Electron leak from NDUFA13 within mitochondrial complex I attenuates ischemia-reperfusion injury via dimerized STAT3

Hengxun Hu,a,b Jingliang Nan,a,b Yong Sun,a,b Dan Zhu,a Changchen Xiao,a Yaping Wang,a Lianlian Zhu,a,b Yue Wu,a,b Jing Zhao,a,b Rongrong Wu,a Jinghai Chen,c,b Hong Yu,a Xinyang Hu,a Wei Zhu,a,b,c and Jian’an Wang,a,b

*Cardiovascular Key Laboratory of Zhejiang Province, Department of Cardiology, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, China; cClinical Research Center, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, China; and Institute of Translational Medicine, Zhejiang University, Hangzhou 310029, China

Edited by J. G. Seidman, Harvard Medical School, Boston, MA, and approved September 1, 2017 (received for review March 23, 2017)

The causative relationship between specific mitochondrial molecular structure and reactive oxygen species (ROS) generation has attracted much attention. NDUFA13 is a newly identified accessory subunit of mitochondria complex I with a unique molecular structure and a location that is very close to the subunits of complex I of low electrochemical potentials. It has been reported that down-regulated NDUFA13 rendered tumor cells more resistant to apoptosis. Thus, this molecule might provide an ideal opportunity for us to investigate the profile of ROS generation and its role in cell protection against apoptosis. In the present study, we generated cardiac-specific tamoxifen-inducible NDUFA13 knockout mice and demonstrated that cardiac-specific heterozygous knockout (cHet) mice exhibited normal cardiac morphology and function in the basal state but were more resistant to apoptosis when exposed to ischemia-reperfusion (I/R) injury. cHet mice showed a preserved capacity of oxygen consumption rate by complex I and II, which can match the oxygen consumption driven by electron donors of N,N,N-,N-tetramethyl-p-phenylenediamine (TMPD)-ascorbate. Interestingly, at basal state, cHet mice exhibited a higher H2O2 level in the cytosol, but not in the mitochondria. Importantly, increased H2O2 served as a second messenger and led to the STAT3 dimerization and, hence, activation of antiapoptotic signaling, which eventually significantly suppressed the superoxide burst and decreased the infarct size during the I/R process in cHet mice.

Author contributions: H.H., J.N., Y.S., D.Z., Y. Wang, Y. Wu, R.W., J.C., H.Y., X.H., W.Z., and J.W. wrote the paper. J.N., Y.S., D.Z., Y. Wang, Y. Wu, J.C., H.Y., X.H., W.Z., and J.W. performed research; J.N., Y.S., D.Z., Y. Wang, J.C., H.Y., X.H., W.Z., and J.W. designed research; H.H., J.N., and Y.S. contributed equally to this work. The authors declare no conflict of interest.

Significance

Reactive oxygen species (ROS) generation due to electron leak from the mitochondria may be involved in physiological or pathological processes. NDUFA13 is an accessory subunit of mitochondria complex I with a unique molecular structure and is located close to FeS clusters with low electrochemical potentials. Here, we generated cardiac-specific conditional NDUFA13 heterozygous knockout mice. At the basal state, a moderate down-regulation of NDUFA13 created a leak within complex I, resulting in a mild increase in cytoplasm localized H2O2, but not superoxide. The resultant ROS served as a second messenger and was responsible for the STAT3 dimerization and, hence, the activation of antiapoptotic signaling, which eventually significantly suppressed the superoxide burst and decreased the infarct size during the ischemia-reperfusion process.
an opportunity to establish a unique profile of ROS generation within the mitochondria when NDUFA13 is down-regulated. It has also been reported that decreased NDUFA13 expression is associated with enhanced STAT3 signaling, which may also account for the augmented survival in tumor cells (14, 16–18). Again, it remains largely unknown whether and how STAT3 activation is related to ROS generation induced by down-regulated NDUFA13.

In the present study, we generated cardiac-specific NDUFA13 knockout mice, which would allow us to investigate the profile of ROS generation when NDUFA13 was moderately down-regulated and how resultant ROS offer the protection for cells, specifically for the myocardium against I/R injury. Further, we aimed to elucidate whether and how the activated STAT3 signaling was also responsible for the protection of the heart.

Results

Moderate NDUFA13 Down-Regulation Confers Protection Against Hypoxia/Reoxygenation-Induced Cell Injury. To test the roles of NDUFA13 in cardiomyocytes, we transfected H9C2 cells with NDUFA13-targeting siRNA and showed that a 100 μmol/L concentration of siRNA-NDUFA13 resulted in a 30% decrease and a 200 μmol/L siRNA-NDUFA13 resulted in a 60% decrease in NDUFA13 expression (Fig. 1A). Note that mitochondrial membrane potential (MMP) was impaired at the high (200 μmol/L) dose of siRNA-NDUFA13, but not at the low dose (100 μmol/L) (Fig. S1A).

Interestingly, the resultant moderate decrease in NDUFA13 expression was associated with a decrease in TUNEL-positive cells when these cells were exposed to hypoxia for 6 h followed by reoxygenation for 18 h (Fig. 1B). However, a severe decrease in NDUFA13 expression failed to elicit any protection against hypoxia/reoxygenation (H/R)-induced apoptosis (Fig. 1B). Consistent with TUNEL staining, the same pattern of changes was observed in cleaved caspase-3 and caspase-9 expression, with the protective effects being present only when NDUFA13 was moderately down-regulated (Fig. 1C). However, cleaved caspase-8, which is involved in the extrinsic apoptotic pathway, did not show significant changes (Fig. 1D). Meanwhile, phosphorylation level of apoptosis signal-regulating kinase at threonine 845 (pASK1 Thr845) and its downstream target p-JNK level were not affected (Fig. S1B). In summary, the data suggested that a moderate decrease in NDUFA13 expression conferred a significant protection against apoptosis obtained from H9C2 cells.

Characterization of Tamoxifen-Inducible Cardiac-Specific NDUFA13 Transgenic Mice. To further investigate how a slight decrease in NDUFA13 offers cardiac protection against apoptosis in vivo, we generated a cardiac-specific conditional NDUFA13 knockout mouse model (see details in Methods). Eight-week-old Myh6Cre\textsuperscript{+}\textsuperscript{-}NDUFA13\textsuperscript{flox/flox} (cHomo) mice and age-matched Myh6Cre\textsuperscript{+}\textsuperscript{-}NDUFA13\textsuperscript{flox/−} (cHet) mice were studied, and Myh6Cre\textsuperscript{+}\textsuperscript{-}NDUFA13\textsuperscript{flox/−} mice were used as controls (CON). i.p. injections of tamoxifen (see Methods for detailed information) were administered to each mouse. To evaluate the time course of changes in NDUFA13 expression after tamoxifen treatment, on days 1, 4, 7, 10, 13, and 16 after tamoxifen administration, three mice from each group were killed, respectively, and the hearts were obtained to test the relationship between NDUFA13 expression and the ATP level. Tamoxifen time-dependently down-regulated NDUFA13 expression in both cHomo and cHet mice. A moderate decrease in NDUFA13 expression was observed on day 16 in cHet mice, whereas a moderate decrease was detected as early as day 1 in cHomo mice, and an almost 80% decrease was observed on day 16 after tamoxifen treatment (Fig. 2A). Interestingly, cHet mice did not exhibit any changes in ATP level in the heart compared with CON mice (Fig. 2B), whereas cHomo exhibited a significant time-dependent decrease in ATP content in the heart, with a significant change detected as early as 4 d after tamoxifen administration (Fig. 2B). Being consistent with quantification of ATP levels in the heart tissue, cHomo mice experienced sudden death, whereas cHet and CON mice shared a similar survival rate as demonstrated by Kaplan–Meyer’s survival curves (Fig. 2C). Based on these data, we used only cHet mice to test whether a moderate NDUFA13 down-regulation had cardioprotective effects against I/R-induced stress. To further confirm that cHet mice did not exhibit any abnormalities in cardiac structure and function, echocardiographic examinations were performed on nine cHet and nine CON mice on day 28 after tamoxifen administration, and the resultant modified echocardiographic examinations were further confirmed in the heart tissue. cHet mice experienced sudden death, whereas cHet and CON mice shared a similar survival rate as demonstrated by Kaplan–Meyer’s survival curves (Fig. S1). The mice were then killed for further analysis. Compared with CON mice, cHet mice did not exhibit any significant changes in cardiac ultrastructure as demonstrated by transmission electronic microscopy (TEM) examination (Fig. 2E), and mitochondrial morphology (Fig. 2E) was similar between cHet and CON mice. Protein expression of several key mitochondrial components, including ATP2A2, NDUFB8, and SDHC (for ATP generation); ATP5A (for ATP consumption); and PGC-1α (a key transcription factor that orchestrates mitochondrial biogenesis) were not altered (Fig. 2F and Fig. S2B). NDUFA13 expression in other tissues, such as the brain, lung, and liver, was similar between cHet and CON mice (Fig. 2F and Fig. S2B), further confirming a reliable cardiac-specific NDUFA13 knock-down mouse model. Using freshly isolated mitochondria from the hearts, substrate-driven mitochondrial respiratory function was analyzed. With the presence of ADP, substrate-driven oxygen consumption rate (OCR) was measured for complex I (addition of pyruvate and malate followed by rotenone, an inhibitor of complex I), complex II (succinate), and complex IV [NAD\textsuperscript{+}/NADH-tetramethyl-p-phenylenediamine (TMPD)/ascorbate followed by azide, an inhibitor of complex IV)]. The data showed a decrease in substrate-driven OCR of complex I in cHet mice; however, OCR by combination of complex I and II could, in compensation, match OCR driven by the electron donor (TMPD/ascorbate) through complex IV (Fig. 2G and Fig. S2C). In summary, we generated a conditional cardiac-specific NDUFA13 knockout mouse model; cHet mice exhibit normal cardiac structure and function at baseline, which was associated with a compensatory normal mitochondrial function and ATP production.

NDUFA13 Down-Regulation Protected Mouse Hearts from I/R Injury. To test whether a moderate down-regulation of NDUFA13 could protect the heart from I/R injury, we generated an in vivo cardiac I/R injury model in both cHet and CON mice, by coronary artery ligation for 45 min followed by 3 h of reperfusion. Interestingly, we observed a significant decrease in the infarct size (IS) in cHet mice.
comparing with CON mice (Fig. 3A). Quantification of apoptotic level at the peri-infarct area showed that TUNEL-positive cardiomyocytes were significantly decreased in cHet mice that underwent I/R intervention compared with CON mice that experienced the same I/R insult (Fig. 3B). The decrease in apoptosis was also confirmed when peri-infarct heart tissue was used for Western blot analysis, showing a significant decrease in cleaved caspase-3 expression in cHet mice compared with CON mice (Fig. 3C). The same pattern of changes was also seen in cytochrome C (cytC) release into the cytosol, showing much less leak in cHet mice following an I/R injury compared with CON mice that were exposed to I/R injury (Fig. 3D). Taken together, these results strongly supported that moderate NDUFA13 down-regulation confers protection against I/R injury through the suppression of apoptosis.

Down-Regulated NDUFA13 Expression Is Associated with Increased Basal ROS Generation. To investigate the profile of ROS generation by a partial loss of NDUFA13, we freshly isolated mitochondria from both cHet and CON mice that were treated with tamoxifen. We measured OCR and \( \Delta H_2O_2 \) levels simultaneously with the Oroboros O2k system. Using different substrates and blockers specific for complex I and complex III, we demonstrated that succinate caused a significant amount of \( \Delta H_2O_2 \) through reverse electron transport (RET) in the mitochondria from CON mice, this RET-induced ROS generation can be blocked by rotenone. In contrast, mitochondria from cHet mice exhibited a much smaller RET-induced \( \Delta H_2O_2 \) generation, resulting in an interrupted RET process. Importantly, however, further addition of pyruvate and malate resulted in an unexpected increase in \( \Delta H_2O_2 \) level in cHet mice, which was not observed in CON mice. A final addition of antimycin A resulted in a similar degree of increment in \( \Delta H_2O_2 \) in both CON and cHet mice (Fig. 4A and Fig. S3A).

To confirm cHet mice mainly generated \( \Delta H_2O_2 \), we used mitoSOX red to detect the superoxide levels within the mitochondria. We cross-bred cHomo (NDUFA13<sup>lox/lox</sup>) mice with wild-type littermates (NDUFA13<sup>WT</sup>) to generate NDUFA13 heterozygous (NDUFA13<sup>lox/+</sup>) mice. The neonatal cardiomyocytes (NCMCs) were then obtained from these mice and transfected with adenovirus-containing Cre recombinase (Ad-Cre) or an empty vector as a normal control (Ad-NC). The effect of Cre recombinase on NDUFA13 expression in NCMCs was confirmed (Fig. S3B). Using mitoSOX Red as a probe, we showed that at basal state, NCMCs treated with Ad-Cre or Ad-NC had a similar level of superoxide (Fig. 4B), importantly, Ad-Cre–treated NCMCs exhibited much lower superoxide levels compared with Ad-NC–treated NCMCs when exposed to H/R (Fig. 4B).

NCMCs obtained from NDUFA13<sup>lox/lox</sup> mice that had been treated with Ad-Cre or Ad-NC as described above were infected adenovirus containing mitochondrial targeting HyPer (mito-Hyper) or cytoplasm-targeting HyPer (cyto-Hyper) for measuring \( \Delta H_2O_2 \) in the mitochondria and in the cytoplasm, respectively (Fig. S3C). At basal state, \( \Delta H_2O_2 \) level detected by cyto-Hyper was higher in Ad-Cre–treated NCMCs than that in Ad-NC–treated cells; however, the differences between these groups were absent when measuring \( \Delta H_2O_2 \) levels in the mitochondria, further confirming that a leak was present within complex I when NDUFA13 was down-regulated. Interestingly, following H/R, a burst of \( \Delta H_2O_2 \) was present in both cytosol and mitochondria of Ad-NC–treated NCMCs, which was much less in the mitochondria in Ad-Cre–treated cells (Fig. 4C).

The Molecular Structure of NDUFA13 in Maintaining the Integrity of Mitochondrial Membrane. We then designed several adenoviruses that contained different truncated NDUFA13 mutants, including Ad-1 (with a deletion of amino acid 40–50), Ad-2 (a deletion of amino acids 70–80), Ad-3 (a deletion of amino acids 110–120), Ad-NDUFA13 (a wild-type full-length NDUFA13 as a normal control), and Ad-Vector (an empty vector as a negative control) (Fig. S4A). By transfecting these various vectors respectively back into NDUFA13-depleted NCMCs (isolated from NDUFA13<sup>lox/lox</sup> mice and pretreated with Ad-Cre with the efficiency confirmed in Fig. S4B), we can measure both MMP and \( \Delta H_2O_2 \) generation to test the role of the TMH in NDUFA13. The NCMCs transfected with Ad-Cre exhibited a significant decrease in MMP compared with NCMCs transfected with Ad-NC, as shown by measuring the fluorescence intensity (Fig. S4C) or by flow cytometry (Fig. S4D) following TMRM staining. Ad-NDUFA13, Ad-1, Ad-2, Ad-3, or Ad-Vector was then transfected into endogenous NDUFA13-depleted NCMCs (with the efficiency of transfection confirmed in Fig. S4E) following TMRM staining. Ad-NDUFA13, Ad-1, Ad-2, Ad-3, or Ad-Vector was then transfected into endogenous NDUFA13-depleted NCMCs (with the efficiency of transfection confirmed in Fig. S4F), whereas Ad-2 and Ad-3 could fully mimic and compensate for wild-type NDUFA13 (Fig. S4F and G). Importantly, the same pattern of changes was seen when cyto-Hyper was cotransfected into NDUFA13-depleted NCMCs to test if increased cytosolic \( \Delta H_2O_2 \) level in Ad-Cre–treated NCMCs was abolished by putting back different truncated NDUFA13 mutants (Fig. S4F). These results strongly supported an essential role for TMH domain in maintaining the MMP, which also might serve as a main source of \( \Delta H_2O_2 \) generation.

\[ \text{Post-TAM} \]

\[ \text{DAYS} \]

\[ \beta\text{-Actin} \]

\[ \text{ATP(mol/liter)} \]

\[ \text{C} \]

\[ \text{OCR of isolated mitochondria (dpm/oilCS activity) } \]

\[ \text{D} \]

\[ \text{E} \]

\[ \text{F} \]

\[ \text{G} \]

\[ \text{H} \]

\[ \text{I} \]

\[ \text{J} \]

\[ \text{K} \]

\[ \text{L} \]

\[ \text{M} \]

\[ \text{N} \]

\[ \text{O} \]

\[ \text{P} \]

\[ \text{Q} \]

\[ \text{R} \]

\[ \text{S} \]

\[ \text{T} \]

\[ \text{U} \]

\[ \text{V} \]

\[ \text{W} \]

\[ \text{X} \]

\[ \text{Y} \]

\[ \text{Z} \]
Both cardiac-specific NDUFA13 heterozygous (Myh6Cre+NDUFA13<sup>flox</sup>STAT3<sup>WT</sup>) and cardiac-specific double heterozygous (Myh6Cre<sup>+</sup>NDUFA13<sup>flox</sup>STAT3<sup>flox</sup>) mice were treated with tamoxifen the same way as described above. Two weeks later, these mice were exposed to I/R injury. The cardiac protection against I/R injury offered by a moderate NDUFA13 was abolished when STAT3 was simultaneously down-regulated in mice as evidenced by an increase in IS (Fig. 6A), which also resulted in a reversal in TUNEL-positive cells in the peri-infarct area (Fig. 6B), as well as the cleaved caspase-3 expression (Fig. 6C). Of note, cardiac-specific STAT3 heterozygous knockout (Myh6Cre<sup>Era<sup>flox</sup>STAT3<sup>flox</sup>) and Myh6Cre<sup>Era</sup>ER<sup>flox</sup>STAT3<sup>WT</sup>) mice that received tamoxifen treatment exhibited a similar degree of I/R injury as evidenced by IS (Fig. 6D), percentage of TUNEL staining-positive cells at peri-infarct area, and levels of cleaved caspase-3 (Fig. 5B and C). Taken together, these data suggest that STAT3 is responsible for the cardioprotective effects against I/R injury when NDUFA13 expression is moderately down-regulated.

**Discussion**

In the present study, we generated a conditional cardiac-specific moderately down-regulated NDUFA13 mice, which were more tolerant to I/R injury, exhibited a smaller IS, and lower apoptotic activity. We then investigated ROS profile induced by mitochondria NDUFA13 down-regulation and detected a moderate increase in the levels of hydrogen peroxide, but not superoxide, in these mice. We have provided strong evidence to show that partial loss of NDUFA13 constitutes a structural substrate allowing for an electron leak such that a small amount of H<sub>2</sub>O<sub>2</sub> would be continuously generated. As a result of a mild increase in hydrogen peroxide, up-regulated PRX2 expression occurred, leading to STAT3 dimerization and, hence, enhanced Bcl-2 expression, which were responsible for the protection offered by NDUFA13 down-regulation. Thus, we have not only elucidated a novel molecular mechanism of cardiac protection against I/R injury offered by a moderate NDUFA13 down-regulation. This study provided strong evidence that partial loss of NDUFA13 constitutes a structural substrate allowing for an electron leak such that a small amount of H<sub>2</sub>O<sub>2</sub> would be continuously generated. As a result of a mild increase in hydrogen peroxide, up-regulated PRX2 expression occurred, leading to STAT3 dimerization and, hence, enhanced Bcl-2 expression, which were responsible for the protection offered by NDUFA13 down-regulation. Thus, we have not only elucidated a novel molecular mechanism of cardiac protection against I/R injury offered by a moderate NDUFA13 down-regulation.
Electron transfer across the different segments of mitochondrial complexes serves as a form of energy transduction, creating an electrochemical gradient across the inner membrane. It also dictates the form of ROS generated in both physiological and pathological states, such as the process of I/R injury, due to the different electrochemical potentials pertaining to the different respiratory moieties (2, 3, 11). With normal oxygen supply, the short electronic effect of FMN is usually surpassed by the strong electron-withdrawing ability of its downstream FeS clusters (7). However, under oxygen deprivation conditions, the failure of electrons to react with their acceptor, oxygen, within complex IV sequentially saturates the FeS clusters and the FMN of complex I with electrons. Once the oxygen supply is restored, O₂ then can react with FIMNH₄FSQ, which is filled with electrons, to generate superoxides (in the form of •O₂⁻). This is a typical hypoxia-reperfusion scenario. •O₂⁻ is highly reactive, and the unpaired electrons on •O₂⁻ will capture any available electrons from molecules they may encounter, thus producing the relatively more stable H₂O₂. Although coenzyme Q is also filled with electrons, its reduction potential is +0.113 V, which is insufficient for the production of O₂⁻ (O₂+•O₂⁻=−0.13 V) but not H₂O₂ (O₂+H₂O₂=+0.70 V). H₂O₂ is a milder type of ROS than •O₂⁻. Under normal physiological conditions, a low level of H₂O₂ generation can mediate a variety of signaling events (20, 21).

Compared with prokaryotic cells, eukaryotic mitochondrial complex I has more nuclear-encoded subunits, which play essential roles in ensuring energy transduction along the mitochondria in a safe and efficient way (22). NDUFA13 is regarded as one of these accessory subunits and located at the heel position of mitochondrial complex I, with its helix segment inserted obliquely into hydrophobic chains ND1 and ND2 of complex I and TMH segment further anchored into the mitochondrial inner membrane (13). Using MOE software and the available database (PDB ID code: 5LDX), we did further analysis and noticed that the first 33 amino acids of NDUFA13 extend along the dorsal side of the CoQ binding chamber after penetrating the inner membrane and are parallel to the last three FeS clusters (N2, N6b, and N6a), which are ∼31 Å apart. The enlarged tail of NDUFA13 remains on the intermembrane side of ND1 and ND2 (Fig. S7). The unique location and structure of NDUFA13 suggest that it may form a channel within complex I that interconnects the matrix with membrane interstitium (23). In the present study, we were mainly focused on elucidating the profile of ROS generated when NDUFA13 were down-regulated. Our data showed that moderate NDUFA13 knockdown resulted in an increase in H₂O₂ without involving in changes in MMP. We applied sequential experiments to test this notion that partial loss of NDUFA13 mainly resulted in an increase in H₂O₂. Data obtained from isolated mitochondria with Amplex red provided direct evidence of H₂O₂ generation secondary to NDUFA13 knockdown, which was confirmed by measuring superoxide within the mitochondria using mitoSOX red, showing no increase in superoxide generation at the basal state. Site-specific detection of H₂O₂ using cyto-HyPer and mito-HyPer further validated that H₂O₂ generated by partial loss of NDUFA13 was localized in the cytosol, but not in the mitochondria. Most importantly, a mild increase in H₂O₂ at the basal state can prevent the burst of superoxide generation following I/R injury. Taken together, we would propose our preliminary idea of a “spillhole theory” for which NDUFA13 serves as a guardian that gauges electron flow across the electron transfer chain, which should be closely related to the location and functional structure of NDUFA13 within mitochondrial respiratory complex I (Fig. S7).

Our present study also provided additional evidence that H₂O₂ promotes the formation of disulfide-linked STAT3 oligomers with the help of PRX2, which regulates the transcriptional activity of STAT3 to up-regulate Bcl2 expression (24), and renders tumor cells more safe and efficient way (22). NDUFA13 is regarded as one of these accessory subunits and located at the heel position of mitochondrial complex I, with its helix segment inserted obliquely into hydrophobic chains ND1 and ND2 of complex I and TMH segment further anchored into the mitochondrial inner membrane (13). Using MOE software and the available database (PDB ID code: 5LDX), we did further analysis and noticed that the first 33 amino acids of NDUFA13 extend along the dorsal side of the CoQ binding chamber after penetrating the inner membrane and are parallel to the last three FeS clusters (N2, N6b, and N6a), which are ∼31 Å apart. The enlarged tail of NDUFA13 remains on the intermembrane side of ND1 and ND2 (Fig. S7). The unique location and structure of NDUFA13 suggest that it may form a channel within complex I that interconnects the matrix with membrane interstitium (23). In the present study, we were mainly focused on elucidating the profile...
resistant to chemotherapy (15). The protection offered by downregulated NDUFA13 through STAT3 signaling did not affect the extrinsic apoptotic pathway, as we did not observe significant changes in cleaved caspase-8 levels. In addition, the key components of the upstream molecule of intrinsic mitochondria-dependent apoptosis signaling pathway, such as ASK and INK, were not affected. These changes indicate that the protection was mainly targeting the mitochondria.

In conclusion, a mild defective structure related to subunits of mitochondrial complex I, such as N2 with low electrochemical potential, can produce only H$_2$O$_2$, which can serve as a second messenger to activate STAT3/Bcl2, an important antiapoptotic signaling pathway. Thus, our study provided another protective mechanism against apoptosis induced by I/R injury.

**Materials and Methods**

**Reagents.** See Supporting Information for details.

**Animals.** Mice with a pair ofloxP sites flanking exon3 of NDUFA13 (Fig. S8 and Table S2) were generated at the Shanghai Biomodel Organisms Center using standard methods and mated with FLP mice to excise the Neo cassette. MYH6-CreERT$^{tm}$ mice (25) (no. 005657) and STAT3$^{lox/lox}$ mice (no. 016923) from The Jackson Laboratory were used for breeding various genotyping mice (see Supporting Information for detailed procedures). All procedures were approved by the Zhejiang University Institutional Animal Care and Use Committee and are in compliance with NIH Publication no. 85-23 (revised 1996). All mice were housed, bred, and maintained under specific pathogen-free (SPF) conditions.

**Animal Model of I/R Injury.** In brief, using a surgical approach, I/R injury was induced with 45 min of ischemia followed by 3 h of reperfusion. Sham surgical procedures were performed on the control group. IS was expressed as the percentage of the infarct area compared with the area at risk (for details, see SI Materials and Methods).

**NMCs with NDUFA13 Knockdown and Putback.** NMCs obtained from NDUFA13$^{lox/lox}$ mice were transfected with adenovirus containing Myh6-Cre to deplete endogenous NDUFA13, then infected with recombinant adenoviruses that expressed either Cyto-HPer or Mito-HPer (27). The intensity of fluorescence was measured by microplate reader. For superoxide measurements, the same designated NMCs were studied and loaded with MitoSOX Red (5 mM for 10 min; thermo Fisher M36008), and the intensity of fluorescence was measured by microplate reader (for details, see SI Materials and Methods).

**For detailed information regarding Western blot, TUNEL staining, siRNA transfection mitochondria isolation, immunostaining, echocardiography, TEM, MMP determination, the quantification of H$_2$O$_2$ and O$_2^-$, native blue PAGE, H/R injury NMC isolation (29), high-resolution respirometry, and NDUF13 knockdown and putback, please refer to SI Materials and Methods.**

**Statistical Analysis.** Data are presented as the mean ± SD. After confirming that all variables were normally distributed using the Kolmogorov-Smirnov test, unpaired Student’s t test was used to determine the differences between two groups. *P* < 0.05 was considered as statistically significant.

**ACKNOWLEDGMENTS.** This work was supported by the National Basic Research Program of China (973 Program, Grants 2014CB965100 and 2014CB965103); National Natural Science Foundation of China (8132010003 and 31371498 (to J.W.), 81700138 and 81801809 (to X.H.), and 81670325 (to W.Z.)); Major Development Projects for Public Welfare, Grant 2013C37054 (to J.W.) and Major Scientific and Technological Projects, Grant 201303094–3 (to Y.S.) from Science and Technology Department of Zhejiang Province; and Grant Y201239862 (to W.Z.) from Education Department of Zhejiang Province.

1. Baradaran R, Bennis MD, Minhas GS, Sazanov LA (2013) Crystal structure of the entire respiratory complex I. Nature 494:443–448.
2. Murphy MP (2009) How mitochondria produce reactive oxygen species. Biochem J 417:1–13.
3. Kuosman L, Hir J (2000) The mechanism of superoxide production by NADH:cubiquinone oxidoreductase (complex I) from bovine heart mitochondria. Proc Natl Acad Sci USA 103:7607–7612.
4. Liu Y, Fiskum G, Schubert D (2000) Generation of reactive oxygen species by the mitochondrial electron transport chain. J Neurochem 70:780–787.
5. Kushnreva Y, Murphy MN, Andreyeva A (2002) Complex I-mediated reactive oxygen species generation: Modulation by cytochrome c and NAD(P)+/oxidation-reduction status. Biochem J 368:545–553.
6. Vityakova TV, Reynolds U (2001) DeltaP/P+ dependent and independent production of reactive oxygen species by rat brain mitochondria. J Bioenerg Biomembr 33:267–277.
7. Sazanov LA (2007) Respiratory complex I: Mechanistic and structural insights provided by the crystal structure of the hydrophilic domain. Biochemistry 46:2275–2288.
8. Hir J, King MS, Pryde KR (2008) The production of reactive oxygen species by complex I. Biochem Soc Trans 36:976–980.
9. Suzuki Y, et al. (2002) Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 (BMK1) and the MEF2C signaling pathway in PC12 cells: Potential role in cell survival following oxidative insults. J Biol Chem 277: 9614–9621.
10. Simon Hu, Haj-Yehia A, Levi-Schaffer F (2000) Role of reactive oxygen species (ROS) in tissue injury through mitochondrial ROS. Nature 405:431–435.
11. Nallar SC, Kalvakolanu DV (2017) GRIM-19: A master regulator of cytokine induced tumor suppression, metastasis and energy metabolism. Cytokine Growth Factor Rev 33:1–18.
12. Fearnley IM, et al. (2001) GRIM-19, a cell death regulatory gene product, is a subunit of bovine mitochondrial NADH:ubiquinone oxidoreductase (complex I). J Biol Chem 276:38345–38348.
13. Kalakonda S, et al. (2013) Monoallelic loss of tumor suppressor GRIM-19 promotes tumorgenesis in mice. Proc Natl Acad Sci USA 109:15212–15217.
14. Angell JE, Lindner DJ, Shapiro PS, Hofmann ER, Kalvakolanu DV (2000) Identification of GRIM-19, a novel cell death-regulatory gene induced by the interferon-beta and retinoic acid combination, using a genetic approach. J Biol Chem 275:33416–33426.
15. Zhang Y, et al. (2011) Downregulation of GRIM-19 promotes growth and migration of human glioma cells. Cancer Sci 102:1991–1998.
16. Nallar SC, et al. (2013) Tumor-derived mutations in the gene associated with retinoid interferon-induced mortality (GRIM-19) disrupt its anti-signal transducer and activator of transcription 3 (STAT3) activity and promote oncogenesis. J Biol Chem 288: 7930–7941.
17. Alchanati L, et al. (2006) A proteomic analysis reveals the loss of expression of the cell death regulatory gene GRIM-19 in human renal cell carcinomas. Oncogene 25: 7138–7147.
18. Nallar SC, et al. (2015) Peroxiredoxin-2 and STAT3 form a redox relay for H2O2 signaling. Nat Chem Biol 11:156–163.
19. Peralta D, et al. (2015) A proton relay enhances H2O2 sensitivity of GADPH to facilitate metabolic adaptation. Nat Chem Biol 11:156–163.
20. Wochenschnur BC, Hampton MB (2015) Redox biology: Signaling via a peroxiredoxin sensor. Nat Chem Biol 11:5–6.
21. Guinebaut V, Schlitt A, Weiss H, Leonard K, Friedrich T (1998) Consistent structure between bacterial and mitochondrial NADH:ubiquinone oxidoreductase (complex I). J Mol Biol 276:105–112.
22. Vinokhukar KM, Zhu J, Hir J (2014) Architecture of mammalian respiratory complex I. Nature 515:80–84.
23. Bhattacharya S, et al. (2005) Selective fluorescent imaging of superoxide in vivo using a novel recycling technique. Proc Natl Acad Sci USA 103:15038–15043.
24. Robinson KM, et al. (2006) Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. Proc Natl Acad Sci USA 103:15038–15043.
25. Zhang N, et al. (2016) Cardiac ankyrin repeat protein attenuates cardiomyocyte apoptosis by upregulation of Bcl-2 expression. Biochim Biophys Acta 1863:3040–3049.