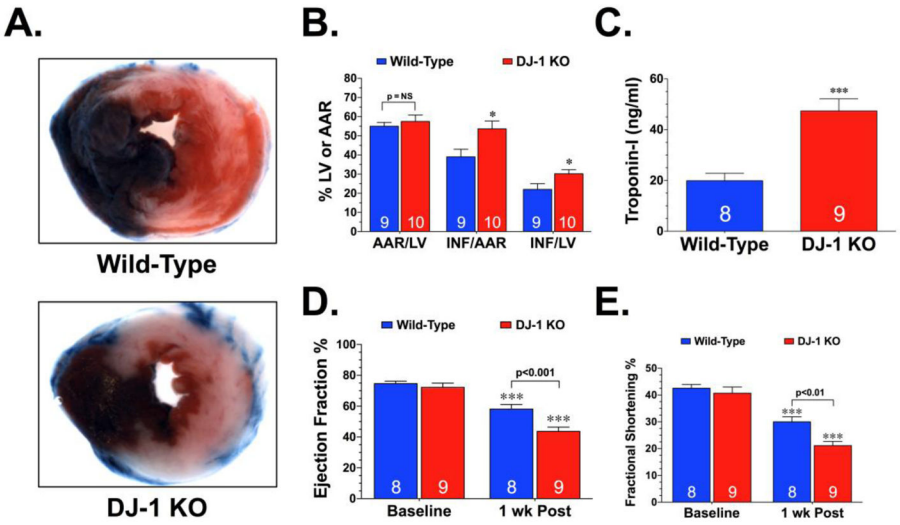

Figure 2.

(A) Representative mid-ventricular photomicrographs of hearts from Wild-Type (WT) and DJ-1 KO mice showing the degree of infarction following 45 minutes of left coronary artery occlusion and 24 hours or reperfusion. **(B)** Myocardial area-at-risk (AAR) as a percentage (%) of total left ventricle (LV) and infarct size (INF) as a percentage of area-at-risk (AAR) and LV. **(C)** Circulating Troponin-I levels (ng/mL) in each group. **(D)** Left Ventricular (LV) Ejection Fraction, and (**E**) Fractional Shortening measured in separate groups of mice using echocardiography images and invasive hemodynamics 1 week following myocardial ischemia and reperfusion (1 wk Post). Values are means ±SEM. *p<0.05 and ***p<0.001 vs. WT or Baseline.

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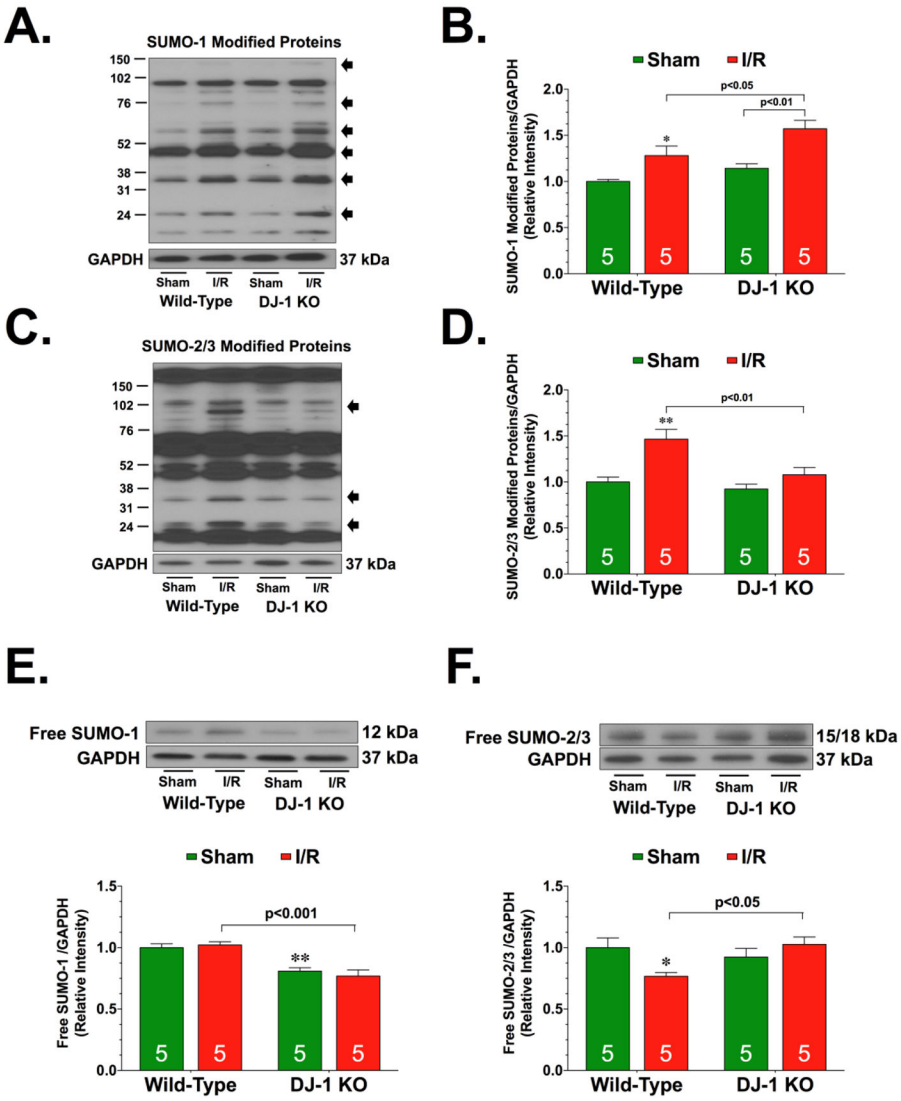

Figure 3.

Representative immunoblots and densitometric analysis of (**A–B**) SUMO-1 modified and (**C–D**) SUMO-2/3 modified proteins in heart homogenates from WT and DJ-1 KO mice. Representative immunoblots and densitometric analysis of (**E**) free SUMO-1 and (**F**) free SUMO-2/3. All samples were prepared from WT and DJ-1 KO hearts collected following 45 minutes of ischemia and 4 hours of reperfusion. Values are means ±SEM. *p<0.05 and **p<0.01 vs. WT Sham.

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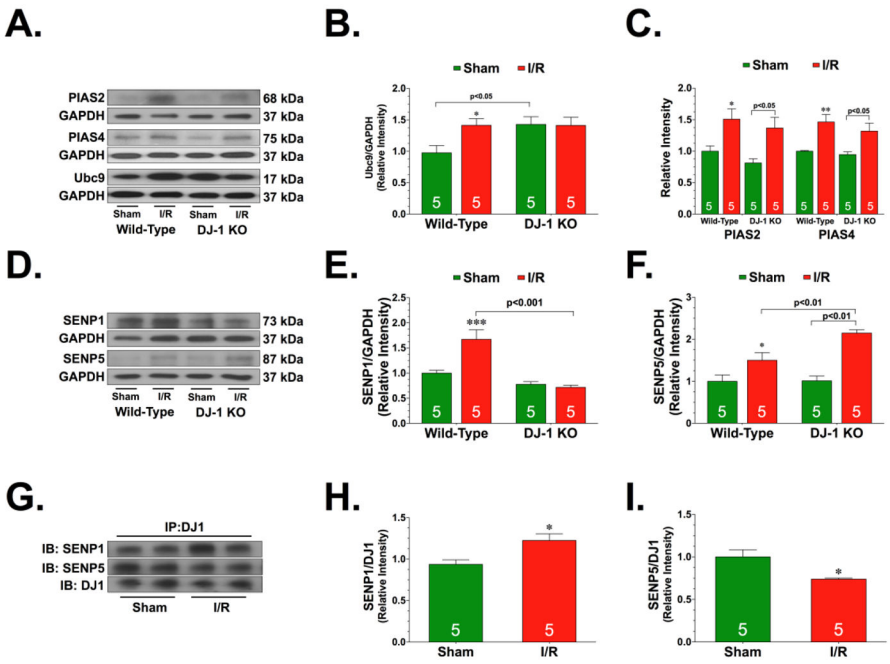

Figure 4.

(**A**) Representative immunoblots and densitometric analysis of (**B**) Ubc9, (**C**) PIAS2 and PIAS4 and in heart homogenates from WT and DJ-1 KO mice. (**D**) Representative immunoblots and densitometric analysis of (**E**) SENP1 and (**F**) SENP5 in heart homogenates from WT and DJ-1 KO mice. (**G**) Representative immunoblots and densitometric analysis of the interaction of DJ-1 with (**H**) SENP1 and (**I**) SENP5 in heart homogenates prepared from WT mice. All samples were collected following 45 minutes of ischemia and 4 hours of reperfusion. Values are means ±SEM. *p<0.05, **p<0.01, and ***p<0.001 vs. WT Sham.

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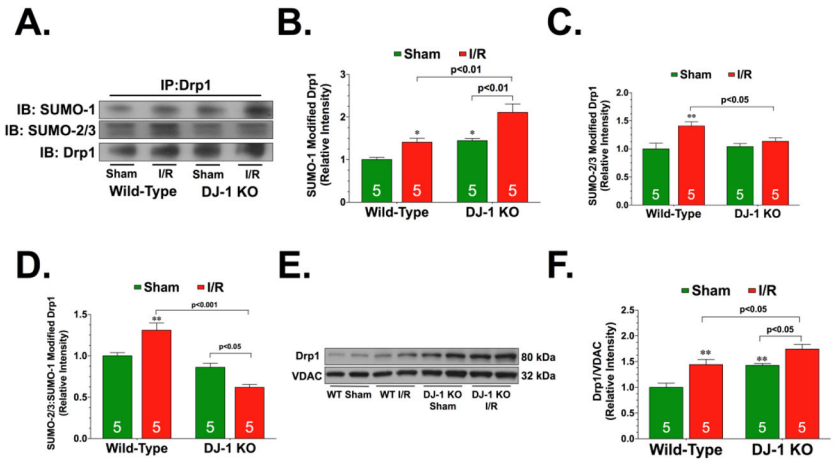

Figure 5.

(**A**) Representative immunoblots and (**B–C**) densitometric analysis of the SUMO status of Drp1. (**D**) Ratio of SUMO-2/3 to SUMO-1 modified Drp1. **(EF)** Representative immunoblots and densitometric analysis of Drp1 in mitochondrial fractions. All samples were prepared from WT and DJ-1 KO hearts collected following 45 minutes of ischemia and 4 hours of reperfusion. Values are means ±SEM. *p<0.05 and **p<0.01 vs. WT Sham.

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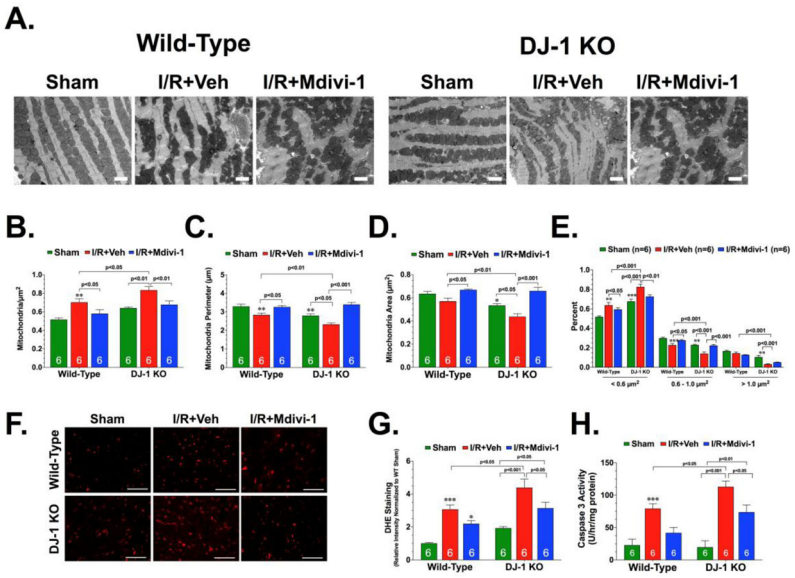

Figure 6.

(A) Representative electron microscopy images of cardiac mitochondria from different groups of WT and DJ-1 KO mice following 45 minutes of ischemia and 4 hours of reperfusion. Mice were administered vehicle (Veh) or the mitochondrial division inhibitor-1 (mdivi-1) 15 minutes prior to the onset of ischemia. Scale bar equals 2.5 μm. **(B)** Number of mitochondria per μm^2 in each field of view. Summary of mitochondria **(C)** perimeter and **(D)** area measurements (μm^2). **(E)** Percentage of mitochondria in a given field that fell into three size categories based on area: <0.6 μm^2, 0.6 μm^2 – 1.0 μm^2, and >1.0 μm^2. (**F–G**) Representative images and quantification of dihydroethidium (DHE) staining of heart sections from WT and DJ-1 KO mice. Scale bar denotes 100 μm. (**H)** Caspase-3 activity. Values are means ±SEM. *p<0.05, **p<0.01 and ***p<0.001 vs. WT Sham.

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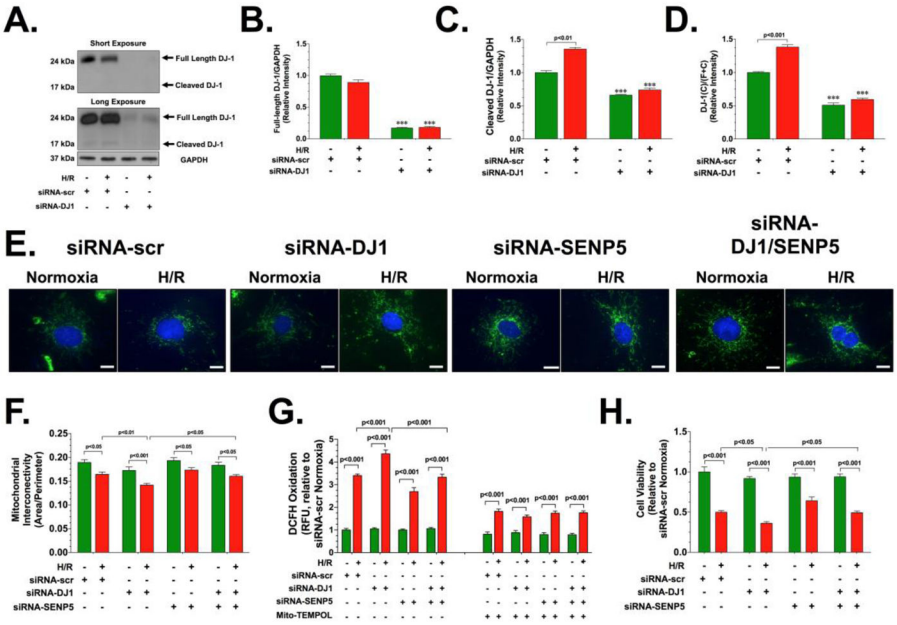

Figure 7.

(A) Representative immunoblots of the full length and cleaved form of DJ-1 in isolated rat neonatal cardiomyocytes subjected to hypoxia/reoxygenation. Densitometric analysis of the (**B**) full-length form of DJ-1, **(C)** the cleaved form of DJ-1, and **(D)** cleaved DJ-1 protein as a percentage of the total level of DJ-1 proteins. Cells were transfected with either siRNA to DJ-1 (siRNA-DJ1) or control siRNA (siRNA-scr). (**E**) Representative images of cells stained with antibodies to TOM20 to visualize mitochondrial networks following H/R (x100 magnification). Scale bar equals 5 μm. Cells were transfected with either siRNA-scr, siRNA-DJ1, siRNA to SENP5 (siRNA-SEPN5) or a combination of siRNA to DJ-1 and SENP5 (siRNA-DJ1/SENP5). (**F**) Quantification of mitochondrial interconnectivity (area/perimeter). (**G**) Intracellular reactive oxygen species levels (mean fluorescence intensity of DCFH oxidation) following H/R in cells treated with vehicle or MitoTempo (10 μM). **(H)** Cell viability following H/R as determined by the Cell Titer Blue assay in cells transfected with siRNA-scr, siRNA-DJ1, siRNA-SENP5, or siRNA-DJ1/SENP5. Values are means ±SEM of three independent experiments of three biological replicates. ***p<0.001 vs. siRNA-scr Normoxia.