Am J Transl Res 2022;14(8):5800-5811
www.ajtr.org /ISSN:1943-8141/AJTR0143323

*Original Article*

# ER stress induces myocardial dysfunction and cardiac autophagy in Sestrin2 knockout mice

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Received April 4, 2022; Accepted July 19, 2022; Epub August 15, 2022; Published August 30, 2022

**Abstract:** Objectives: Sestrin2 is an essential regulator of the cellular adaptive response against various stresses. The endoplasmic reticulum (ER) is critical in maintaining normal cardiac function by controlling intracellular $Ca^{2+}$ accumulation, as well as protein folding and processing. Autophagy contributes to stress-associated heart dysfunction. AMP-activated protein kinase (AMPK) is important in energy homeostasis in cardiomyocytes. However, the function of Sestrin2 (Sesn2) in ER stress-induced autophagy that induces myocardial dysfunction has not been clarified. In this study, mice and cardiac tissues were treated with tunicamycin (TN), an inducer of ER stress. We then explored the roles of Sesn2 and the AMPK pathway associated with autophagy in ER stress-induced myocardial dysfunction in mice. Methods: Echocardiography, contractile function analysis, intracellular $Ca^{2+}$ status, and immunoblot analysis of AMPK pathway were performed, ER stress and autophagy markers were examined. Results: The study revealed that ER stress caused significant heart dysfunction and cardiotoxicity in the mouse heart and cardiomyocytes. Biochemical analysis indicated enhanced cardiac autophagy mediated by ER stress and AMPK/mTOR activation. Sesn2 knockout exacerbated ER stress-related myocardial dysfunction due to the failed response of cardiac autophagy and AMPK/mTOR pathway activation. Further, pharmacological inhibition of AMPK or autophagy worsened TN-induced cardiac dysfunction. Conclusion: Taken together, loss of the Sesn2 protein exacerbates ER stress-induced cardiac dysfunction through the AMPK/mTOR signaling cascade and loss of autophagy response.

**Keywords:** Sestrin2, ER stress, autophagy, cardiac dysfunction

## Introduction

Sestrins belong to stress-inducible proteins with high conservation across species. Sestrin-2 (Sesn2) is involved in several cellular events such as cell survival, anti-inflammatory processes, energy metabolism and homeostasis [1-3]. Sesn2 protects dysfunctional cells against sepsis-induced necrosis [4]. AMP-activated protein kinase (AMPK), controlling energy metabolism, represents a major regulator in cardiovascular disorders and cell stress [5]. Sesn2 decreases cell ROS levels via AMPK-dependent autophagy and exerts cardioprotective effects in cardiovascular disorders [6]. The mammalian target of rapamycin complex 1 (mTORC1) plays important roles in intracellular signaling pathways controlling growth, metabolism and aging [7]. Evidence supports roles for Sesn2 and AMPK in autophagy induction in response to exercise [8]. Sesn2 silencing exacerbates atherosclerosis by enhancing inflammation and ER stress in endothelial cells via AMPK signaling.

Autophagy is mostly involved in maintaining a balance between production of cell components and degradation of flawed or useless organelles and other cell components. It is important in maintaining cardiac homeostasis and is upregulated during stress [9]. Constitutive autophagy is critical in the maintenance of heart structure and function. Many studies have reported that enhancing autophagy in the

myocardium is cardioprotective, whereas superabundant autophagy could increase injury and induce heart failure [10, 11]. Many pathways control autophagy, e.g., mTOR signaling. mTOR activity is regulated by AMPK that senses the levels of cell nutrients and energy. Downregulation of mTOR stimulates protein biosynthesis and is associated with autophagy [12].

The endoplasmic reticulum (ER) is critical in protein synthesis, calcium homeostasis, and lipid biosynthesis. ER is highly sensitive to changes in cell homeostasis. Several extracellular stimuli, including ischemia, hypoxia and enhanced protein biosynthesis can induce ER stress. In turn, ER stress triggers a cytoprotective response termed unfolded protein response (UPR). With prolonged ER stress, cells decrease protein production for preventing the accumulation of UPR proteins as detected in heart and liver disorders [13, 14]. Sesn2 can be upregulated by ER stress activators, including tunicamycin and thapsigargin [15]. Globular adiponectin markedly enhances ER stress in primary peritoneal macrophages. Sesn2 induction contributes to ER stress-induced autophagy activation, regulation of inflammation and cell survival.

Given the physiological relevance of the association between Sesn2 and AMPK, we hypothesized that both proteins have important roles in the process of ER stress, which is related to myocardial function. The precise molecular mechanism by which Sesn2 and AMPK regulate autophagy still needs to be elucidated, especially in the context of ER stress. In the present study, we harvested cardiac tissues from mice treated with tunicamycin (TN), an inducer of ER stress. Echocardiography, pathological analysis, contractile function analysis, intracellular $Ca^{2+}$ assessment, and immunoblot analysis of ER stress were performed and autophagy biomarkers were examined.

## Material and methods

### *Animals and tunicamycin treatment*

The animal study had approval from the Animal Care and Use Committee of the Shandong Provincial Hospital affiliated to Shandong First Medical University (Shandong, China) (NSFC: NO.2021-226). Sex-matched C57BL/6J wild type (WT) and Sestrin2 knockout (Sesn2 KO) mice were provided by Dr. Ji Li, Department of Surgery, University of South Florida. Mice were maintained under standard conditions with food and water available at will. Both C57BL/6J WT and Sesn2 KO animals underwent intraperitoneal administration of 1 mg/kg of the ER stress inducer tunicamycin (TN, Sigma, USA) once a day for two days. Tunicamycin dosage was based on previously reported findings of evident myocardial dysfunction with no substantial mortality [16]. The treated animals were monitored until use in the study. Two days after tunicamycin administration, euthanasia was carried out by cervical dislocation.

### *Echocardiography*

Transthoracic echocardiography was carried out with resting mice, which were anesthetized with 1.5-2.0% isoflurane, on a Vevo 3100 imaging system (Visual Sonics). M-mode echocardiography was performed along the short axis of the left ventricle at the chordae tendineae level. Heart rate, left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD) and left ventricular diastolic interventricular septum thickness were detected, as well as left ventricular ejection fraction (EF). Three-to-five successive cardiac cycles were analyzed [17].

### *Purification of mouse cardiomyocytes*

Each mouse was administered heparin (100 U, i.p.) for anticoagulation before anesthesia with sodium pentobarbital (100 mg/kg). The heart was extracted, fixed to a cardiomyocyte perfusion apparatus (Radnoti, USA), and perfused at 37°C with $Ca^{2+}$-free Krebs-Henseleit based buffer (pH 7.3) containing $KH_2PO_4$ (0.6 mM), $Na_2HPO_4$ (0.6 mM), HEPES (10 mM), KCl (14.7 mM), $MgSO_4$ (1.7 mM), NaCl (120.3 mM), $NaHCO_3$ (4.6 mM), taurine (30 mM), glucose (10 mM) and 2,3-butanedione monoxime (10 mM), bubbled with a gas mixture comprising 95% $O_2$ and 5% $CO_2$. Upon equilibration, heart digestion was performed with the above buffer supplemented with 0.067 mg/mL Liberase Blendzyme 4 (Roche, USA). Then, the cardiac tissue underwent mincing, before addition of extracellular $Ca^{2+}$ at 1 mM. Cardiomyocytes were next administered vehicle (DMSO), tunicamycin (3 μg/mL), Compound C (10 μM; Enzo Life Sciences, USA) and 3-methyladenine (3-MA, 3 mM; Selleckchem, USA) for 4 h.

*Cell shortening and relengthening*

The mechanical features of cardiomyocytes were evaluated on a SoftEdge MyoCam system (IonOptix, USA). Cardiomyocytes in a chamber were applied a 0.5 Hz current. IonOptix SoftEdge was utilized for capturing the alterations of sarcomere length during shortening and relengthening. Cell shortening and relengthening parameters were evaluated, i.e., peak shortening (PS), indicating peak contractility; time-to-PS (TPS), indicating contraction duration; time-to-90% relengthening ($TR_{90}$), indicating relaxation duration; and maximal velocities of shortening/relengthening (±dL/dt), indicating maximal pressure development and decline [18].

*Intracellular $Ca^{2+}$ assessment*

Intracellular $Ca^{2+}$ assessment used a dual-excitation, single emission photomultiplier system (IonOptix, USA). Cardiomyocytes loaded with fura 2-AM (2 μM) underwent exposure to light from a 75-W halogen lamp via a 340- or 380-nm filter during stimulation to contract at 0.5 Hz. Fluorescence was then measured. Qualitative change of rise in intracellular $Ca^{2+}$ clearance was reflected by the fura-2 fluorescence intensity ratio of 360 nm to 380 nm. Fluorescence decay time (Single and Bi-Exponential) was calculated as an indicator of intracellular $Ca^{2+}$ clearance.

*Immunoblot*

Upon treatment, total protein extraction was performed from mouse heart samples. A bicinchoninic acid (BCA) protein assay kit (Pierce, USA) was utilized for protein quantitation as directed by the manufacturer. Equal amounts of total protein underwent separation by SDS-PAGE and electro-transfer onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Blocking was carried out with 5% skimmed milk in TBST for 1 h at 37°C. Protein levels of Sestrin2; phosphorylated AMPK, ACC, mTOR, p-S6 and ULK; ER stress biomarkers Gadd153, Grp78, phosphorylated eIF2α, and phosphorylated IRE1α; autophagy biomarkers Atg5, p62 and LC3 I/II were examined. Primary antibodies targeting the above proteins were added overnight to the membranes at 4°C, respectively. An enhanced chemiluminescence substrate kit (Pierce, USA) was utilized for detection, and quantitation used the Quantity One software (Bio-Rad Laboratories, USA) [19].

*Statistical analysis*

Data are mean ± SEM. GraphPad Prism 8.0 was utilized for data analysis. Analysis of variance (ANOVA) with Tukey's *post hoc* test or Student's t test was carried out for comparing groups. $P < 0.05$ indicated statistical significance.

## Results

*Morphological features of WT and Sesn2 KO mice administered tunicamycin*

TN was administered by the intraperitoneal route in mice for inducing myocardial dysfunction. Echocardiography showed heart weights and interventricular septum thicknesses were comparable in C57BL/6J WT and Sesn2 KO mice. Heart rate was decreased in TN treated WT and Sesn2 KO mice, with a more pronounced effect in TN treated Sesn2 KO mice. Moreover, TN treatment overtly increased myocardial volume in LVESD, with reduced EF (**Figure 1**) in Sesn2 KO animals. Sesn2 KO aggravated TN-dependent alterations of LVESD and EF, with the remaining geometric and functional factors showing minimal alterations. These findings suggested Sesn2 knockout impaired cardiac protection under TN-induced ER stress.

*Effect of TN-induced ER stress on contractile response and intracellular $Ca^{2+}$ features of mouse cardiomyocytes*

To assess the regulatory role of Sesn2 in myocardial function, the effects of TN-induced ER stress on contractile function were examined in cardiomyocytes obtained from mouse heart samples. As shown in **Figure 2A-F**, the average representative cell shortening values were determined with the IonOptix system. Neither TN treatment nor Sesn2 knockout impacted resting cell length (**Figure 2A**). However, TN remarkably decreased peak shortening (PS) and ±dL/dt in Sesn2 KO cardiomyocytes compared with WT cardiomyocytes (**Figure 2B-D**). TPS tended to be prolonged in WT and Sesn2 KO cardiomyocytes after TN treatment (**Figure 2E**). $TR_{90}$ was prolonged in WT cardiomyocytes

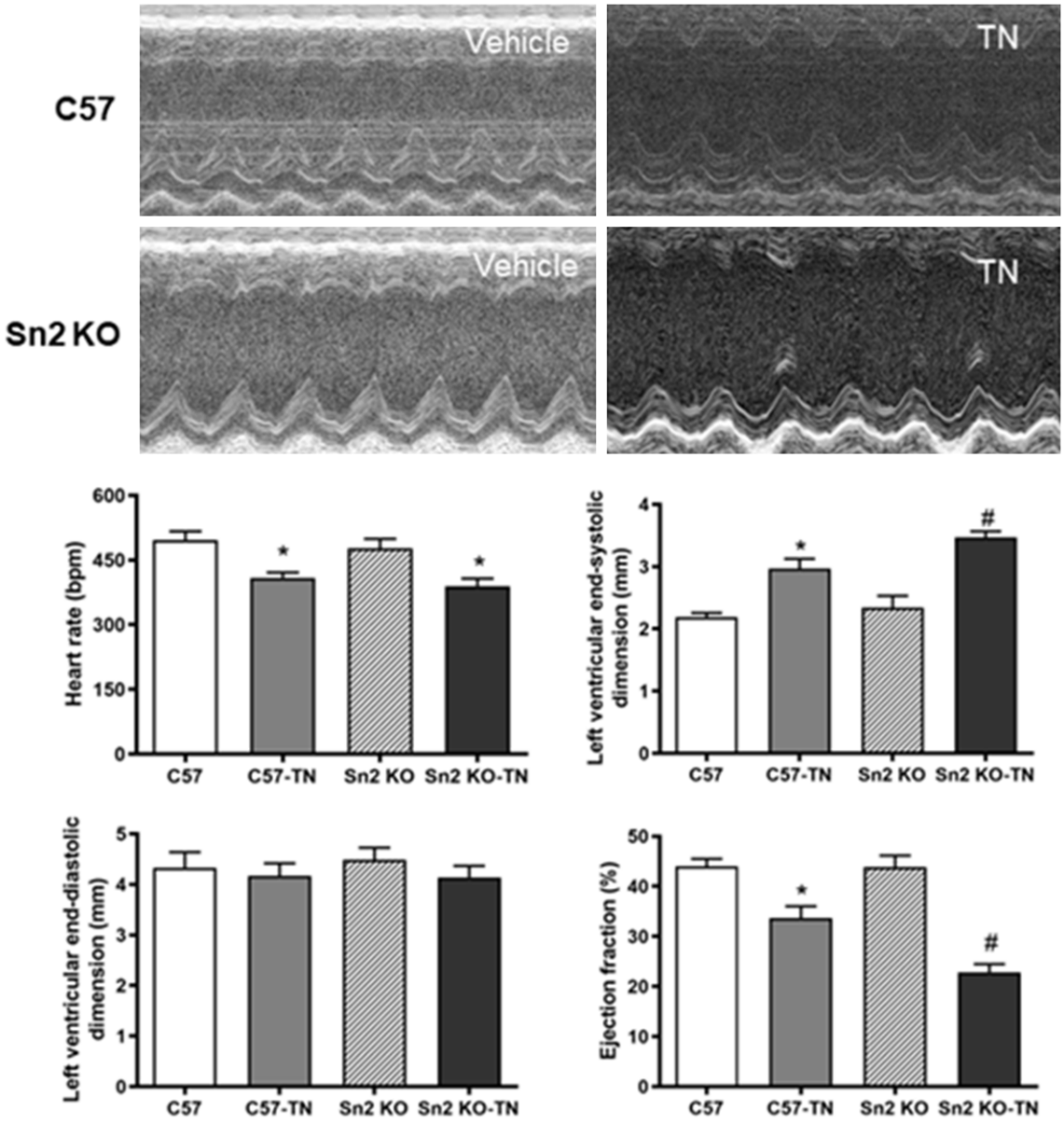


**Figure 1.** Representative echocardiograms in different groups, including the C57, C57-TN, Sn2 KO and Sn2 KO-TN groups. Mean ± SEM, n = 3-4 per group, *P < 0.05 vs. C57 group; #P < 0.05 vs. C57-TN group.

obviously but not in Sesn2 KO cardiomyocytes (**Figure 2F**). The above findings suggested a detrimental effect for ER stress-triggered contractile defect in cardiomyocytes.

To explore the mechanism underpinning ER stress-dependent mechanical abnormality, intracellular $Ca^{2+}$ amounts were assessed by the Fura-2 fluorescence method. The results demonstrated TN administration caused no alterations in resting intracellular $Ca^{2+}$ amounts (**Figure 3A**) although intracellular $Ca^{2+}$ elevation (**Figure 3B**) and intracellular $Ca^{2+}$ clearance delay were markedly decreased (**Figure 3C**, **3D**). Nevertheless, the above effects were exacerbated by Sesn2 knockout. These data indicated that calcium disorders induced by ER stress were involved in cardiomyocyte contractile dysfunction.

*Effects of Sesn2 knockout on TN-elicited activation of ER stress and autophagy*

We assessed Sestrin2 expression after tunicamycin treatment in both C57BL/6J WT and Sesn2 KO mice. The expression of Sestrin2

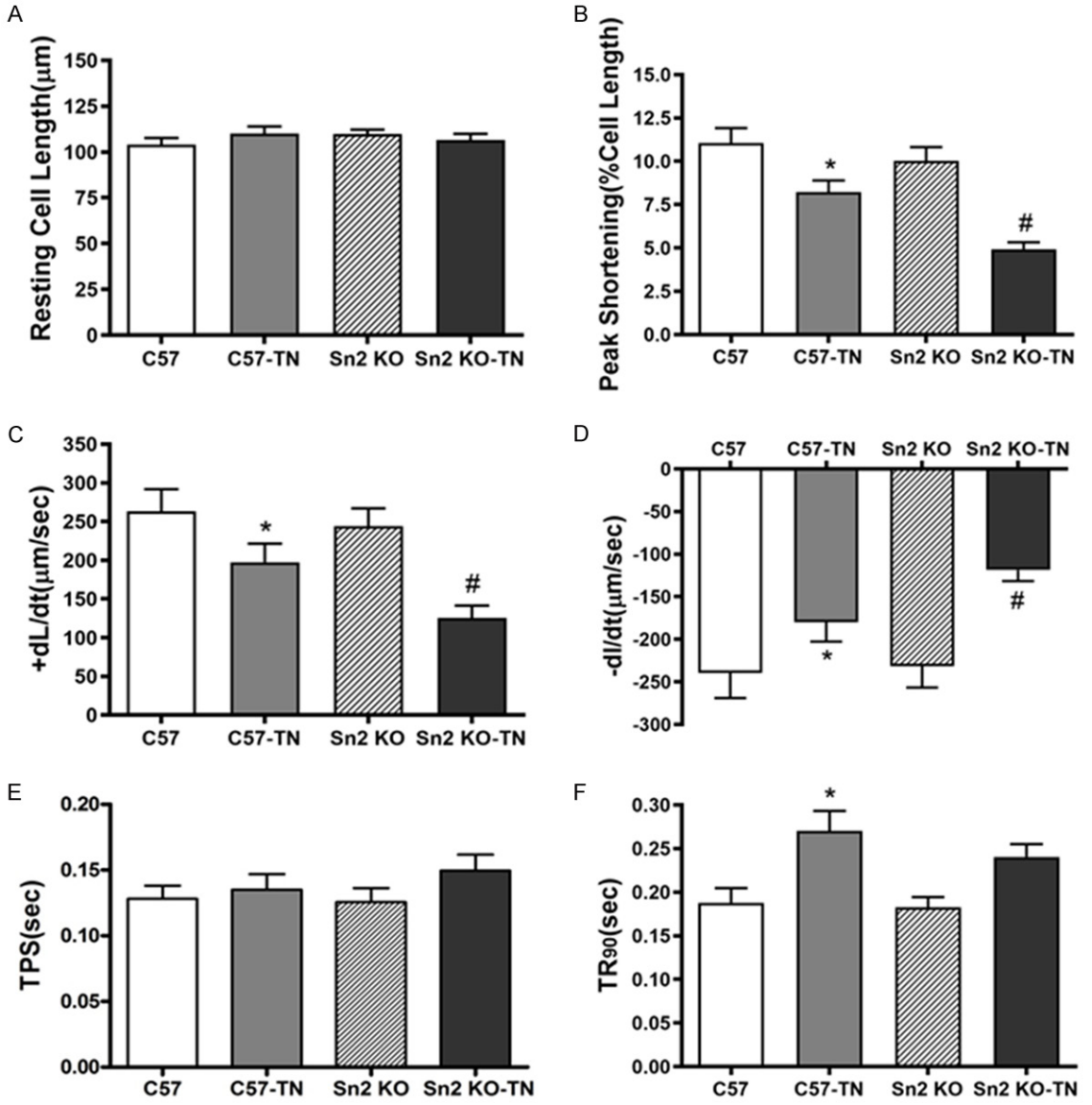


**Figure 2.** Cardiomyocyte contractile properties in C57 and Sestrin2 knockout mice treated with or without tunicamycin. A. Resting cell length; B. Peak shortening (PS, normalized to cell length); C. Maximal velocity of shortening (+dL/dt); D. Maximal velocity of relengthening (-dL/dt); E. Time-to-PS (TPS); F. Time-to-90% relengthening ($TR_{90}$). Mean ± SEM, 3-4 mice per group, *P < 0.05 vs. C57 group; #P < 0.05 vs. C57-TN group.

increased in WT after tunicamycin treatment. Our results showed the ER stress biomarkers Gadd153, Grp78, phosphorylated eIF2α and phosphorylated IRE1α were markedly upregulated in heart samples from C57BL/6J WT mice after TN treatment, and these effects were more pronounced in Sesn2 knockout animals compared with TN treated WT mice (**Figure 4A**, **4B**). To assess whether autophagy was involved in TN-triggered ER stress that caused contractile dysfunction, the amounts of the autophagic biomarkers Atg5, p62 and LC3 I/II were assessed by immunoblotting. The data demonstrated that tunicamycin remarkably elevated Atg5 and p62 amounts, as well as LC3 II/I ratios in C57BL/6J WT mouse hearts compared with the Sesn2 KO group (**Figure 5A**, **5B**). These findings indicated that Sesn2 KO mouse hearts were very sensitive to TN-induced ER stress but not to autophagy.

*TN-induced ER stress modulates cardiac AMPK-mTOR pathway*

Since AMPK plays a critical regulatory role in heart function in diverse pathologies, the AMPK

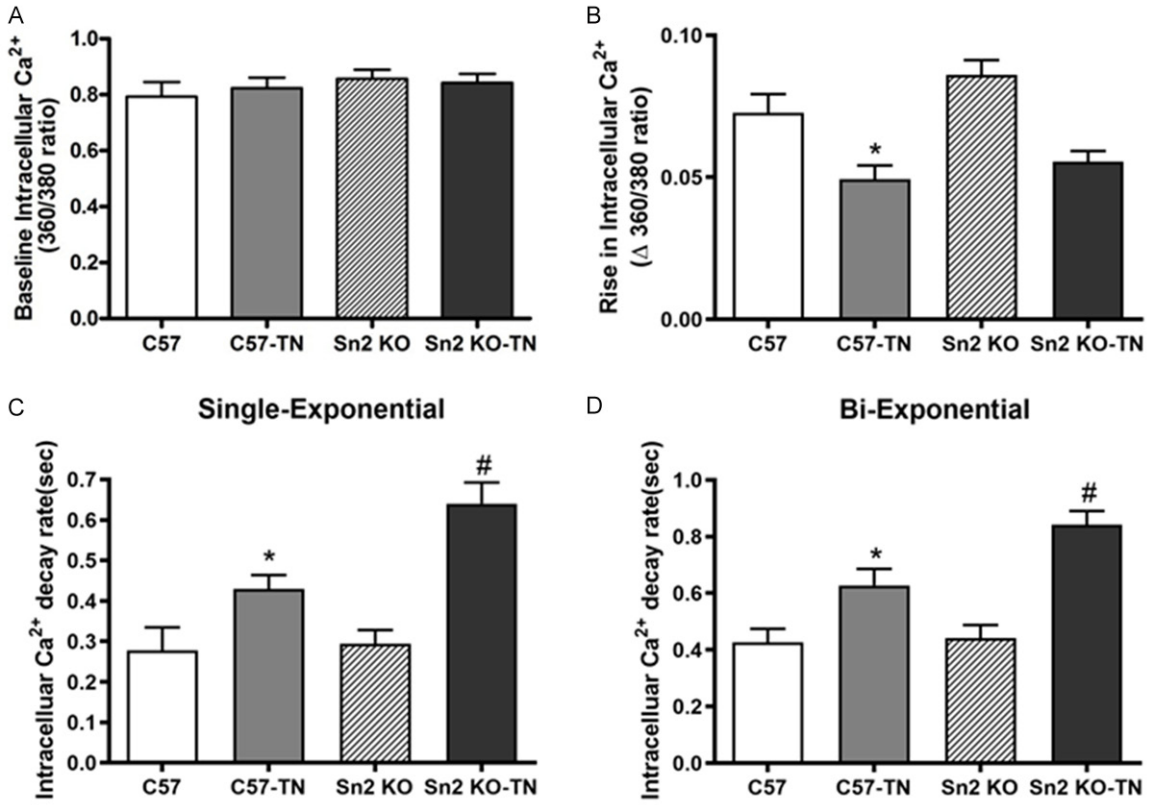


**Figure 3.** Intracellular $Ca^{2+}$ levels in cardiomyocytes from C57 and Sestrin2 knockout mice treated with or without tunicamycin. A. Baseline intracellular $Ca^{2+}$; B. Electrically-stimulated intracellular $Ca^{2+}$; C and D. Single and bi-exponential intracellular $Ca^{2+}$ decay rate. Mean ± SEM, 3-4 mice per group, *P < 0.05 vs. C57 group; #P < 0.05 vs. C57-TN group.

signaling pathway and other related proteins were examined (**Figure 6A**, **6B**). The immunoblotting results showed that phosphorylated AMPK and the downstream target acetyl-CoA carboxylase (ACC) had markedly elevated amounts after tunicamycin administration in mouse heart specimens, particularly in WT animals versus Sesn2 KO animals. Intriguingly, phosphorylated mTOR and p-S6, regulated by AMPK, showed higher levels in TN-treated Sesn2 KO hearts but not in WT hearts; ULK phosphorylation only occurred in WT but not in Sesn2 KO hearts.

*Effects of AMPK and autophagy inhibitors on cardiomyocyte contractile response*

To examine the causal relationship between AMPK and autophagy in TN-induced ER stress associated with contractile response in isolated cardiomyocytes, C57BL/6J WT mouse cardiomyocytes were administered tunicamycin with or without Compound C (AMPK suppressor) and/or 3-MA (autophagy suppressor). Firstly, the AMPK or autophagy suppressor alone did not influence resting cell length or time-to-PS. Secondly, AMPK suppression and/or autophagy inhibition significantly increased the depressed peak shortening, maximal velocity of shortening/relengthening and prolonged relengthening in tunicamycin treated C57BL/6J WT mouse cardiomyocytes (**Figure 7**).

## Discussion

The present work examined the impact of Sesn2 knockout on autophagy-related cardiac dysfunction in mice with ER stress. We found a significant elevation in autophagy in C57BL/6J mouse heart samples after administration of TN. In addition, enhanced AMPK activation was detected in mice with ER stress, indicating that AMPK is important in ER stress-induced cardiac dysfunction. Considering both AMPK phosphorylation and autophagy stimulated cardiomyocyte contractility and intracellular $Ca^{2+}$

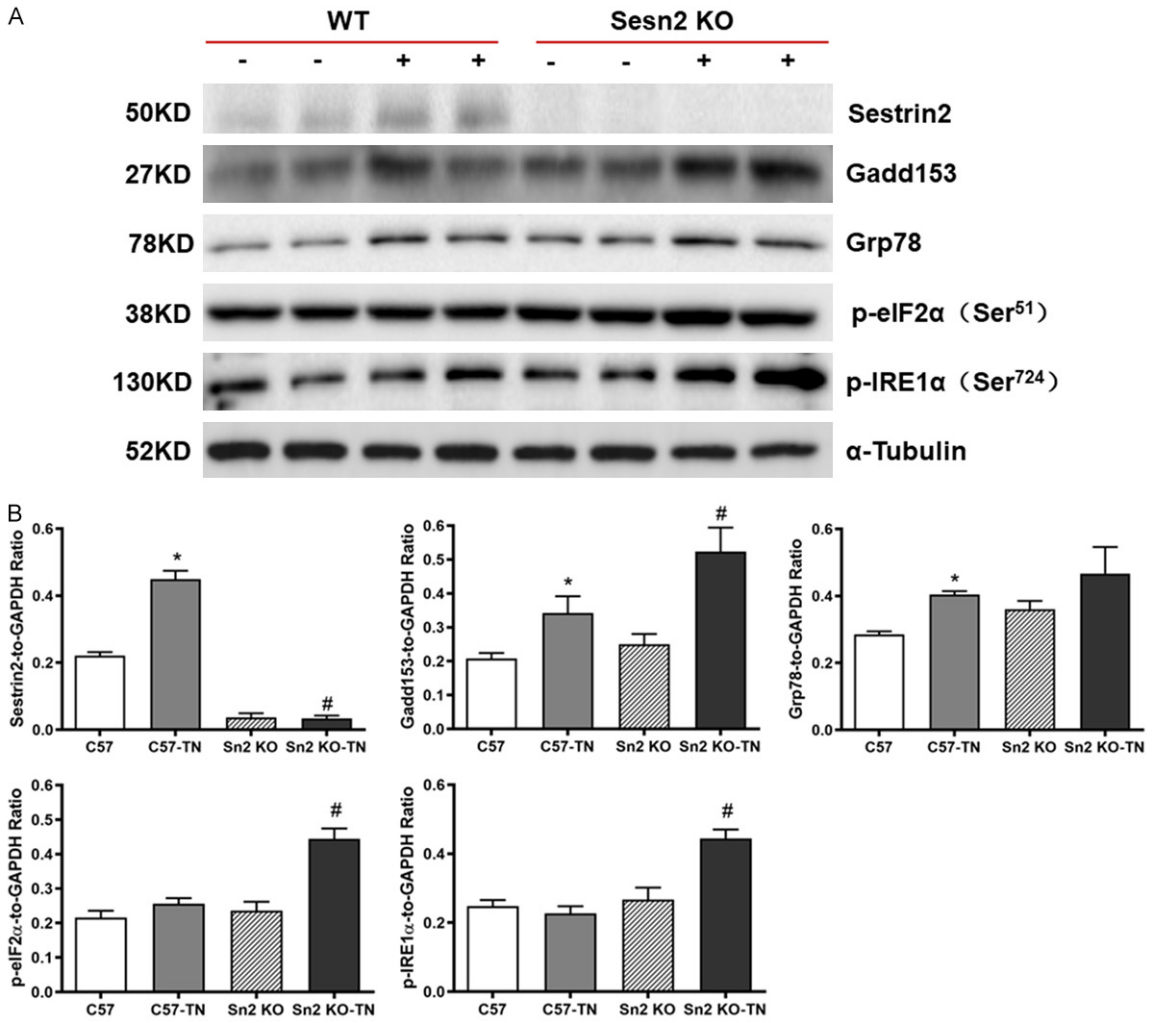


**Figure 4.** Western blot analysis of myocardial samples from C57 and Sestrin2 knockout mice treated with or without tunicamycin. A. Protein expression levels of Sestrin2 and the ER stress markers Gadd153, Grp78, p-eIF2α and p-IRE1α, determined with specific antibodies; B. Pooled data of the above proteins normalized to α-tubulin. Mean ± SEM, n = 3-4 per group, *P < 0.05 vs. C57 group; #P < 0.05 vs. C57-TN group.

homeostasis, we wondered whether AMPK and cardiomyocyte contractility are associated. In addition, treatment with compound C, an inhibitor of AMPK, worsened cardiomyocyte contractility. This study further demonstrated that blockage of autophagy under ER stress in the mouse heart could not rescue myocardial contractile dysfunction. Interestingly, *in vivo* data also showed that Sesn2 knockout mice had decreased autophagy and increased damage of contractile response and intracellular $Ca^{2+}$ levels in mouse cardiomyocytes after tunicamycin treatment. Several studies suggested AMPK is an important downstream effector of Sesn2-mediated activity [20, 21]. It was shown that Sesn2 enhances autophagy and decreases insulin resistance via AMPK pathway regulation in C2C12 myotubes [22]. This study demonstrated an association of Sesn2 with abnormal cardiac response to ER stress through AMPK-related autophagy.

Autophagy is critical for cell homeostasis, as a catabolic process that delivers cytoplasmic cargo to lysosomes for degradation. This study showed that Sesn2 knockout exacerbates ER stress-induced myocardial dysfunction due to the failure response of cardiac autophagy. The function of autophagy in cardiovascular injury in diverse experimental models remains controversial. Autophagy alleviation might have a therapeutic benefit in cardiac diseases [23]. On

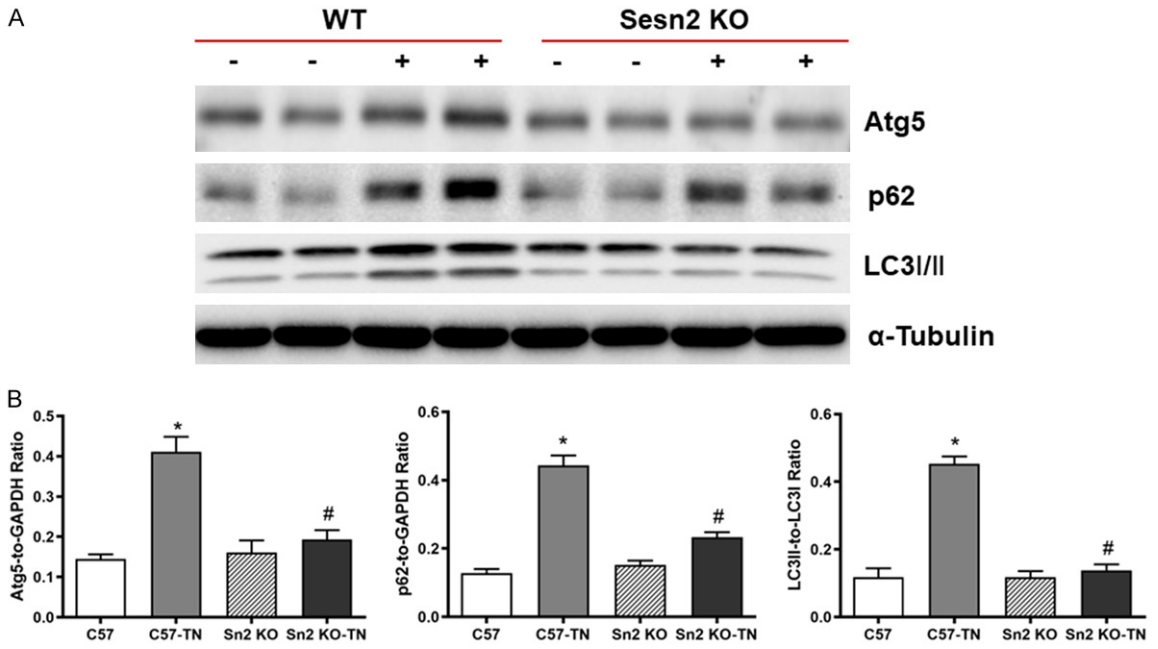


**Figure 5.** Western blot analysis of autophagy markers in myocardial samples from C57 and Sestrin2 knockout mice treated with or without tunicamycin. A. Expression levels of Atg5, p62 and LC3I/II; B. Pooled data of the above proteins normalized to α-tubulin. Mean ± SEM, n = 3-4 per group, *P < 0.05 vs. C57 group; #P < 0.05 vs. C57-TN group.

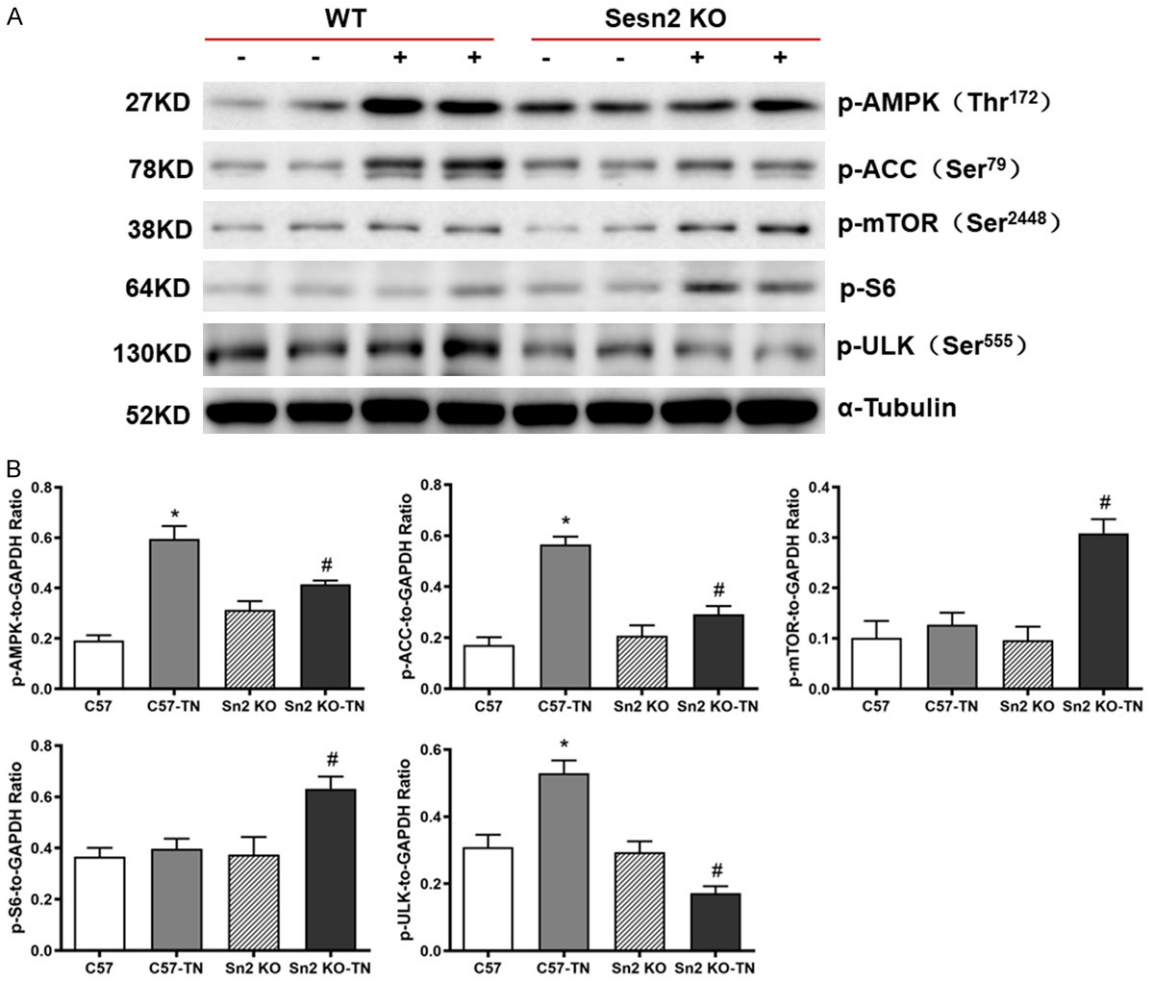

**Figure 6.** Western blot analysis of myocardial samples from C57 and Sestrin2 knockout mice treated with or without tunicamycin. A. Expression levels of p-AMPK, p-ACC, p-mTOR, p-S6 and p-ULK; B. Pooled data of above proteins normalized to α-tubulin. Mean ± SEM, n = 3-4 per group, *P < 0.05 vs. C57 group; #P < 0.05 vs. C57-TN group.

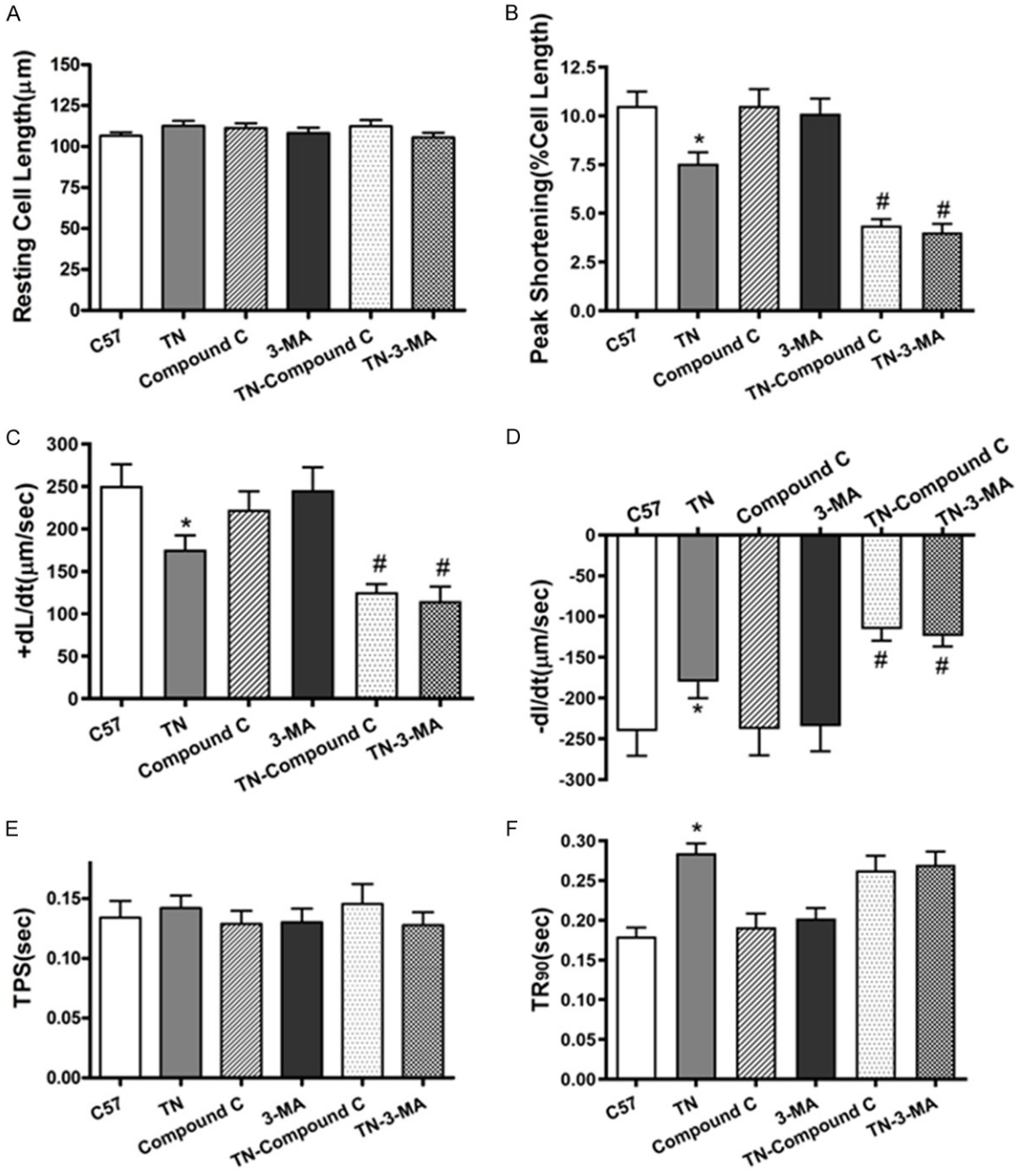


**Figure 7.** Effect of tunicamycin on cell shortening in cardiomyocytes from C57 mice. Cardiomyocytes were incubated with tunicamycin (3 μg/ml) for 4 h in vitro prior to assessment of mechanical properties. A cohort of cardiomyocytes was coincubated with the AMPK inhibitor compound C (10 mM) or the autophagy inhibitor 3-methyladenine (3-MA, 3 mM) alone with TN. A: Resting cell length; B: Peak shortening (PS, normalized to cell length); C: Maximal velocity of shortening (+dL/dt); D: Maximal velocity of relengthening (-dL/dt); E: Time-to-PS (TPS); F: Time-to-90% relengthening (TR90). Mean ± SEM, 3-4 mice per group, *P < 0.05 vs. C57 group; #P < 0.05 vs. TN group.

the other hand, enhanced autophagy induces cell death. In this work, Atg5, p62 and LC3 were upregulated after tunicamycin treatment in the mouse heart. The exact cellular events or sig-

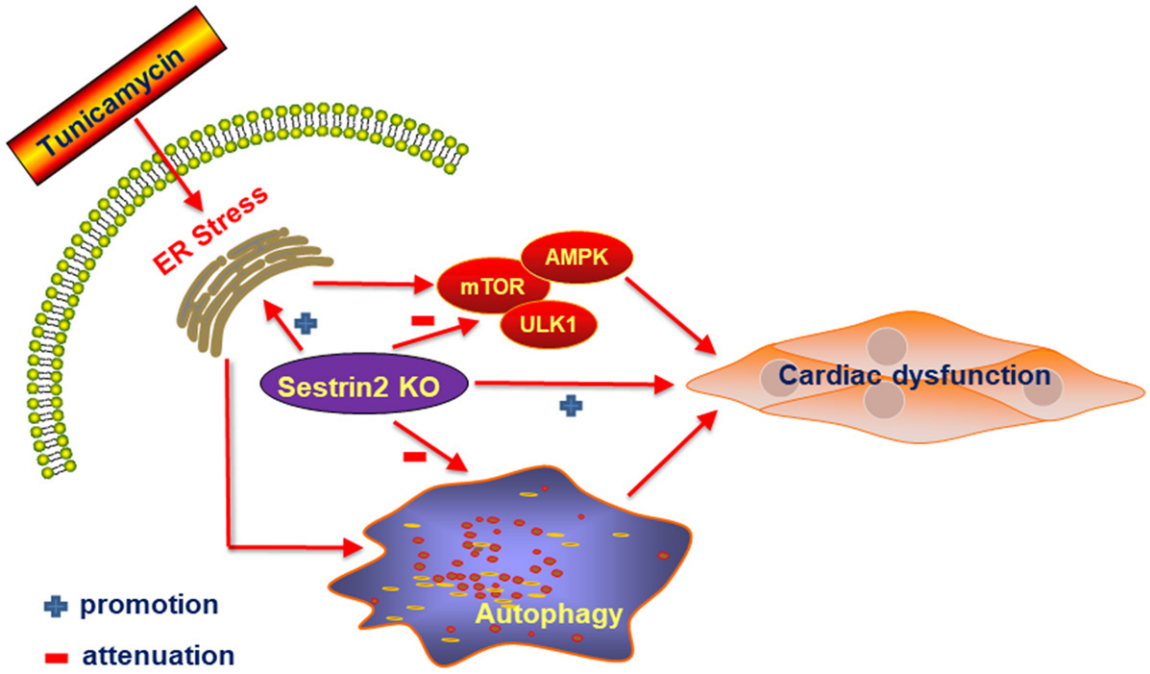


**Figure 8.** Schematic diagram of tunicamycin induced ER stress in C57 and Sestrin2 knockout mice. ER stress led to activation of AMPK pathway and autophagy. Autophagic response leads to cardiac dysfunction. Sestrin2 knockout exacerbates ER stress induced myocardial dysfunction due to the failure response of cardic autophagy and AMPK/mTOR activation. +: promotion; -: attenuation.

naling pathways explaining autophagy initiation by stimulus responses are unknown. This may be because given stimuli often interact with diverse molecules, triggering multiple types of injury [24]. These interactions induce stress responses, e.g., ER stress discussed in this study. It is further required to determine the functions of autophagy in ER stress-induced cardiac dysfunction. The Sestrin2-AMPK pathway contributes to multiple biological processes, protecting cells by metabolic adaptation [25]. Sesn2 promotes the stabilization of the LKB1/AMPK complex that regulates AMPK activation under ischemic conditions [26]. Sesn2 regulates mTOR under environmental stress, progressively downregulating mTOR, an important autophagy modulator and a critical nutrient sensor. Sesn2 upregulation contributes to autophagy enhancement, whereas its downregulation suppresses autophagy triggered by many stimuli [27], which is consistent with the results of this study.

AMPK is critical for the maintenance of energy homeostasis by regulating diverse homeostatic mechanisms such as autophagy and protein degradation. The current findings demonstrated that mTOR and ULK are the major factors regulating this process. Furthermore, mTOR controls cardiomyocyte survival and modulates cell proliferation, apoptosis, cell migration and metabolism. Further studies indicated that AMPK suppression increases the amounts of p-mTOR protein. As shown above, AMPK was one of the factors protecting from ER stress-induced autophagy, corroborating a report by Kim et al., which revealed that AMPK can be induced to positively regulate autophagy through mTOR suppression and ULK phosphorylation. Altered p-ULK in association with AMPK induction upon stress response has been widely observed [28]. As demonstrated above, ER stress induced phosphorylation of mTOR and ULK, which are downstream targets of AMPK and mTOR, respectively. Since AMPK acts as both an energy level sensor and an autophagy inducer, we hypothesized that AMPK/mTOR/ULK pathway countering ER stress participated in myocardial dysfunction.

Several follow up studies are required. First, further investigation should determine whether autophagic response is a “double-edged sword” in cardiac function in the long run. Secondly, autophagy effectors are presently examined as novel therapeutic targets for drug

development in diverse human disorders [29]. Therefore, more proteins that regulate autophagy need further clarification. More experiments should be performed using Sesn2 overexpression mice to elucidate the function of Sestrin2 and the role of AMPK/mTOR signaling. Thirdly, more patients with ER stress related heart diseases should be assessed to confirm the hypothesis that AMPK-mTOR signaling could be targeted for modulating autophagy.

In summary, the above findings suggest autophagy contributes to cardiomyocyte contractile dysfunction, and AMPK has an important function in ER stress-induced autophagy activation. Moreover, these effects induced by Sestrin2 could result from changes in AMPK-mTOR signaling that interacts with ER stress (**Figure 8**). Therefore, Sestrin2 may represent an efficient therapeutic target in ER stress-mediated cardiovascular disorders.

## Acknowledgements

This work was supported by grants from the Provincial Natural Science Foundation of Shandong province, China (ZR2020QH019, ZR2021MH066), Traditional Chinese Medicine Science and Technology Project of Shandong Province (2020M041), Clinical Medicine Science and Technology Innovation Plan of Jinan City (202019093).

## Disclosure of conflict of interest

None.

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