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Research Paper

Sestrin2 maintains OXPHOS integrity to modulate cardiac substrate metabolism during ischemia and reperfusion

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ABSTRACT

Sestrin2 (Sesn2) is a stress-inducible protein that declines with aging in the heart. We reported that rescue Sesn2 levels in aged mouse hearts through gene therapy improves the resistance of aged hearts to ischemia and reperfusion (I/R) insults. We hypothesize that Sesn2 as a scaffold protein maintains mitochondrial integrity to protect heart from ischemic injury during I/R. Young C57BL/6 J (3–6 months), aged C57BL/6 J (24–26 months), and young Sesn2 KO (3–6 months, C57BL/6 J background) mice were subjected to *in vivo* regional ischemia and reperfusion. The left ventricle was collected for transcriptomics, proteomics and metabolomics analysis. The results demonstrated that Sesn2 deficiency leads to aging-like cardiac diastolic dysfunction and intolerance to ischemia reperfusion stress. Seahorse analysis demonstrated that Sesn2 deficiency in aged and young Sesn2 KO versus young hearts lead to impaired mitochondrial respiration rate with defects in Complex I and Complex II activity. The Sesn2 targeted proteomics analysis revealed that Sesn2 plays a critical role in maintaining mitochondrial functional integrity through modulating mitochondria biosynthesis and assembling of oxidative phosphorylation (OXPHOS) complexes. The RNA-Seq data showed that alterations in the expression of mitochondrial compositional and functional genes and substrate metabolism related genes in young Sesn2 KO and aged versus young hearts. Further immunofluorescence and immunoprecipitation analysis demonstrated that Sesn2 is translocated into mitochondria and interacts with OXPHOS components to maintain mitochondrial integrity in response to I/R stress. Biochemical analysis revealed that Sesn2 is associated with citrate cycle components to modulate pyruvate dehydrogenase and isocitrate dehydrogenase activities during I/R stress. Thus, Sesn2 serves as a scaffold protein interacting with OXPHOS components to maintain mitochondrial integrity under I/R stress. Age-related downregulation of cardiac Sesn2 fragilizes mitochondrial functional integrity in response to ischemic stress.

1. Introduction

Coronary heart disease (CHD), is also called ischemic heart disease (IHD), remains the primary cause of death globally and the prevalence of IHD increases with aging [1,2]. Moreover, it is observed that elderly patients sustain higher mortality and elevated myocardial damage in both clinical and experimental settings of IHD [3]. The detrimental effects of IHD to myocardium is usually due to the acute ischemia and reperfusion injury [4]. Myocardial ischemia injury is induced by the depletion of blood because of the occlusion of the coronary artery in the heart leading to the abrupt of biochemical and metabolic changes within the myocardium [5]. The deprivation of oxygen and energy supply induces various intracellular damage, such as disrupted oxidative phosphorylation in mitochondria, ATP depletion, disturbance of cellular ion homeostasis, elevated glycolysis, increased permeability of mitochondrial membrane in myocardium, and impaired myocardial contractility [4,6–8]. The most effective intervention to minimize myocardial injury is to reperfuse the heart with either thrombolytic therapy or percutaneous coronary intervention [4,9,10]. However, coaxed cardiomyocyte death is always accompanied by reperfusion namely reperfusion injury [9,10]. During reperfusion, the reactivated electron transport chain,

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Nonstandard abbreviations and acronyms:

AMPK	5' AMP-activated protein kinase
CHD	Coronary heart disease
DEGs	Differential expressed genes
E/A	Early to late ventricular filing velocities ratio
E/E′	Mitral flow E wave/tissue Doppler mitral annulus velocity
GO	Gene ontology
IDH3A	Isocitrate dehydrogenase
IPA	Ingenuity pathway analysis
KEGG	Kyoto encyclopedia of genes and genomes
LAD	Left anterior descending coronary artery
MPTP	Mitochondrial permeability transition pore
mTORC1	Mammalian target of rapamycin complex 1
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
PE	Paired-end
SDHA	Succinate dehydrogenase complex flavoprotein subunit A
TCA	Tricarboxylic acid cycle

washed out lactic acid, restored pH, and the mitochondrial permeability transition pore (MPTP) opening leads to excessive oxidative stress, hypercontracture, inflammation as well as cell death [4,8]. Despite the timely efficient and successful process of reperfusion, there is still no effective therapy for protecting IHD patients, especially elderly patients, from myocardial ischemia and reperfusion injury [4].

Sestrin2 (Sesn2), a conserved stress inducible protein, plays important roles in repressing reactive oxygen species (ROS) as an antioxidant and modulating signaling pathways in responding to hypoxia and nutritional stress through AMP-dependent protein kinase (AMPK) and mammalian target of rapamycin complex 1 (mTORC1) [11–13]. Loss of Sestrin in *Drosophila* (dSesn) results in age-associated and obesity-associated pathologies including cardiac dysfunction and fat accumulation [14]. In mice, we reported that Sesn2 expression decreases in the aging heart leading to intolerance to ischemia reperfusion (I/R) stress [15]. Furthermore, the rescue of Sesn2 in aged heart improves cardiac performance during I/R, indicating Sesn2 is an age-related cardioprotective effector and a new therapeutic target in cardiovascular disease for the aging population [12,15,16]. However, the comprehensive mechanisms by which Sesn2 protects the heart, especially aged heart, from I/R injury remains unclear.

To understand the mechanisms underlying enhanced myocardial injury induced by ischemia and reperfusion in the aged heart and the critical roles of Sesn2 contributing to this phenomenon, we employed comparative transcriptomics, metabolomics, and proteomics analysis among young, aged, Sesn2 KO mice. In this study, we show that Sesn2 is a stress inducible protein that accumulates in mitochondria in response to cardiac I/R stress. In addition, Sesn2 maintains mitochondrial integrity and function to modulate substrate metabolism as an adaptive response to I/R stress. We demonstrate that Sesn2 deficiency induces mitochondrial impairment, cardiomyocyte contractility defects, and maladaptive metabolomic response in heart, leading to exacerbated myocardial cell death and cardiac dysfunction upon I/R stress. Our results provide novel mechanistic insights to the roles of Sesn2 in protecting against cardiac I/R injury.

2. Materials and methods

The authors declare that all supporting data are available within the article and its online supplementary files.

2.1. Animals

Young C57BL/6J mice (3–6 months, young-WT), Aged-WT C57BL/6 J mice (24–26 months, aged-WT) were provided by National Institute on Aging (NIA), the Sesn2 knockout (Sesn2 KO) (3–6 months, C57BL/6 J background), and its littermate mice were bred in our laboratory [13,16,17]. Cardiomyocyte specific deletion of Sesn2 gene was generated by breeding $Sesn2^{flox/flox}$ mice (3–6 months, C57BL/6 J background) with transgenic mice that carried an autosomally integrated Cre gene driven by the cardiac-specific alpha-myosin heavy chain promoter (αMHC) (Jackson Laboratory, 018,972) in our laboratory. All animal protocols in this study were approved by the Institutional Animal Care and Use Committee of the University of South Florida and conform to the NIH Guide for the care and use of laboratory animals.

2.2. In vivo regional ischemia/reperfusion surgery

Mice were subjected for anesthesia, intubation and ventilation as we previously described [15,18]. Reginal ischemia/reperfusion and sham-operation were performed with ligating and non-ligating the left anterior descending coronary artery (LAD) for 45 min and subsequently release for 24 h with a left lateral thoracotomy, respectively.

2.3. Transcriptomics analysis

Total RNA extractions of the left ventricles from young-WT, aged-WT, and Sesn2-KO groups under both sham and I/R challenged conditions were performed and subjected to RNA-sequencing with Illumina NextSeq 500-mRNA-Seq platform in a 2 × 100 bp paired-end (PE) at Molecular and Genomics Core of University of Mississippi Medical center (Jackson, MS). A customized bioinformatics pipeline was applied for alignment, transcript assembly, read-counting as well as comparative analysis to generate differential expressed genes (DEGs) between each two groups [19,20].

2.4. Proteomics analysis

Young-WT, aged-WT, and Sesn2-KO mice were subjected to either sham and I/R challenged conditions and total protein extraction. Immunoprecipitated proteins with Sesn2 antibody were digested and subjected to mass spectrometric analysis with Thermo Q-exactive-HF mass spectrometer coupled to a Thermo Easy nLC 1200. Output data was searched against Uniprot reviewed Mouse database using Thermo Proteome Discoverer 2.2 software. Max quant analysis was then applied to each group among biological replicates (n = 3) and Welch's *t*-test was performed to determine the z-score cut off between each pair comparison. Enrichment analysis was performed with ClueGO app in Cytoscape [21]. Furthermore, ingenuity pathway analysis (IPA) powered by QIAGEN was utilized to identify the involved pathways of Sesn2 associated proteins and upstream transcriptional regulators that can explain the observed alterations of the Sesn2-protein association in response to the I/R stress in the dataset.

2.5. Metabolomics analysis

Frozen left ventricular tissues from the young-WT-sham, aged-WT-sham, Sesn2-KO-sham, young-WT-I/R, aged-WT-I/R, Sesn2-KO-I/R mice were homogenized to extract polar metabolites for 2DLC-MS/MS analysis with Thermo Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled with a Thermo DIONEX UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) [22]. Each sample was respectively analyzed by 2DLC-MS in positive mode (+) and negative mode (−) to obtain the full MS data. For 2DLC-MS data analysis, XCMS software was used for spectrum deconvolution, and MetSign

software was used for metabolite identification, cross-sample peak list alignment, normalization, and statistical analysis [23–25]. Enrichment analysis of significantly altered metabolites were performed with MBROLE 2.0 by searching against KEGG, UniPathway, and BioCyc SMPDB database to obtain significantly regulated pathways [26].

2.6. Statistics analysis

Statistical analysis results of cardiac function, immunoblotting, mtDNA level, oxygen consumption rate, cardiomyocyte contractile function, immunofluorescent staining signal, glucose/fatty acid oxidation rate were performed and expressed as means ± standard error of the means (SEM). Welch's *t*-test, one-way ANOVA with Tukey's test and Kruskal-Wallis test were used to perform the statistics comparison among a set of samples with Prism 8.0 (GraphPad Software). $P < 0.05$ was considered as significant difference.

The data, analytic methods, study materials will be made available to other researchers for purpose of reproducing the results or replicating the procedures. Expanded detailed materials and methods can be found in the Supplemental Materials.

3. Results

3.1. Sesn2 deficiency causes cardiac diastolic dysfunction and augments transcriptomic sensitivity to ischemia reperfusion stress

Sesn2 is a stress inducible protein playing crucial roles in repressing oxidative stress and it is marked to be an age-related protein because of the depletion of Sesn2 in *Drosophila* leading to age-related pathologies [14,15]. Cardiac diastolic dysfunction is commonly associated with cardiac hypertension, obesity, metabolic syndrome, and aging while the patients have preserved systolic function [27]. Impaired Diastolic function is often reported with the decreased ratio between early and late filing to left ventricular (E/A ratio) and increased E/e' ratio (mitral flow E wave/tissue Doppler mitral annulus velocity) which is correlated with left atrial pressure [27]. Echocardiography results showed that Sesn2 KO group demonstrated impaired cardiac diastolic function, similar to that seen in the aged-WT group, as evidenced by decreased E/A ratio in both physiological and I/R injured conditions (Fig. S1A). These results indicated that Sesn2 plays important roles in maintaining myocardial relaxation and ventricular stiffness in aging. In addition, cardiac systolic functions were also assessed in young-WT, aged-WT, and Sesn2 KO mice under physiological and I/R stressed condition. The results showed that I/R leads to cardiac systolic dysfunction in young-WT group with significantly decreased ejection fraction (EF) and fraction shortening (FS), stroke volume, cardiac output, and other parameters (Fig. S1B and Table S1). Furthermore, these effects were exacerbated in aged-WT and Sens2 KO groups (Fig. S1B and Table S1). This suggested that stress inducible protein Sesn2 is critical for the resistance of heart to I/R stress.

Sesn2 was demonstrated to regulate AMPK and mTORC1 signaling pathway under various stress conditions, such as oxidative stress and ischemic stress, which are critical for mediating cellular energetic, metabolic, and translational process [11,15,16]. To explore the roles of Sesn2 as an age-related regulator in adaptation to I/R stress, RNA-Seq was performed to assess the gene expression alteration pattern in myocardium under cardiac I/R stress in young-WT, aged-WT and Sesn2 KO mice. Comparative analysis results demonstrated an astonishing finding that Sesn2 KO heart exhibits 1323 unique DEGs after I/R stress (Fig. 1A). By visualizing the clustered KEGG pathway results in Fig. 1B, the remarkable pattern was illustrated with 28 unique metabolic pathways significantly downregulated in the Sesn2 KO heart in response to I/R stress (Fig. 1B). Gene Ontology (GO) clustering analysis demonstrated these unique DEGs are associated with substrate catabolic and metabolic processes (GO: 0009062; GO: 0009063; GO: 0006103; GO: 0009081) which are extensively down-regulated in Sesn2KO heart under I/R stress (Fig. 1C, red frames highlighted). Furthermore, significant down-regulation of mitochondrial matrix (GO: 0005759) was observed in in Sesn2KO heart after I/R stress, in which 190 DEGs (18.5%) overlapped with 1, 027 known genes with a p-value as 7.1E-43 as well as the mitochondrial inner membrane (GO: 0005743), in which 85 DEGs (27.5%) overlapped with 309 known genes and a p-value as 6.1E-32 (Fig. 1D, highlighted in red frames).

These transcriptomics analyses illustrated the impaired expression of mitochondrial associated proteins in Sesn2 KO heart under I/R stress, indicating that Sesn2 plays important roles in maintaining mitochondrial transcriptional integrity and function in adaptation to cardiac I/R injury. Besides, multiple inflammatory related biological processes and cellular components were up-regulated in both aged-WT and Sesn2 KO heart compared to young-WT group under I/R injury (Fig. 1C–D, highlighted in black frame) indicated that Sesn2 deficiency resulted in the elevated inflammatory response to I/R stress.

3.2. Sesn2 maintains the integrity of OXPHOS complexes in mitochondria under I/R stress

In cardiomyocytes, mitochondria are responsible for more than 95% of cellular energy production, which are critical for maintaining cardiomyocyte contractility, mediating cellular signaling, substrate metabolism and ion homeostasis [28]. Mitochondria inner membrane is folded into cristae and embedded with the oxidative phosphorylation (OXPHOS) complexes I to V, which is responsible for ATP generation through mediating electron transportation [28,29]. Mitochondrial proteins are encoded by the nuclear and mitochondrial DNA (mtDNA), which contains 37 genes and encodes 13 OXPHOS complex subunits [29].

To investigate the roles of Sesn2 in mitochondrial biosynthesis, the transcriptional alteration of mitochondrial biosynthesis associated genes (Reactome database) were clustered in six groups. The results showed that Nr5a2 (Nuclear hormone receptor), Atp5s (ATP synthase), Celsr2 (Cadherin EGF LAG Seven-Pass G-Type Receptor 2), Perm 1 (PGC1 And ERR Induced Regulator), and Prkab1 (AMPK) were significantly down-regulated in aged-WT and Sesn2 KO group under I/R stress, suggesting Sesn2 deficiency affects the expression of the mitochondrial biosynthesis associated genes (Fig. 1E). Furthermore, immunoblotting results demonstrated that major mitochondrial biogenesis regulator, PGC-1α (peroxisome-proliferator-activated receptor γ co-activator-1α) protein is significantly up-regulated in young-WT heart after I/R stress but the effects were blunted in aged-WT and Sesn2 KO heart (Fig. 1F). In addition, the accumulation of NFR2 (nuclear respiratory factor 2), downstream factor of PGC-1α was significantly lower in aged-WT and Sesn2 KO hearts than that in young hearts (Fig. 1F). These results indicated that Sesn2 is critical for modulating the expression of PGC-1α and NFR2 in heart under I/R stress.

To study the role of Sesn2 in mediating mitochondrial DNA integrity, mtDNA copy were assessed in the six groups. The results showed significantly decreased mtDNA content after I/R in aged-WT and Sesn2 KO heart compared to young-WT group, indicating the greater loss of mtDNA in aged-WT and Sesn2 KO hearts versus young-WT hearts (Fig. 1G). Furthermore, gene expression alteration profiles of OXPHOS complex subunits among young-WT, aged-WT, and Sesn2 KO heart in response to I/R stress were extracted and analyzed from RNA-seq data. The results showed the expression of majority subunits in Complex I (Ndufa10, Ndufa1, Ndufaf1, Ndufaf3, Foxred1, Nubpl), Complex II (Sdha, Sdhb, Sdhc), Complex III (Uqcrc2, Uqcrfs1, Cyc 1), and Complex V (Atp5a1, Atpaf2, Tmem 70) were significantly down-regulated in Sesn2-KO heart under normal physiological conditions and the effect was elevated after I/R stress (Fig. 2A–E). In addition, immunoblotting results of representative OXPHOS Complex subunits showed the accumulation of Complex II subunit SDHA, Complex V subunit ATP5A, Complex III UQCRC2 in aged-WT and Sesn2 KO heart were significantly lower compared to the young-WT heart after I/R insults, indicating the

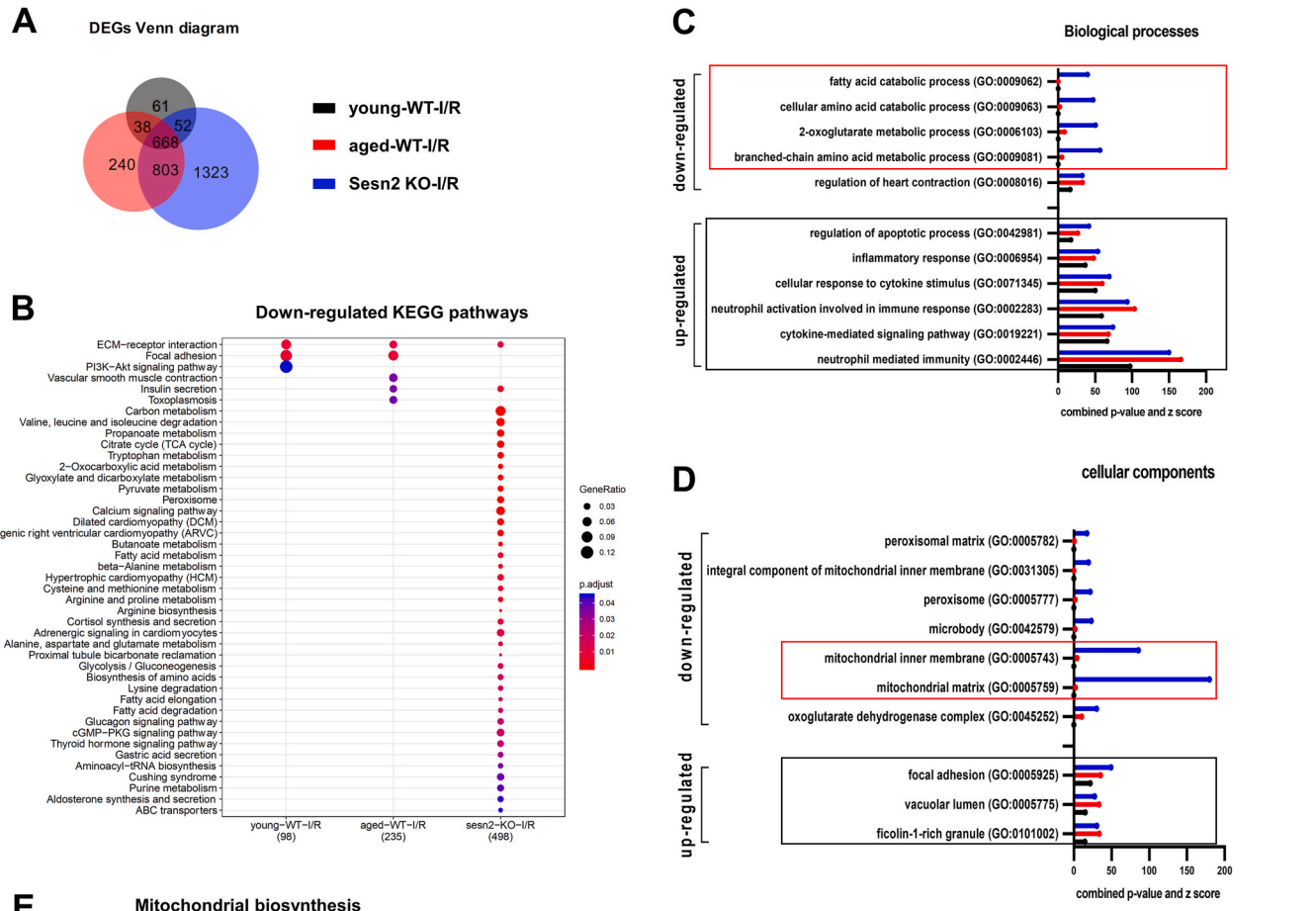

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Fig. 1. Loss of Sesn2 leads to transcriptomic hypersensitivity and impaired mitochondria biosynthesis in responding to ischemia reperfusion stress. (A) Venn diagram of differently expressed genes (DEGs) in young-WT-I/R, aged-WT-I/R, and Sesn2 KO–I/R groups normalized by young-WT-sham group (n = 3, adjusted p value < 0.05), DESeq analysis. (B) Significantly down-regulated KEGG signaling and disease pathways of young-WT, aged-WT, and Sesn2 KO groups in response to I/R stress (n = 3, adjusted p value < 0.05), clustered with clusterProfiler from all significantly down-regulated DEGs generated from DESeq analysis. Color represent p-value from lowest (red) to highest (blue) and the bubble size represent the significantly shifted gene ratio in the individual pathway. (C) GO term enriched significantly altered biological functions in young-WT, aged-WT, and Sesn2 KO groups in response to I/R stress (n = 3, adjusted p value < 0.05), clustered with REVIGO from all significantly altered DEGs generated from DESeq analysis. (D) GO term enriched significantly altered cellular components in young-WT, aged-WT, and sesn2-KO groups in response to I/R stress (n = 3, adjusted p value < 0.05), clustered with REVIGO from all significantly altered DEGs generated from DESeq analysis. (E) Relative transcriptional abundance of mitochondrial biosynthesis associated genes in young-WT, aged-WT and Sesn2 KO hearts vs. young-WT-sham under physiological and I/R stressed conditions (n = 3), DESeq analysis. Color represent the increase (red) and decrease (blue) of each gene, alteration ratios are presented in log2 form according to the legend panel. (F) Western blot analysis of mitochondrial biogenesis regulatory protein levels in young-WT, aged-WT and Sesn2 KO hearts *vs.* young-WT-sham under physiological and I/R stressed conditions (n = 5, values are means ± SEM, *p < 0.05 *vs.* young-WT Sham; †p < 0.05 *vs.* young-WT I/R; ‡p < 0.05 *vs.* young-WT Sham, One-way ANOVA). (G) Relative mtDNA copy was determined in young-WT, aged-WT and Sesn2 KO heart *vs.* young-WT-sham under physiological and I/R stressed conditions (n = 5, †p < 0.05 *vs.* young-WT I/R, values are means ± SEM, One-way ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

exacerbated mitochondrial OXPHOS impairment in Sesn2 KO heart after I/R stress (Fig. 2F). Of interest, the accumulation of Complex I subunit NDUFB8, Complex II subunit SDHB and SDHA, Complex III subunit UCQRC2, and Complex V subunit ATP5A were significantly decreased in Sesn2 KO sham operated heart compared to young-WT group, suggesting that Sesn2 is required to maintain mitochondrial OXPHOS structural integrity in terms of Complex I, II, III, and V in physiological heart.

3.3. *Sesn2 deficiency leads to impaired mitochondrial respiratory function in heart*

OXPHOS (oxidative phosphorylation) coupling with the electron transfer chain driven by substrates (e.g., glucose and fatty acids) through the electron transmembrane gradient is the major mechanism for ATP generation utilizing oxygen in mitochondria [30,31]. Given the defective OXPHOS complexes subunit in aged-WT and Sesn2-KO hearts under I/R stress, we next performed the coupling assay and electron flow assay using Seahorse X96 analyzer with isolated cardiac mitochondria to investigate the roles of Sesn2 in mediating mitochondrial respiratory function.

The effect of aging and Sesn2 on basal respiration (state 2), phosphorylating respiration in the presence of ADP (state 3), resting respirations with oligomycin (state 4_0), maximal uncoupling respiration in the presence of FCCP (state 3μ), and the response to antimycin A was determined using 5 μg isolated cardiac mitochondria from young-WT, aged-WT, and Sesn2-KO groups (Fig. 3A). Under physiological condition, basal oxygen consumption rate (OCR) of young-WT group was higher than the aged-WT and Sesn2 KO groups (Fig. 3B). Meanwhile, we observed significantly elevated State 3 phosphorylating respiration induced by addition of ADP in young-WT compared to aged-WT and Sesn2 KO groups (Fig. 3B). There was non-significant difference among three groups in state 4_0 respiration induced by oligomycin (Fig. 3B). The maximal respiration in response to uncoupling by FCCP was higher in young-WT group versus aged-WT and the response to complex III inhibitor antimycin A was similar among the groups (Fig. 3B). In the coupling assay, pyruvate and malate was used to drive the mitochondria respiration via complex I, while the state 2 respiration rate and maximal respiration with FCCP injection are uncoupled in Sesn2 KO group, indicating that Complex I is impaired in Sesn2 KO heart (Fig. 3B).

To investigate the roles of Sesn2 in modulating mitochondrial respiratory function after I/R stress, the cardiac mitochondria from I/R groups were isolated after 45 min ischemia and 24 h reperfusion. Basal OCR of young-WT-I/R group was significantly decreased compared to young-WT-Sham group, indicating the presence of damaged mitochondria after I/R stress (Fig. 3B). Intriguingly, Sesn2 KO group resembled the aged-WT group with significantly lower State 2 basal respiration, State 3 phosphorylating respiration, and state 3μ maximal respiration rate compared young-WT group, indicating the exacerbated mitochondrial damage with defected Sesn2 (Fig. 3B).

To further demonstrate the effect of aging and Sesn2 on individual mitochondrial respiratory complexes, we performed the electron flow experiment with pyruvate and malate as substrates to drive initial respiration from complex I (Fig. 3A). There was a significant decrease in the basal OCR values of mitochondria from aged-WT mice heart compared to young-WT mice indicating the impaired Complex I activity in the Sesn2 defected heart (Fig. 3C). When rotenone was injected the OCR came to baseline level indicating inhibition of Complex I driven respiration (Fig. 3C). When Complex II driven respiration was initiated by sequential addition of succinate, the OCR in young-WT mice was significant higher compared to aged-WT and Sesn2 KO mice, indicating that Complex II function is significantly compromised in the Sesn2 deficiency heart (Fig. 3C). The function of Complex IV was determined after blocking of complex III function by addition of antimycin A. There were no differences among three groups in Complex IV function when ascorbate + TMPD was used to drive complex IV-mediated oxygen consumption (Fig. 3C). These results demonstrated that ischemia reperfusion impairs Complex I and Complex II activity in cardiac mitochondria and these effects are exacerbated in aged-WT and Sesn2 KO heart, indicating the crucial role of Sesn2 in modulating mitochondria respiratory function (Fig. 3C). Combined with the mitochondrial integrity and respiratory function results, Sesn2 is demonstrated to be a critical regulator in maintaining cardiac mitochondrial structural and functional integrity in terms of Complex I, II and III and V.

3.4. *Cardiomyocyte Sesn2 defects results in impaired contractile property*

Mitochondria play a crucial role in providing energy for maintaining the contractile property of cardiomyocyte [32]. It is well known that the impaired mitochondrial function is associated with various cardiovascular disease due to defective cardiac energetics and its related signaling regulation pathway [32]. In line with our previous observation that Sesn2 deficiency in heart leads to mitochondrial functional and structural defects, we proposed to evaluate the effects of on the contractile properties of isolated cardiomyocytes from young/aged-WT as well as cardiac-specific Sesn2 knockout Cre-Sesn2$^{flox/flox}$ (cSesn2$^{-/-}$) and the Sesn2$^{flox/flox}$ (Sesn2$^{f/f}$) control hearts under normoxia or hypoxia/reoxygenation (H/R) conditions. During normoxia condition, contractile property of aged-WT and cSesn2$^{-/-}$ isolated cardiomyocyte is impaired demonstrated by significantly reduced sarcomere shortening length, percentage of shortening, and the rate of shortening were impaired versus young-WT and Sesn2$^{f/f}$ group, respectively (Fig. 3D). Consistent with the results from seahorse analysis of mitochondrial function, we found that the defects of Sesn2 resulted in both mitochondrial function and cardiomyocytes contractile function impairment under normoxia condition (Fig. 3D). Analysis of the transient calcium flux in cardiomyocytes by measuring the fura-2 signal alteration during contraction showed that cardiac specific Sesn2 deletion elevated calcium flux during cardiomyocyte contraction in comparison with Sesn2$^{f/f}$ cardimyocytes with upregulated calcium shortening peak, the percentage of shortening, and the rate of shortening (Fig. 3E). However, there is no

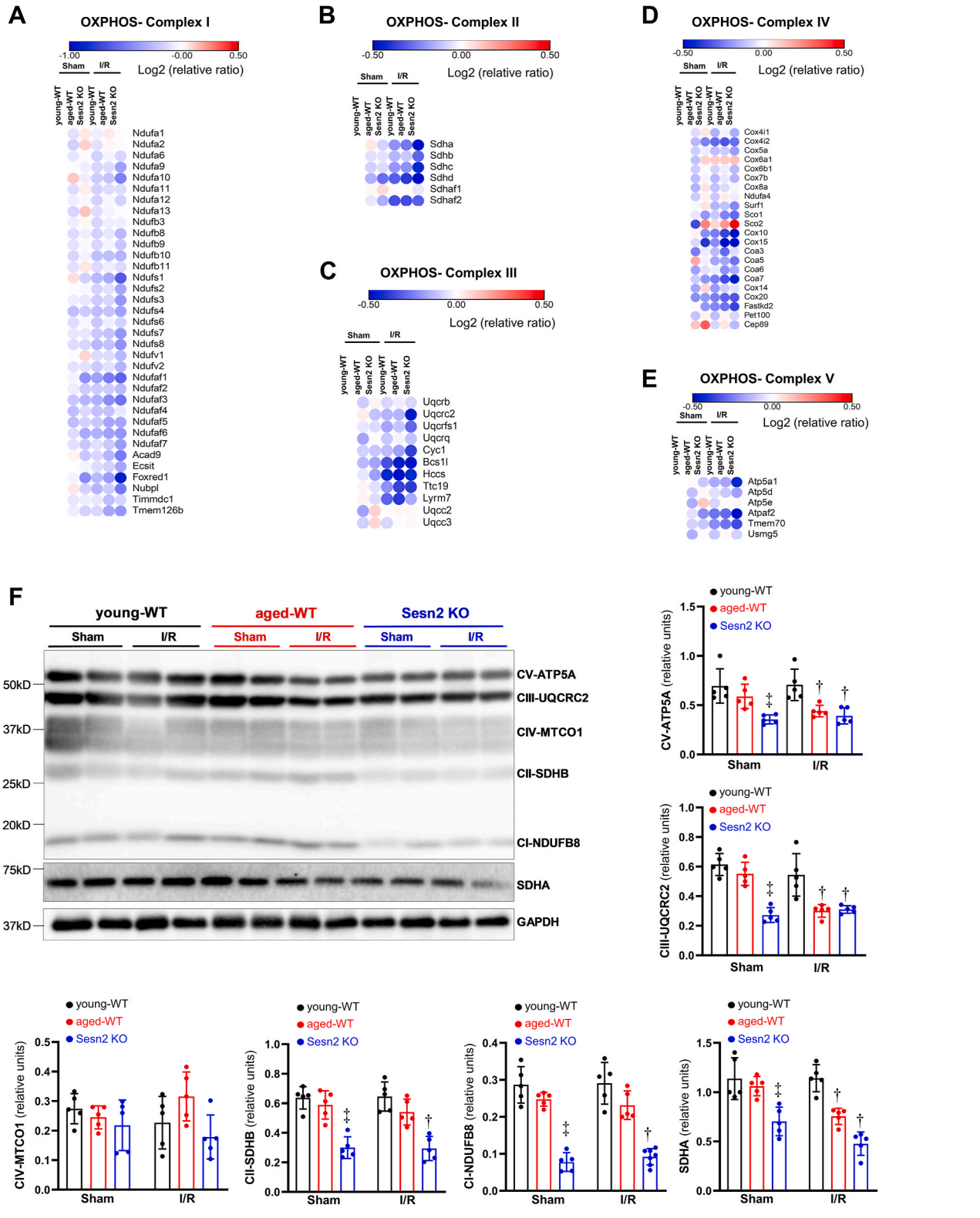

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Fig. 2. Sesn2 is critical to maintain OXPHOS complexes integrity in heart under I/R stress. (A) Relative transcriptional abundance of mitochondrial OXPHOS Complex I subunits in young-WT, aged-WT and Sesn2 KO hearts *vs.* young-WT Shamunder physiological and I/R stressed conditions (n = 3), DESeq analysis. Color represent the increase (red) and decrease (blue) of each gene, alteration ratios are presented in log2 form according to the legend panel. (B) Relative transcriptional abundance of mitochondrial OXPHOS Complex II subunits in young-WT, aged-WT and Sesn2 KO hearts *vs.* young-WT Sham under physiological and I/R stressed conditions (n = 3), DESeq analysis. Color represent the increase (red) and decrease (blue) of each gene, alteration ratios are presented in log2 form according to the legend panel. (C) Relative transcriptional abundance of mitochondrial OXPHOS Complex III subunits in young-WT, aged-WT and Sesn2 KO heart *vs.* young-WT Sham under physiological and I/R stressed conditions (n = 3), DESeq analysis. Color represent the increase (red) and decrease (blue) of each gene, alteration ratios are presented in log2 form according to the legend panel. (D) Relative transcriptional abundance of mitochondrial OXPHOS Complex IV subunits in young-WT, aged-WT and Sesn2 KO hearts *vs.* young-WT Sham under physiological and I/R stressed conditions (n = 3), DESeq analysis. Color represent the increase (red) and decrease (blue) of each gene, alteration ratios are presented in log2 form according to the legend panel. (E) Relative transcriptional abundance of mitochondrial OXPHOS Complex V subunits in young-WT, aged-WT and Sesn2 KO heart *vs.* young-WT Sham under physiological and I/R stressed conditions (n = 3), DESeq analysis. Color represent the increase (red) and decrease (blue) of each gene, alteration ratios are presented in log2 form according to the legend panel. (F) Western blot analysis of OXPHOS complex I, II, III, IV, V subunits accumulation levels in young-WT, aged-WT and Sesn2 KO hearts under physiological and I/R stressed conditions (n = 5, values are means ± SEM, †p < 0.05 *vs.* young-WT I/R; ‡p < 0.05 *vs.* young-WT Sham, One-way ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

difference between young-WT and aged-WT in terms of calcium flux during cardiomyocyte contraction.

After 20 min hypoxia and 20 min reoxygenation stress, the cardiomyocyte contractile and calcium flux properties were defected in young-WT and Sesn2$^{f/f}$ group versus normoxia condition (Figs. 3D and 3E). This effect was exacerbated in aged-WT and cSesn2$^{-/-}$ cardiomyocyte due to the impairment of Sesn2 while compared to young-WT and Sesn2$^{f/f}$ group, respectively (Figs. 3D and 3E). In addition, the contractile-calcium flux loop relation showed that H/R stress shifted the loop towards left and up indicating the reduction of contractile function and calcium flux (Fig. 3F). The shift in aged-WT and cSesn2$^{-/-}$ cardiomyocytes was aggravated compared to young WT and Sesn2$^{f/f}$ group which is consistent with the representative sarcomere length and calcium fura signal dynamic figures (Fig. 3D and 3E). With the impaired mitochondrial function and OXPHOS complexes accumulation in Sesn2 defected heart, it is unsurprisingly that we also observed that Sesn2 deficiency induced cardiomyocyte contractile property decline.

3.5. Age-related Sesn2 is associated with OXPHOS and TCA components in response to I/R stress

To determine the mechanism of Sesn2 in maintaining mitochondrial integrity and function, we used Sesn2 specific antibody and the lysate of whole left ventricular to perform immunoprecipitation and followed proteomics analysis of young-WT, aged-WT, and Sesn2-KO groups with sham and I/R operations. GO term enrichment analysis was performed out of the 55 proteins that were associated with Sesn2 normalized by negative control group (Sesn2 KO). It was demonstrated that Sesn2 is associated with mitochondrial protein complex, respiratory chain complex, tricarboxylic acid cycle (TCA) enzymes, and substrate metabolism related proteins (Fig. 4A). Comparative proteomics analysis with Integrated pathway (IPA) supplied by QIAGEN was utilized between I/R and Sham group in young heart showed the up-regulated association of Sesn2 and proteins involved in TCA cycle, PDH complex, OXPHOS complex, and 2-ketoglutamrate dehydrogenase complex (Fig. 4A). These effects were observed blunted in aged-WT heart indicated defected Sesn2 regulatory mechanism in aged heart in response to I/R stress (Fig. 4A).

Furthermore, immunoblotting results of the Sesn2 immunoprecipitated proteins demonstrated that Sesn2 directly interacts with OXPHOS Complex I subunit NDUFB8, Complex II subunit SDHB, and Complex III subunit UQCRC2 (Fig. 4B). Moreover, the association of Sesn2 with Complex II subunit SDHB was significantly enhanced in young-WT heart in response to I/R stress, indicating Sesn2 could modulate OXPHOS Complex II accumulation through direct association (Fig. 4B). Intriguingly, the association of Sesn2 with SDHB and Complex I subunit NDUFB8 were significantly decreased in aged-WT versus young-WT heart in response to I/R stress (Fig. 4B). Combined with the mitochondrial OXPHOS activity results generated from seahorse analyzer, these results suggested that the impaired Complex I and Complex II in aged-WT heart under I/R stress might be ascribed to their defected association of Sesn2.

In addition, comparative proteomics analysis between I/R and Sham condition in young-WT heart reported that the association of four proteins with Sesn2 were up-regulated in response to I/R, which were pyruvate dehydrogenase (PDH) complex E3 subunit (DLDH), succinate dehydrogenase subunit (SDHA) and cytochrome b-c complex (ETC component) (Table 1). However, these effects were abolished in aged-WT. On contrary, in aged-WT heart, there are three Sesn2 associated proteins significantly up-regulated in adaptation to I/R, which were isocitrate dehydrogenase (IDH3A), stress- 70 protein, and cytochrome b-c complex (ETC component) (Table 1). These results indicated the potential different roles and mechanisms of Sesn2 in young and aged heart during I/R stress which need further investigation.

3.6. Sesn2 accumulates in myocardial mitochondria in response to I/R stress

Integrating with above results, Sesn2 plays critical role in maintaining mitochondria integrity by direct association with OXPHOS complexes components under both physiological and I/R stressed conditions. To determine whether the distribution of Sesn2 in mitochondria is altered in response to I/R stress, mitochondrial fractionations were extracted and Sesn2 accumulation in mitochondria were detected (Fig. 5A). First, we detected the accumulation of Sesn2 in mitochondrial fraction in young-WT and aged-WT heart (Fig. 5A). Intriguingly, the accumulation of Sesn2 was significantly elevated in young-WT I/R group compared to the young-WT sham group indicating that ischemia reperfusion stress triggered accumulation of Sesn2 in mitochondria, leading to its increased association with mitochondrial OXPHOS complexes and TCA related enzymes (Figs. 5A and 4B). However, the accumulation of Sens2 in mitochondria in response to I/R stress were diminished in the aged heart, indicating that the impaired Sesn2 accumulation in mitochondria in the aged heart could result in defected mitochondrial integrity and function under I/R stress (Figs. 5A, 4B and 3). To confirm the expression of Sesn2 in myocardial mitochondria, immunofluorescence staining was performed by detect the colocalization of Sesn2 and MitoTracker DeepRed in heart cryostat sections and isolated cardiomyocytes (Fig. 5B–C). The colocalization of Sesn2 and MitoTracker DeepRed were significantly increased in young-WT heart and cardiomyocytes after I/R or H/R stress, respectively (Fig. 5B–C). Furthermore, significantly less Sesn2 and MitoTracker DeepRed staining were observed in aged-WT and Sesn2-KO groups compared to young-WT-I/R group in I/R or H/R stressed heart (Fig. 5B–C). It confirmed the impairment of Sesn2 in aged heart which leading to the exacerbated mitochondria damage in Sesn2 defected heart under ischemia reperfusion stress (Fig. 5B–C). This study demonstrated that Sesn2 is localized in mitochondria and directly associated with OXPHOS complexes, which play important roles in adaptation to ischemia reperfusion stress.

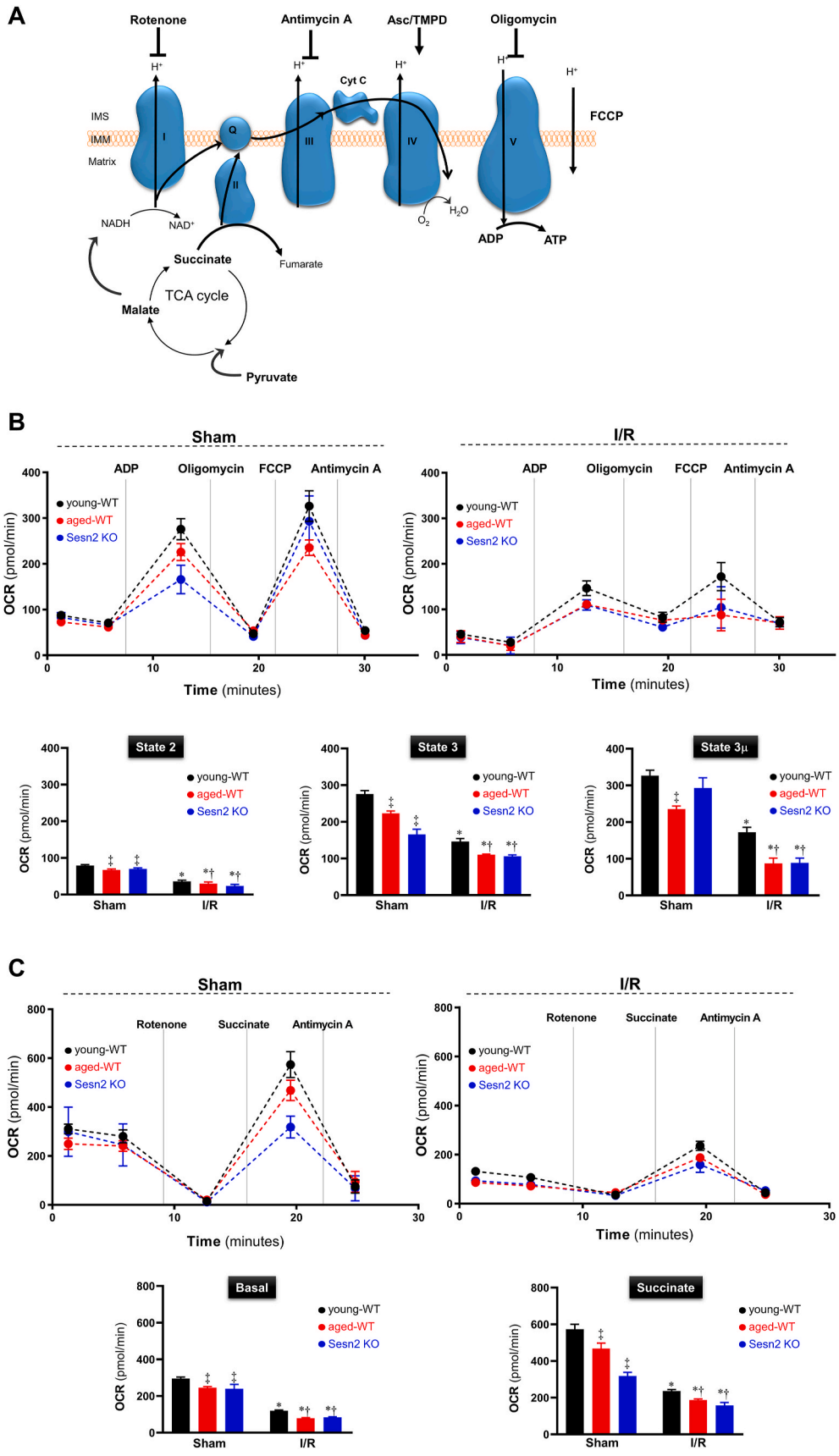


Fig. 3. Sesn2 deficiency in aging causes impaired mitochondrial respiratory function in heart. (A) The scheme depicts the distinct substrate and compounds entry points into the respiratory chain. (B) Oxygen consumption rate (OCR) was measured using XFe 96 Seahorse bioanalyzer in Complex I coupling assay using isolated heart mitochondria from young-WT, aged-WT and Sesn2 KO heart under physiological and I/R stressed conditions in response to ADP, oligomycin, FCCP and antimycin A using point-to-point measurements. OCR of state 2, state 3, and state 3μ respirations were measured and compared among groups (n = 5, values are

means ± SEM, *p < 0.05 *vs.* Sham, respectively; †p < 0.05 *vs.* young-WT I/R; ‡p < 0.05 *vs.* young-WT Sham, One-way ANOVA). (C) Electron flow in mitochondria isolated from mouse hearts were determined by oxygen consumption rate (OCR) using isolated mitochondria in young-WT, aged-WT and Sesn2 KO groups. OCR of electron flow driven by Complex I with pyruvate and malate as substrates, and the effect of rotenone, succinate, and antimycin A were measured and compared among groups (n = 5, values are means ± SEM, *p < 0.05 *vs.* Sham, respectively; †p < 0.05 *vs.* young-WT I/R; ‡p < 0.05 *vs.* young-WT Sham, One-way ANOVA). (D) The contractile properties of isolated cardiomyocytes from young/aged-WT and Sesn2$^{f/f}$/cSesn2$^{-/-}$ mouse hearts under normoxia or H/R stress conditions. n > 5, *p < 0.05 *vs.* young-WT, respectively; †p < 0.05 *vs.* Normoxia, respectively. (E) The transient calcium signal response of the isolated cardiomyocytes from young/aged WT and Sesn2$^{f/f}$/cSesn2$^{-/-}$ mouse under normoxia or H/R stress condition. n > 5, *p < 0.05 *vs.* young-WT, respectively; †p < 0.05 *vs.* Normoxia, respectively. (F)The representative traces of transient calcium signal and the relationship loop between sarcomere length and calcium signal of cardiomyocytes from young/aged WT and Sesn2$^{f/f}$/cSesn2$^{-/-}$ mouse under normoxia or H/R stress conditions.

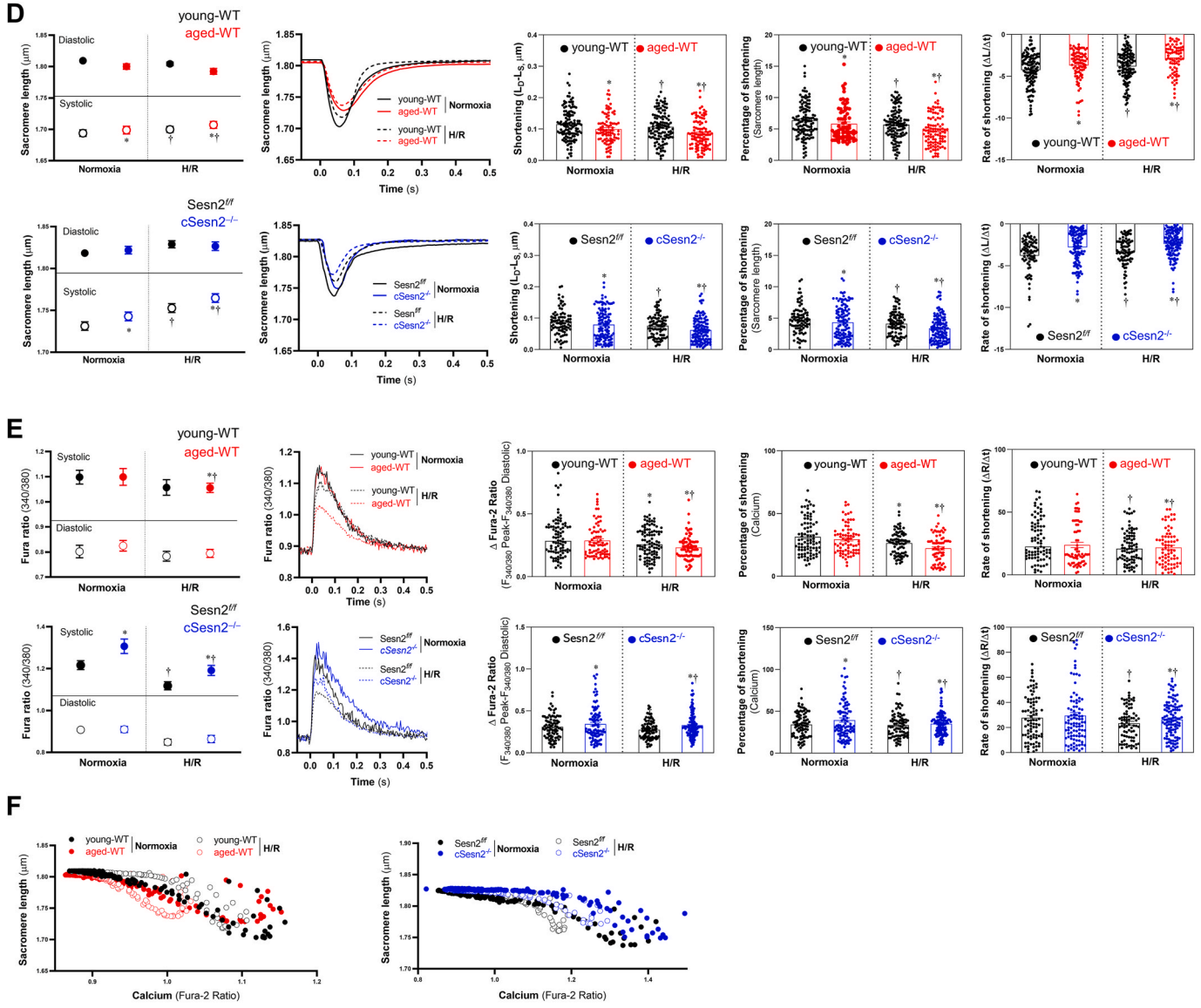


Fig. 3. (*continued*).

3.7. Age-related Sesn2 modulates PDH and IDH activity in responding to I/R stress

From the proteomics analysis results, Sesn2 was demonstrated to associate with metabolic related enzymes besides OXPHOS complexes in mitochondrial inner membrane suggested that Sesn2 modulates substrate metabolism in response to I/R stress through the key enzymes (PDH and IDH) in mitochondria matrix (Table 1 and Fig. 4A). To explore whether Sesn2 modulates the PDH and IDH activity in mitochondria, the activity-inhibited form, phosphorylated PDHE1α at site 293 were examined as well as the pyruvate dehydrogenase kinase (PDK1) in young-WT, aged-WT and Sesn2 KO groups. Interestingly, PDK1 and phosphorylated PDH were significantly increased in Sesn2 KO heart in both normal physiological and I/R injured heart compared to young-WT heart (Fig. 6A). Increased PDK1 accumulation was also observed in aged-WT normal physiological heart compared to young-WT and the effect was exacerbated after I/R insults (Fig. 6A). It demonstrated that Sesn2 is critical for regulating PDK1 accumulation in heart which affects PDHE1α activity and downstream metabolic processes. Isocitrate was used to determine IDH activity in fresh homogenized left ventricular from young-WT, aged-WT and Sesn2 KO groups. In response to I/R stress, Sesn2 KO heart showed significantly decreased NAD^+ and $NADP^+$ dependent IDH activity indicating that Sesn2 depletion impairs IDH activity under I/R stress (Fig. 6B). However, these effects were not observed in aged-WT group suggesting the association of Sesn2 with IDH in aged heart is critical to maintain IDH activity (Fig. 6B, Table 1).

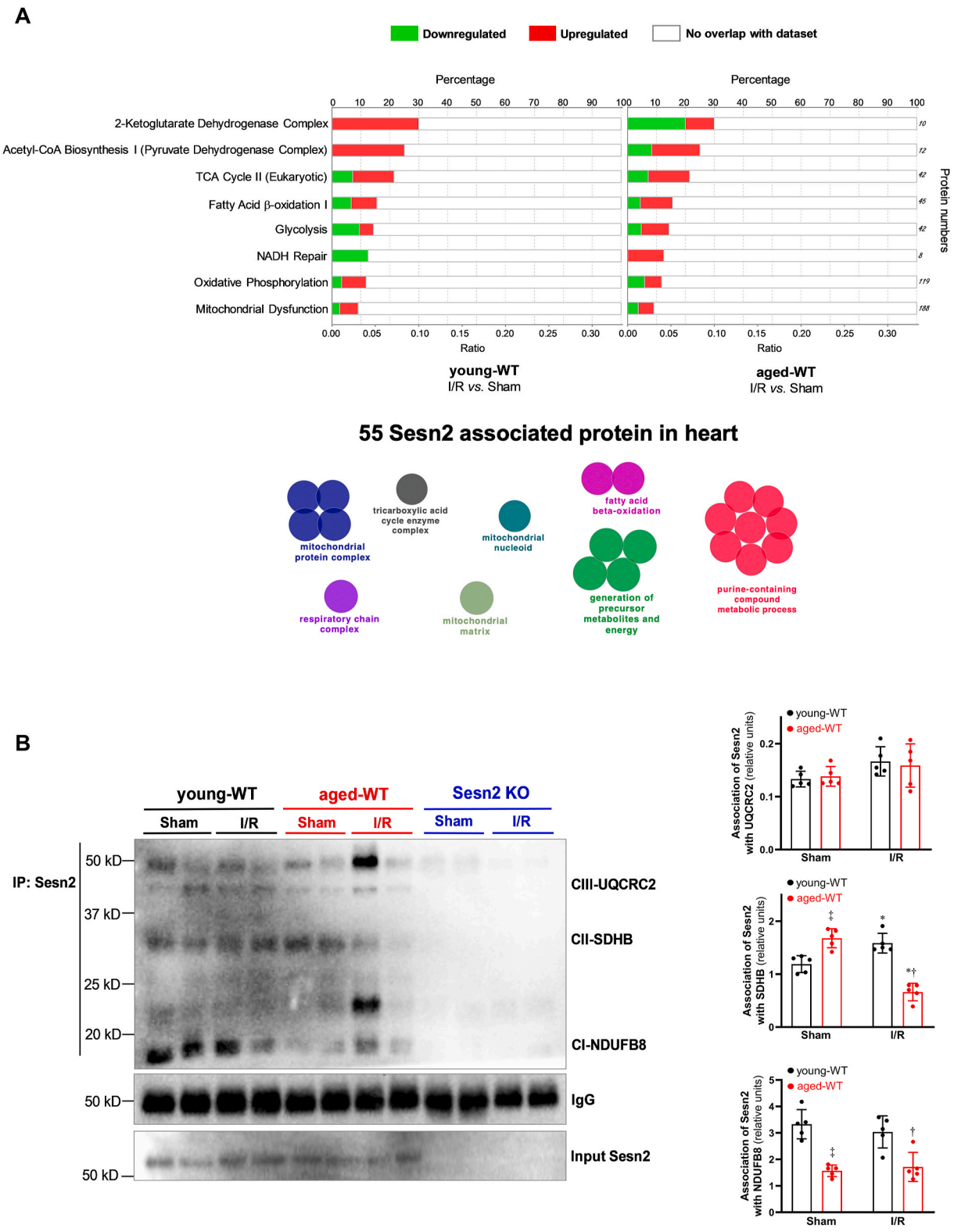


Fig. 4. Sesn2 is associated with OXPHOS and TCA components in responding to I/R stress. (A) Upper: Ingenuity pathway analysis (IPA) enrichment analysis of the dynamics of Sesn2 associated proteins in young-WT and aged-WT hearts in response to I/R stress, respectively. Green bars are representing the percentage of genes in the pathway were down-regulated in response to I/R in young-WT and aged-WT hearts *vs.* sham condition. Red bars are representing the percentage of genes in the pathway were up-regulated in response to I/R in young-WT and aged-WT hearts *vs.* sham condition. White bars are representing the percentage of genes in the pathway were not altered in response to I/R or not associated with Sesn2 in young-WT and aged-WT hearts *vs.* sham condition. Lower: GO term enrichment analysis of Sesn2 associated proteins in young-WT and aged-WT clustered with ClueGO, Welch's *t*-test. (B) Western blot analysis of OXPHOS complexes subunits interacting with Sesn2 in young-WT, aged-WT and Sesn2 KO hearts under physiological and I/R stressed conditions (n = 5, values are means ± SEM, *p < 0.05 *vs.* Sham, respectively; †p < 0.05 *vs.* young-WT I/R; ‡p < 0.05 *vs.* young-WT Sham, One-way ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Sesn2 associated protein in response to I/R stress in young-WT and aged-WT heart.

Symbol ID	Proteins	young-WT I/R *vs.* Sham		aged-WT I/R *vs.* Sham	
		p-value	ratio	p-value	ratio
DLDH	Dihydrolipoyl dehydrogenase	0.019	2.068	>0.05	0.849
QCR1	Cytochrome b-c1 complex subunit 1	0.028	1.542	>0.05	1.117
AT2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	0.033	1.246	>0.05	1.108
SDHA	Succinate dehydrogenase [ubiquinone] flavoprotein subunit	0.049	1.532	>0.05	1.095
QCR6	Cytochrome b-c1 complex subunit 6	>0.05	0.804	0.023	1.126
IDH3A	Isocitrate dehydrogenase [NAD] subunit gamma 1	>0.05	0.965	0.025	1.472
GRP75	Glucose regulated protein 75	>0.05	1.010	0.046	1.233

Note: ratio is refereeing to the normalized protein abundance associated with Sesn2 (n = 3).

3.8. Age-related Sesn2 is critical to maintain metabolic homeostasis during I/R stress

Metabolomics analysis were further performed to characterize the roles of Sesn2 in modulating substrate metabolisms considering Sesn2 modulating metabolism related complexes, such as TCA, OXPHOS, PDH, and IDH. Representative relative abundance of metabolites presented in the heatmap showed the comparable pattern between aged-WT and Sesn2 KO heart in response to I/R stress (Fig. 7A). To explore the substrate metabolism alteration pattern in young-WT, aged-WT, and Sesn2 KO heart, specific glycolysis, TCA cycle and amino acid metabolism related metabolites were extracted and compared between I/R and physiological condition (Fig. 7B). Glycolytic metabolites were visualized to present a clear insight into the effect of I/R on metabolite profiles in young-WT, aged-WT, and Sesn2 KO heart (Fig. 7C). In response to I/R stress, the increased metabolites accumulation pattern was observed in Sesn2 KO heart compared to young-WT and aged-WT heart indicating elevated glycolysis in Sesn2 defected heart after I/R stress (Fig. 7C). As to TCA cycle, it was showed the elevated pyruvate, α-ketoglutarate, fumarate, and malate in young-WT heart in response to I/R stress *versus* aged-WT and Sesn2 KO heart, indicating that Sesn2 deficiency leads to a compromised metabolic adaptive response in terms of TCA cycle (Fig. 7D). Furthermore, the significantly shifted metabolic modules and pathways were then clustered (Figs. 7E–7G) from significantly up-regulated and down-regulated metabolites, respectively. In response to I/R stress, TCA pathway, several ribonucleotides salvage pathways and beta-Alanine biosynthesis pathway were significantly augmented in young-WT heart (Fig. 7E, highlighted in black frame). Amino acid metabolism and glutathione biosynthesis were found significantly regulated as well (Fig. 7E). These findings indicated the metabolic adaptive response in young-WT heart with increased citrate acid flux and cardiac remodeling processes after I/R stress. However, compared to young-WT hearts, the metabolic adaptive responses related to salvage ribonucleotides and amino acid biosynthesis were impaired in aged-WT heart (Fig. 7F, highlighted in red frame). Furthermore, nearly every metabolic process in young-WT hearts as adaptive response to I/R stress included amino acid biosynthesis, salvage of nucleotides processes, and citrate cycle were significantly downregulated in Sesn2 KO hearts (Fig. 7G, highlighted in blue frame). Intriguingly, Sesn2 KO hearts exhibited elevated glycolysis and pyruvate metabolism compared to young-WT hearts in response to I/R stress (Fig. 7D). These results demonstrated that Sesn2 is required to prompt metabolic adaptive response by elevating amino acid biosynthesis and citrate cycle to assist cardiac remodeling after I/R insults.

3.9. Sesn2 is critical for metabolic adaptive response to I/R stress

To validate the critical roles of Sesn2 in mediating the metabolic adaptive response to ischemia reperfusion stress, the isolated hearts from young/aged-WT and $Sesn2^{f/f}$/$cSesn2^{-/-}$ mice were perfused in the *ex vivo* working heart perfusion system (non-recirculating manner) for 20 min no-flow induced ischemia and 30 min reperfusion with a preload of 12.5 mmHg and an afterload of 50 mmHg as previously described [33,34]. Simultaneously, glucose oxidation and fatty acid oxidation were analyzed by measuring [^{14}C]-glucose incorporation into $^{14}CO_2$ and the ^3H_2O from [9, 10-^{3}H]-oleate released in the perfusate. The results demonstrated that ischemia reperfusion stress induce significant reduction of glucose oxidation, oxygen consumption and systolic function in terms of aortic flow and left ventricle developed pressure in isolated young-WT and $Sesn2^{f/f}$ heart (Fig. 7H, Table 2). While Sesn2 defects in heart from aged-WT and $cSesn2^{-/-}$ lead to exacerbated impairment of glucose oxidation, oxygen consumption and systolic function in terms of aortic flow and left ventricle developed pressure during reperfusion after 20 min ischemia stress (Fig. 7H, Table 2). On the contrary, there was no significant difference observed in fatty acid oxidation rate between ischemia and reperfusion in all four groups (Fig. 7I). Above results indicated that Sesn2 is critical to maintain the glucose oxidation rate in response to I/R stress. Together, after I/R stress, Sesn2 is required to maintain glucose oxidation and mediate TCA cycle as energy source in order to preserve the cardiac functional property as adaptive response to I/R stress.

4. Discussion

Ischemia reperfusion injury is known to accompany with the progress and treatment of coronary artery disease which induce greater damage in the aged heart with no effective strategy [8,35]. Sesn2 is a stress inducible protein which was upregulated in response to various stimuli, such as hypoxia and oxidative stress [12]. Several clinical reports announced that the expression of Sesn2 in myocardium and in plasma was significantly up-regulated in chronic heart failure (CHF) patients and coronary artery disease (CAD) patients [36,37]. Furthermore, the high concentration of Sesn2 in plasma were positively correlated with the high risk of incidence of major adverse cardiac events. Sesn2 is predicted not only as a novel biomarker but also potential therapeutic target for the treatment of cardiovascular disease [12]. Our lab reported that Sesn2 is age-related protein with declined accumulation in heart with aging [15]. Sesn2 administration in aged heart via AAV overexpression increased the tolerance of aged heart to I/R injury [15]. However, the comprehensive mechanism of how Sesn2 protecting heart from I/R stress remains unclear. To determine the cardioprotective roles and mechanisms of Sesn2 in adapting to cardiac I/R stress, comparative transcriptomics, proteomics, metabolomics as well as biochemical means were applied to achieve the goal in this study.

This study first elucidates that Sesn2 is critical to maintain metabolomics homeostasis and mitochondrial integrity in transcriptional level after I/R stress demonstrated by the 28 significantly altered metabolism associated signaling pathways and 195 significantly expressed mitochondrial components genes in Sesn2 depletion heart versus wild type heart. Of interest, mitochondria is the primary organelle responsible for performing substrate metabolism and energy production and modulating the synthesis of phospholipids and heme, calcium homeostasis, apoptotic activation and cell death [28,29,31]. Meanwhile, mitochondria is also postulated as the factor contributing to the increased injury in the aged heart with defected OXPHOS complexes activity and excessive oxidative stress [8]. In this study, we demonstrated that critical link between Sesn2 and mitochondrial integrity included the modulation of expression of PGC1α signaling and the maintenance of OXPHOS complexes transcription and accumulation of Complex I, II, III, and V under I/R stress.

Subsequently, the defected OXPHOS complex I and II activity in

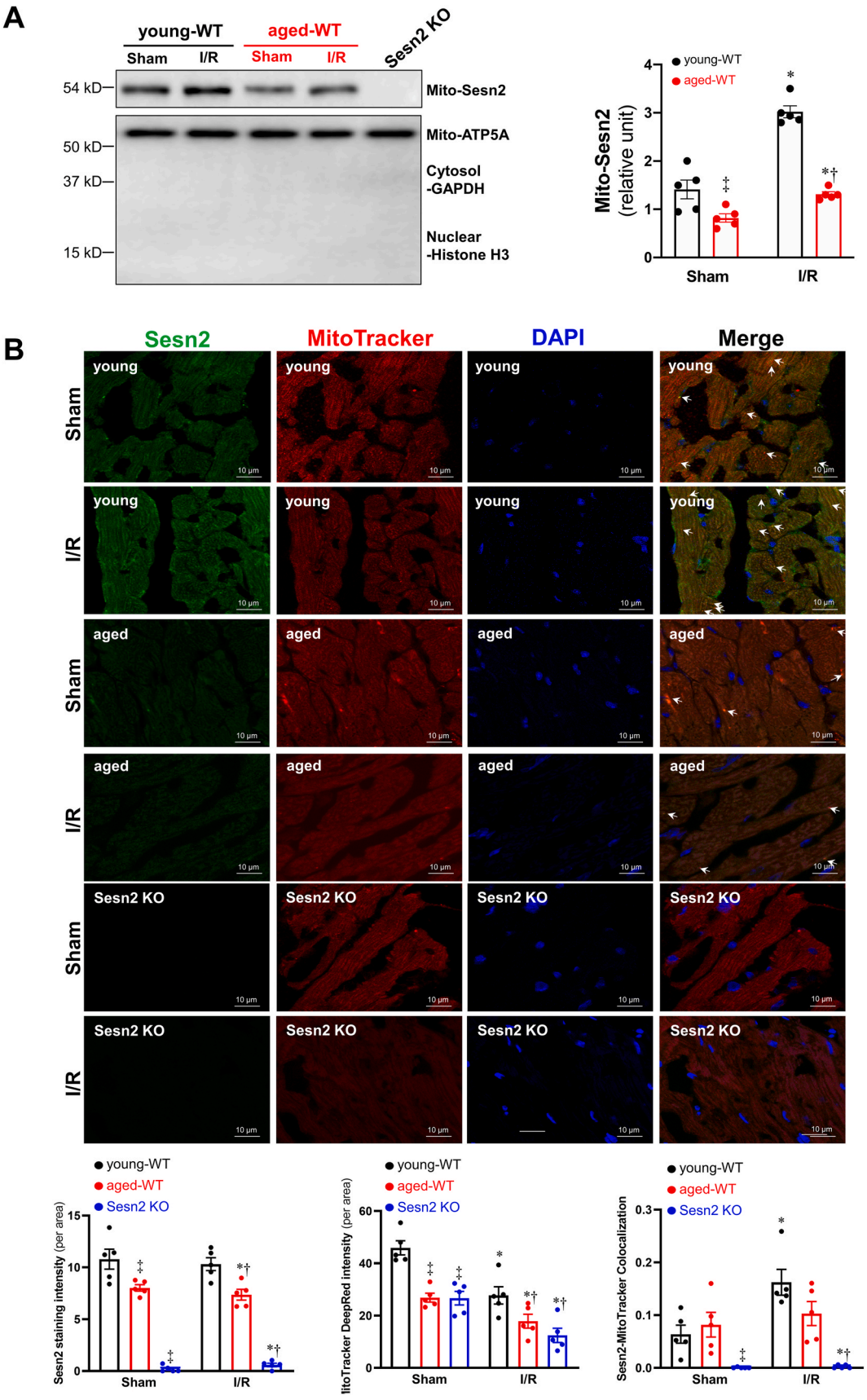
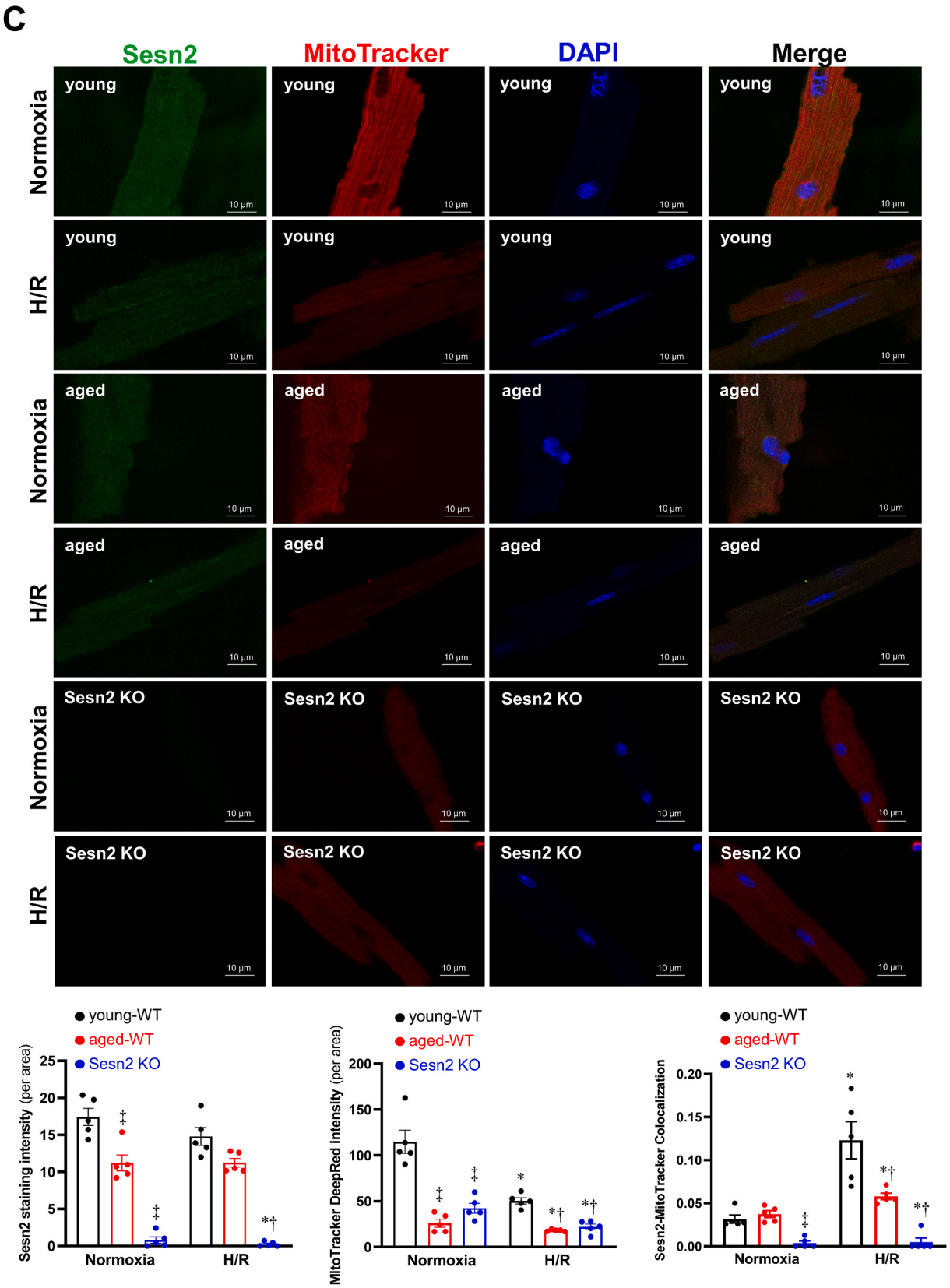


Fig. 5. Sesn2 accumulation in mitochondria in response to I/R stress. (A) Western blot analysis of mitochondrial Sesn2 in young-WT, aged-WT and Sesn2 KO heart under physiological and I/R stressed conditions (n = 5, values are means ± SEM, *p < 0.05 *vs.* Sham, respectively; †p < 0.05 *vs.* young-WT I/R; ‡p < 0.05 *vs.* young-WT Sham, One-way ANOVA). (B) Upper: Representative immunofluorescence staining images of Sesn2 and mitochondria in young-WT, aged-WT and Sesn2 KO heart under physiological and I/R stressed conditions. White arrows were utilized to highlight the yellow colored overlay of Sesn2 (green) and Mitochondria (red) in myocardium. Lower: Statistical analysis of Sesn2, MitoTracker DeepRed staining and Sesn2 and MitoTracker colocalization ratio in young-WT, aged-WT and Sesn2

KO heart under physiological and I/R stressed conditions (n = 5, values are means ± SEM, *p < 0.05 *vs.* Sham, respectively; †p < 0.05 *vs.* young-WT I/R; ‡p < 0.05 *vs.* young-WT Sham, One-way ANOVA). (C) Upper: Representative immunofluorescence staining images of Sesn2 and mitochondria in young-WT, aged-WT and Sesn2 KO isolated cardiomyocytes under normoxia and hypoxia 20 min/reoxygenation 20 min (H/R) stressed conditions. Lower: Statistical analysis of Sesn2, MitoTracker DeepRed staining and Sesn2-MitoTracker colocalization ratio in young-WT, aged-WT and Sesn2 KO heart under normoxia and hypoxia/reoxygenation (20′/20′) stressed conditions (n = 5, values are means ± SEM, *p < 0.05 *vs.* Sham, respectively; †p < 0.05 *vs.* young-WT I/R; ‡p < 0.05 *vs.* young-WT Sham, One-way ANOVA). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



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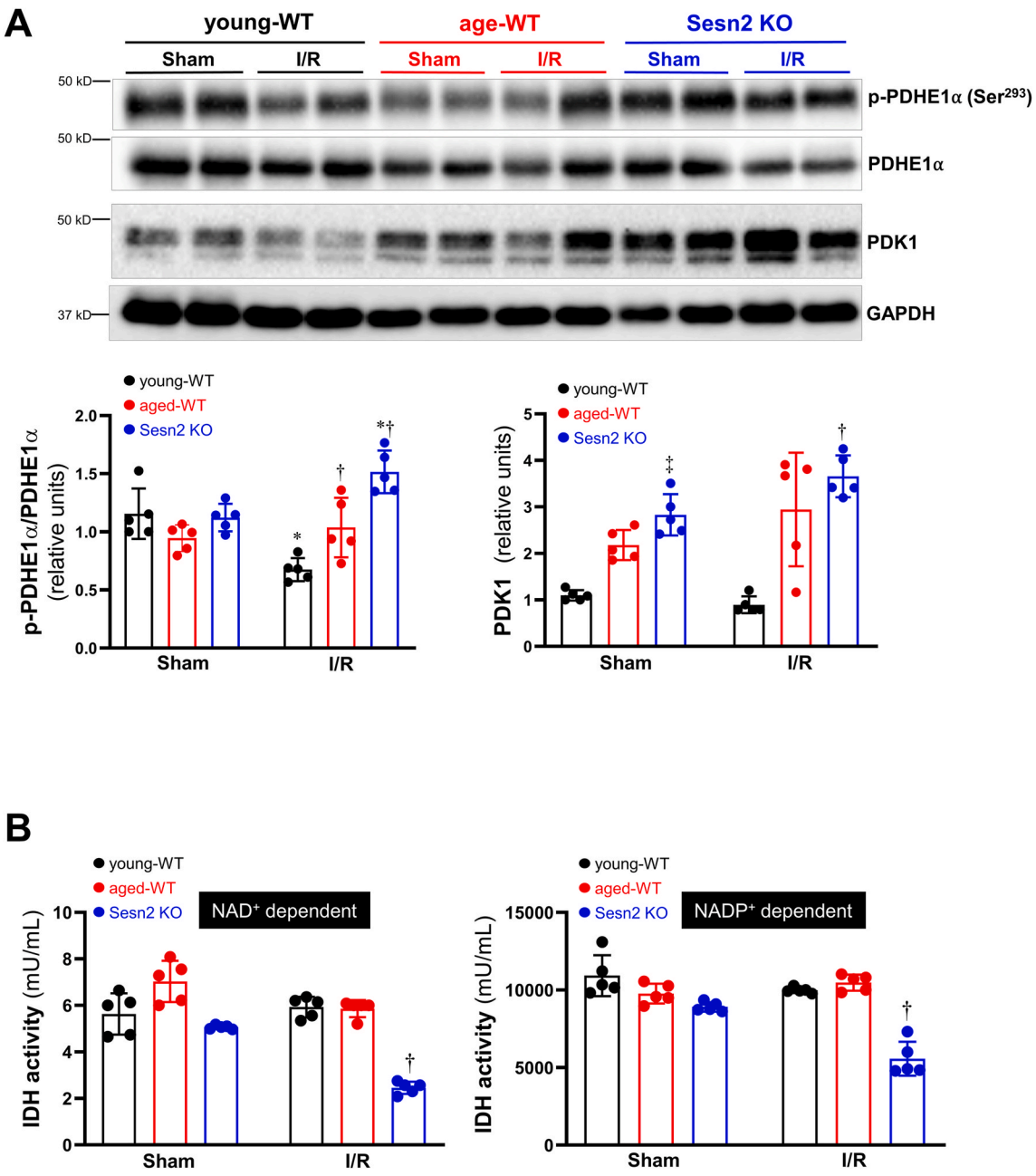


Fig. 6. Sesn2 play a role in modulating PDH and IDH activity in responding to I/R stress. (A) Western blot analysis of total and phosphorylated PDHE1α subunit of PDH complex and the kinase PDK1 accumulation levels in young-WT, aged-WT and Sesn2 KO heart under physiological and I/R stressed conditions (n = 5, values are means ± SEM, *p < 0.05 *vs.* Sham, respectively; †p < 0.05 *vs.* young-WT I/R; ‡p < 0.05 *vs.* young-WT Sham, One-way ANOVA). (B) IDH (NAD^+ dependent and $NADP^+$ dependent) activity in young-WT, aged-WT and Sesn2 KO heart under physiological and I/R stressed conditions (n = 5, values are means ± SEM, †p < 0.05 *vs.* young-WT I/R, One-way ANOVA).

aged-WT and Sesn2 KO hearts compared to young-WT group suggested that Sesn2 is critical for myocardium to maintain the mitochondrial functional integrity. Cardiomyocyte contractile function analysis demonstrated that Sesn2 is required to maintain the proper cardiomyocyte contractility under normoxia and H/R stressed condition. Next, glucose/fatty acid oxidation analysis and systolic function analysis with perfused working heart system illustrated the exacerbated impairment of glucose oxidation, oxygen consumption, and cardiac systolic function in Sesn2 defected heart after I/R stress. Combined evidence indicated Sesn2 is also required to regulate OXPHOS complexes activity and glucose oxidation rate to preserve the high-energy demanding cardiomyocyte contractile property, especially under I/R stress. However, the cardiac specific Sesn2 deletion induce elevated calcium flux during cardiomyocyte contraction, which could be a compensation mechanism or impaired calcium channel exchange system.

To investigate the mechanism of how Sesn2 modulate OXPHOS complexes activity, proteomics analysis of Sesn2 associated protein in myocardium revealed that Sesn2 is directly interacting with OXPHOS complexes on mitochondrial inner membrane and TCA cycle enzymes in the mitochondrial matrix. However, the dynamic association of young and aged heart in responding to I/R stress is distinguished. Ischemia reperfusion injury induce the upregulation of Sesn2-mitochondiral related proteins in young heart, which is blunted in aged heart leading to the defected PDH activity but normal LDH activity. Eventually, in response to I/R stress, young heart showed elevated TCA cycle and

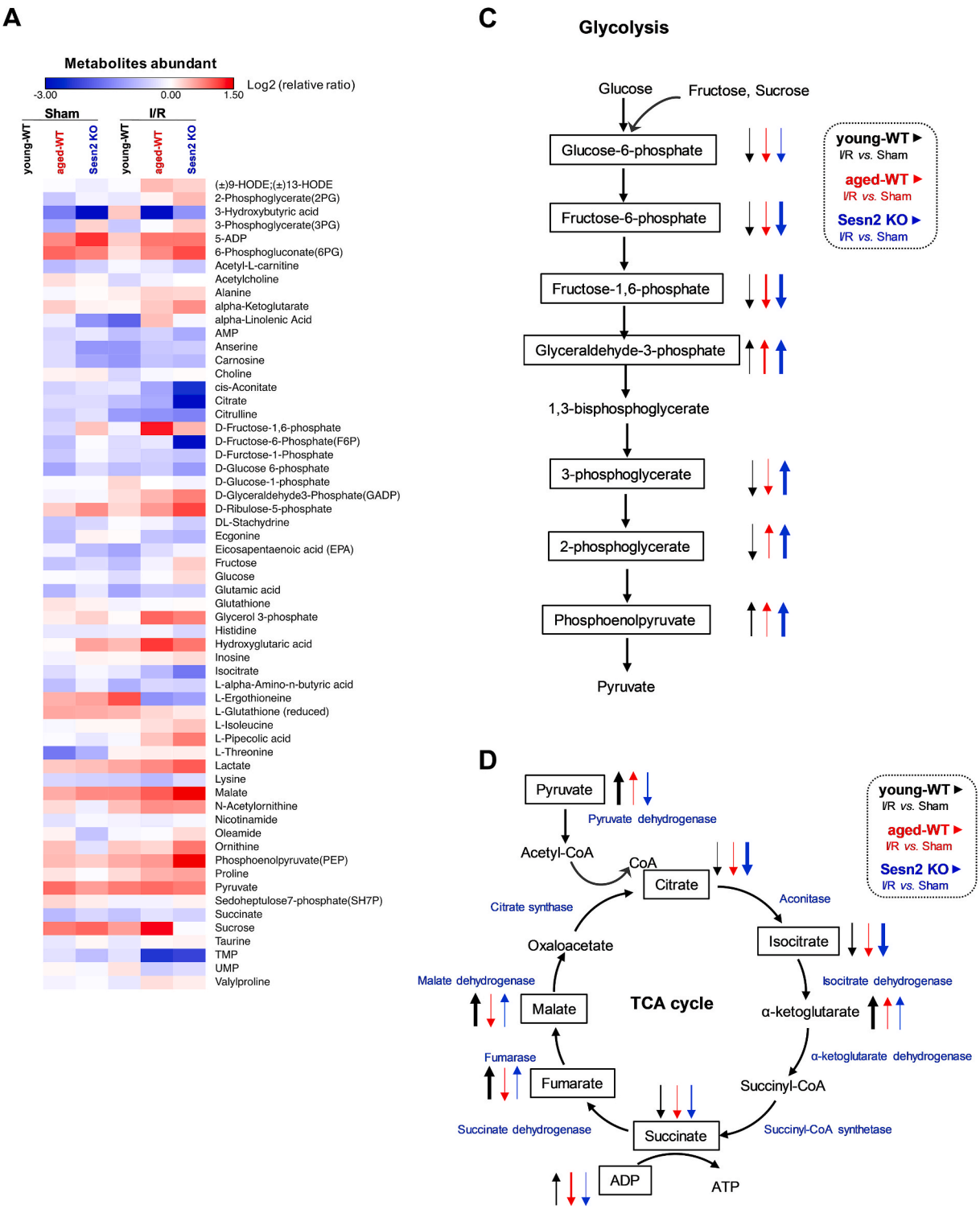
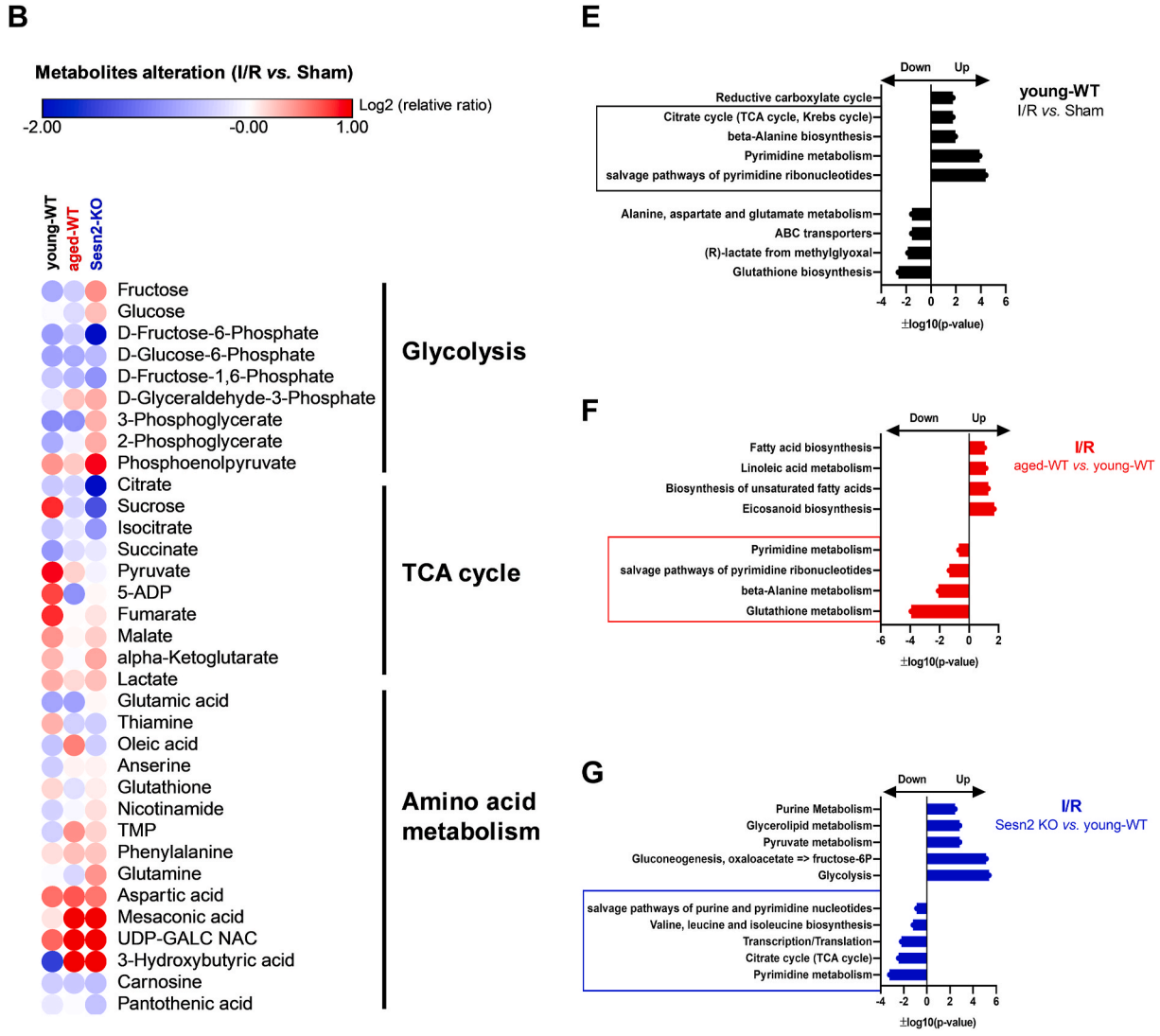


Fig. 7. Sesn2 is critical to elevate cardiac remodeling related metabolism and TCA cycle in responding to I/R stress. (A) Heatmap shows relative abundance of representative significantly altered metabolites in responding to I/R stress in Young-WT, Aged-WT and Sesn2-KO heart *vs.* young-WT-sham under physiological and I/R stressed conditions (n = 3), Grubbs' test. Color represent the increase (red) and decrease (blue) of each gene, alteration ratios are presented in log2 form according to the legend panel. (B) Heatmap of relative abundance of modulated metabolites which were clustered to significantly altered TCA, glycolysis, and amino acid metabolism pathway in young-WT-I/R *vs.* young-WT-sham heart (n = 3), Grubbs' test. Color represent the increase (red) and decrease (blue) of each gene, alteration ratios are presented in log2 form according to the legend panel. (C) Glycolysis metabolism pathway representing changes in abundance of metabolites in responding to I/R stress in young-WT, aged-WT, Sesn2-KO heart (n = 3). Grubbs' test. Color represents the alteration between young-WT-I/R *vs.* young-WT-sham (black), aged-WT-I/R *vs.* aged-WT-sham (red), and Sesn2-KO-I/R *vs.* Sesn2-KO-sham (blue). Arrows direction indicates up-regulation (up) or down-regulation (down) and lines thickness is positively correlated with the alteration ratios. (D) TCA cycle metabolism pathway representing changes in abundance of metabolites in responding to I/R stress in young-WT, aged-WT, Sesn2-KO heart (n = 3). Grubbs' test. Color represents the alteration between young-WT-I/R *vs.* young-WT-sham (black), aged-WT-I/R *vs.* aged-WT-sham (red), and Sesn2-KO-I/R *vs.* Sesn2-KO-sham (blue). Arrows direction indicates up-regulation (up) or down-regulation (down) and lines thickness is positively correlated with the alteration ratios. (E) Metabolism pathway analysis representing the significantly up-regulated and down-

regulated substrate pathways in young-WT-I/R *vs.* young-WT-Sham heart (n = 3), MBROLE 2.0 enrichment analysis. Significantly up-regulated pathways were presented on the right side with positive log10 form of p-value. Significantly down-regulated pathways were presented on the left side with negative log10 form of p-value. (F) Metabolism pathway analysis representing the significantly up-regulated and down-regulated substrate pathways in aged-WT-I/R *vs.* young-WT-I/R heart (n = 3), MBROLE 2.0 enrichment analysis. Significantly up-regulated pathways were presented on the right side with positive log10 form of p-value. Significantly down-regulated pathways were presented on the left side with negative log10 form of p-value. (G) Metabolism pathway analysis representing the significantly up-regulated and down-regulated substrate pathways in Sesn2-KO-I/R *vs.* young-WT-I/R heart (n = 3), MBROLE 2.0 enrichment analysis. Significantly up-regulated pathways were presented on the right side with positive log10 form of p-value. Significantly down-regulated pathways were presented on the left side with negative log10 form of p-value. (H) Glucose oxidation was analyzed by measuring [^{14}C]-glucose incorporation into $^{14}CO_2$ in the *ex vivo* young/aged WT and Sesn2$^{f/f}$/cSesn2$^{-/-}$ mouse hearts subjected to 20 min ischemia and 30 min reperfusion. n = 5, *p < 0.05 *vs.* young WT or Sesn2$^{f/f}$, respectively; †p < 0.05 *vs.* young-WT or Sesn2$^{f/f}$ reperfusion, respectively. (I) The oleate oxidation was analyzed by measuring incorporation of [9,10-^{3}H] oleate into $^{3}H_2O$ in the *ex vivo* young/aged WT and Sesn2$^{f/f}$/cSesn2$^{-/-}$ mouse hearts subjected to 20 min ischemia and 30 min reperfusion. n = 5. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



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cardiac remodeling pathways as adaptive response which is attenuated in aged heart and further exacerbated in Sesn2 KO heart with metabolomics analysis. We conclusively confirmed that Sesn2 play comprehensive roles in modulating mitochondrial OXPHOS and substrate metabolic function which is critical to adapt to I/R stress.

Intriguingly, we observed that ischemia reperfusion stress triggered the elevated accumulation of Sesn2 in mitochondria in young heart but not the case in aged heart. The impaired mitochondrial Sesn2 in aged heart could be the attribution of Sesn2 associating differently with targets in aged heart versus young heart. Immunoprecipitation and immunofluorescence analysis confirmed the association of Sesn2 with OXPHOS complex I, II, and III as well as the localization of Sesn2 in mitochondria of both young and aged heart. Furthermore, proteomics analysis showed that Sesn2 is associated with glucose regulated protein 75 (GRP75), which is a mitochondrial chaperone Hsp 70 family protein playing critical roles in assisting the mitochondrial precursor proteins transporting into mitochondrial compartments (Table 1) [38,39]. Moreover, Sesn2 is reported to localize in mitochondria without the targeting signal sequence in Human Mitochondrial Protein Database with Mito ID MT001579 as well as it was evidenced that Sesn2 is localized in mitochondrial to regulate mitochondria functions *in vitro* [40,41]. Meanwhile we provided a probable mechanism that chaperone GRP75 could facilitate the translocation of Sesn2 into myocardium mitochondria in response to ischemia reperfusion stress to modulate the

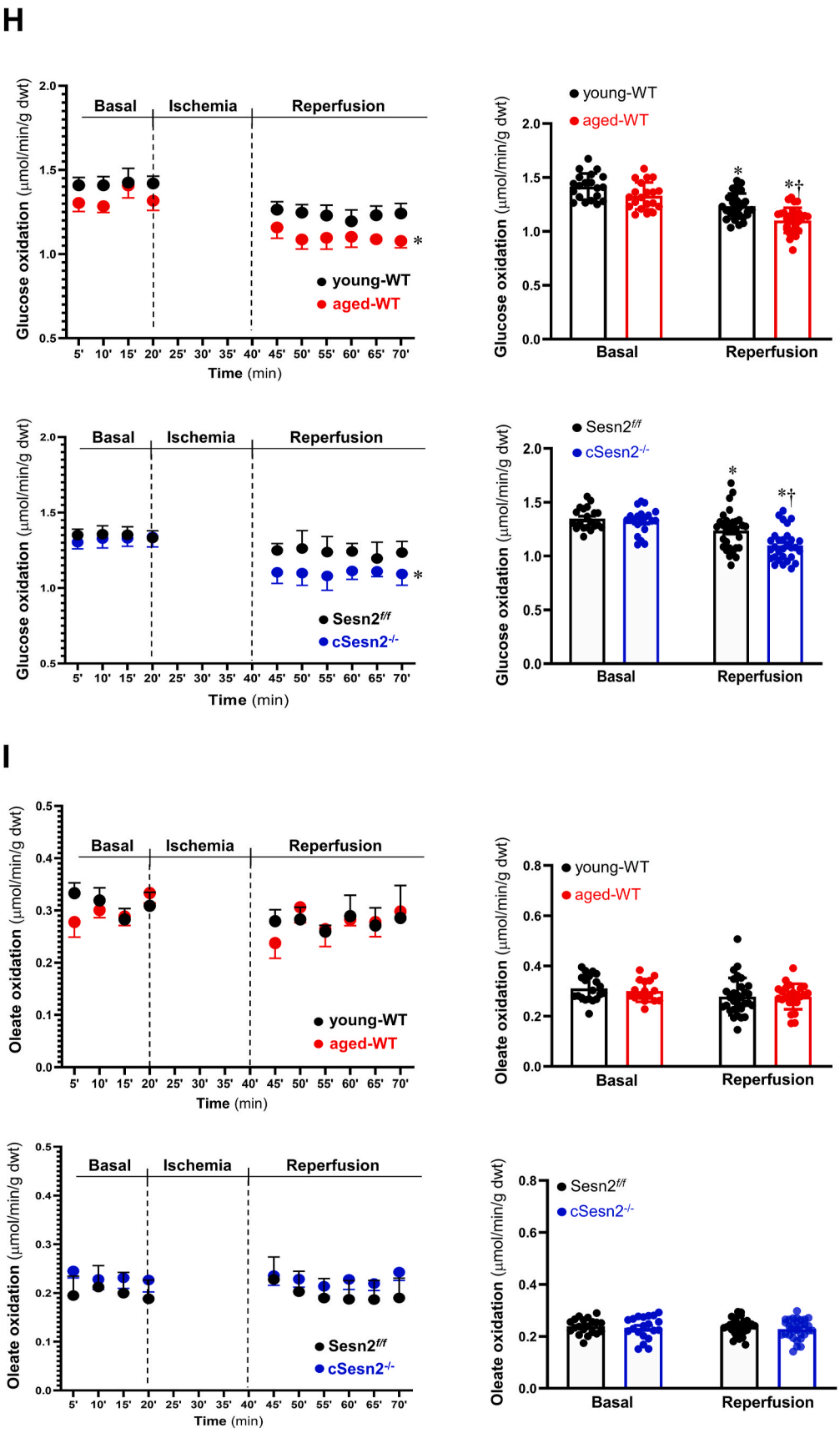

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Table 2
Hemodynamic parameters of working heart perfusion system.

Parameter	young-WT		Aged-WT		$Sesn2^{f/f}$		$cSesn2^{-/-}$	
	Basal	I/R	Basal	I/R	Basal	I/R	Basal	I/R
HR (min^{-1})	293 ± 30.5	196 ± 14.5*	256 ± 34.9	183 ± 18.4*	281 ± 23.6	175 ± 19.8*	280 ± 33.2	153 ± 28.3*#
CF (ml min^{-1})	2.6 ± 0.2	2.4 ± 0.2	2.6 ± 0.1	2.5 ± 0.2	2.4 ± 0.2	2.5 ± 0.2	2.9 ± 0.2	2.7 ± 0.1
AF (ml min^{-1})	1.05 ± 0.06	0.98 ± 0.06*	0.98 ± 0.04	0.70 ± 0.11*#	1.10 ± 0.04	0.87 ± 0.03*	1.10 ± 0.03	0.51 ± 0.02*#
CO (ml min^{-1})	3.64 ± 0.17	3.38 ± 0.18*	3.58 ± 0.11	3.20 ± 0.16*#	3.50 ± 0.20	3.37 ± 0.22*	4.00 ± 0.23	3.21 ± 0.18*#
LVDP (mmHg)	20.7 ± 3.2	17.3 ± 2.8*	20.0 ± 2.2	12.5 ± 2.3*#	22.7 ± 2.7	15.9 ± 2.9*	20.6 ± 3.2	13.7 ± 2.0*#
MVO_2 (μl/g dw/min)	40.7 ± 5.4	30.8 ± 5.5*	38.7 ± 3.4	25.8 ± 5.5*	43.3 ± 5.8	29.2 ± 4.6*	38.5 ± 3.7	23.4 ± 3.2*#

Note: HR: heart rate; CF: coronary flow; AF: aortic flow; CO: cardiac output; LVDP: left ventricle developed pressure; MVO_2: myocardial oxygen consumption. *$p < 0.05$ *vs.* basal group within the same colony group. #$p < 0.05$ *vs.* young-WT I/R group and $Sesn2^{f/f}$ I/R group, respectively.

mitochondrial function.

Sesn2 is well known as a stress inducible protein and an ROS repressing factor [42]. Moreover, our lab reported that Sesn2 is a signaling regulator which is critical for AMPK activation by increasing its association with LKB1 as a scaffold protein in cytoplasm [13]. Furthermore, our lab also reported that Sesn2 modulate mitochondrial biosynthesis through PGC1-α signaling and affect myocardium apoptosis in the post-myocardium infarction model [16]. The comprehensive roles and mechanisms of Sesn2 require further investigation. This study is the first time to demonstrate that I/R stress could trigger Sesn2 to accumulate in mitochondria and Sesn2 is required to maintain mitochondrial functional integrity through directly interacting with OXPHOS complexes I, II, and III, and multiple TCA cycle related enzymes, such as PDH and IDH. It was demonstrated by our lab that PDHE1α deficiency leading to the increased association of Sesn2 with PDH E2/E3 subunit which disturbs the interaction of Sesn2 with LKB1 [43]. Here, we found that Sesn2 associated with PDH E3 subunit and Sesn2 deficiency induced PDH kinase and the PDHE1α inhibited form accumulation. It implicates that Sesn2 interacted with PDH complex in a dynamic feedback manner and their association modulates LKB1, AMPK, and TCA cycle in ischemia heart. Besides, IDH activity of converting NAD^+/$NADP^+$ to NADH/-NADPH was significantly compromised in Sesn2 knockout heart under both physiological and I/R stressed condition. Combined with the finding that IDH associated with Sesn2 in heart, Sesn2 is postulated to play critical roles in modulating NAD signaling in ischemic heart disease. NAD is well known as a coenzyme in redox reaction and modulate sirtuins activity which is depending on the cellular NAD^+/NADH ratio resulting in plays critical roles in modulation the posttranslational modification as the deacetylation enzyme [44]. It was reported impaired longevity Sirt1 in aged heart increased sensitivity to ischemia insults [45]. This study expanded the mechanistic and insightful view of Sesn2 in protecting aged heart against cardiac ischemia reperfusion injury. The limitation of this study is lack of demonstrating the mechanism of how Sesn2 was translocated into mitochondria under I/R stress as an adaptive response.

5. Conclusions

Age-related Sesn2 serves as a scaffold protein interacting with OXPHOS components to maintain mitochondrial integrity in the heart under I/R stress. The downregulation of Sesn2 in aged heart fragilizes mitochondrial functional integrity in response to ischemic stress. Thus, Sesn2 is crucial for protecting heart from I/R injury through modulating the substrate metabolism and maintaining mitochondrial functional integrity under pathological stress conditions. The implication of this study could encourage the researchers to pay attention in Sesn2 for developing the new therapeutic strategies of the cardiac diseases, especially for the aging population.

Author contributions

D.R. and J.L. designed and conducted the study; D.R., Z.H., J.F., J.Z., E.W. and X.Z. performed data collection and analysis; D.R., Z.H., J.F., J. Z., D.E.K. and J.L. interpreted data; and D.R. and J.L. wrote the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101824.

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