E3 Ubiquitin Ligase TRIM9 Ubiquitin Modified-Beclin1 Regulates Autophagy to Relieve Myocardial Ischemia Reperfusion Injury

Limeiting Wang

Kunming Medical University

Nan Wang

Kunming Medical University

Peng Rao

Kunming Medical University

Changyan Li

Kunming Medical University

Xiang Liu

Kunming Medical University

Wenting Song

Kunming Medical University

Lin Sun

Kunming Medical University

Di Lu (✉ ludi20040609@126.com)

Kunming Medical University <https://orcid.org/0000-0001-5716-8451>

Research Article

Keywords: TRIM9, ischemia reperfusion injury, Beclin1, autophagy

Posted Date: January 25th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1262427/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

TRI partite motif (TRIM) family proteins are RING finger domain-containing proteins, which are related with the maintenance of cellular functions. TRIM9, a pivotal E3 ubiquitin ligase, has been implicated in the pathogenesis of many diseases. However, the role of TRIM9 in myocardial ischemia-reperfusion injury (MIRI) remains unknown. In this study, we found that TRIM9 expression is up-regulated in MIRI *in vivo* and *in vitro* model. The inhibition of TRIM9 expression further increased the protein levels of LC3II and p62, while they were elevated in the process of MIRI, suggesting the disruption of autophagic flow. Mechanistically, TRIM9 down-regulated the expression of Beclin1, a crucial protein in autophagy, and promoted the ubiquitination of Beclin1, followed by the decreased expression of LC3II and p62. Collectively, our data revealed that TRIM9 can protect against MIRI and might be a novel and potential therapeutic target for cardiovascular disease.

1. Introduction

Ischemic cardiomyopathy remains a globally prevalent disease characterized by high morbidity and mortality [1]. At present, for patients who subjected to myocardial infarction, rapidly reconstruction of blood flow in ischemic myocardial tissue has been approved widely to be most effective treatment. However, a large number of clinical and experimental evidences have confirmed that reconstructing blood flow of ischemic myocardial tissue results in myocardial tissue injury, which is known as myocardial ischemia-reperfusion injury (MIRI) [2]. MIRI causes a series of pathophysiological changes in the heart, and among these changes, excessive myocardial cell death is a principal and basic pathological symptom of MIRI [3]. However, the mechanism underlying MIRI-induced cell death remains vague.

Autophagy, as an evolutionarily conserved catabolic mechanism, plays an important process for maintenance of energy and protein homeostasis, which digests and degrades damaged organelles and misfolded proteins to maintain normal cellular function via fusing with lysosomes [4]. Emerging evidences have suggested that the disruption of autophagy is content to pathogenesis and progress of a variety of human diseases [5, 6]. Recent studies have demonstrated that autophagy is defective during ischemia/reperfusion (I/R) [7]. However, the molecular underpinnings that regulates autophagy have not yet been fully elucidated. Beclin1, one of the earliest autophagy effector discovered in mammals [8] and a core component of the class (III) phosphatidylinositol 3-kinase (PI3K)/Vps34 complex, is involved in the regulation of autophagosome formation as an autophagy initiation factor [9–11]. Despite of the role of Beclin1 reported in MIRI, what Beclin1 plays a beneficial and harmful effects on MIRI remain controversial. Therefore, further analyzing the role and the regulatory mechanism of Beclin1 in MIRI are of great value.

Accumulative evidences have demonstrated that autophagy and the ubiquitin-proteasome system (UPS) are two major degradation mechanism in eukaryotic cells [12]. Autophagy includes microautophagy, macroautophagy and chaperone-mediated autophagy. Here, we focus on macroautophagy/autophagy [13]. The UPS is a selective proteolytic system in which the conjugation of ubiquitin to substrates induces degradation by the proteasome [14]. Ubiquitination is mediated by the cooperative action of three

enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin-transferring enzyme (E2), and the ubiquitin ligase (E3) [15]. The ubiquitin chain can then be recognized by the proteasome, which degrades the target substrate in an ATP-dependent manner [16]. A recent study from our lab has demonstrated that increased UBE2D3 expression, one of members of the ubiquitin-binding enzyme 2 family, arises the ubiquitination of p62/SQSTM1, subsequently disrupting autophagic flux and exacerbating myocardial ischemia-reperfusion injury in dependence of mTOR-Beclin1 pathway [17]. In addition, some studies from other groups also have demonstrated that ubiquitination play a crucial role in the pathogenesis of cardiovascular diseases, for example ischemia-reperfusion injury [18]. As a member of the tripartite motif-containing protein (TRIM) family of E3 ubiquitin ligases, TRIM9 plays a significant role in the central nervous system of developing adults, and affects a variety of biological processes, such as inflammation, immunity, cell proliferation, apoptosis, and antiviral mechanisms [19, 20]. Several studies have demonstrated that TRIM proteins can recruit ULK1 and BECN1 complexes and bind to mammalian Atg8-family paralogs via an LC3-interacting region act as both autophagy cargo receptors and platforms assembling autophagosome-formation machinery [21, 22]. Moreover, in non-small-cell lung cancer, TRIM59 inhibits Beclin1 expression to block autophagy via inhibiting the NF- κ B pathway [23]. However, whether and how TRIM9 regulates autophagy remains unclear.

In this study, we found that TRIM9 expression was up-regulated in in vivo and in vitro MIRI model. And TRIM9 down-regulated the expression of Beclin1 and promoted the ubiquitination of Beclin1, subsequently inhibiting the expression of LC3II and p62 and blocking autophagy to ameliorating MIRI. Taken together, our study revealed that TRIM9 can protect against MIRI and may represent a promising valuable therapeutic target for MIRI.

2. Materials And Methods

2.1 Animals

Adult male SD rats weighing 230 g ~ 250 g were purchased from the Experimental Animal Center of Kunming Medical University (Kunming, China). The animals were kept under standard laboratory conditions with 12 h of light and provided plenty of food and water. All animal feeding and experimental protocols were performed according to the ethical standards set out in the Declaration of Helsinki and were approved by the Institutional Committee for Animal Protection and Utilization of Kunming Medical University.

2.2 Cardiac ischemia-reperfusion model

A rat model of myocardial ischemia reperfusion was constructed as described previously [24]. In brief, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 ml/100 g), subjected to endotracheal intubation and connected to a ventilator. Electrocardiography was performed throughout the whole procedure. After thoracotomy, the anterior descending branch of the left coronary artery was ligated with 6-0 silk thread. After 45 min of ischemia, the silk thread was cut, and reperfusion was initiated.

2.3 2,3,5-Triphenyltetrazolium chloride (TTC) staining

Heart samples were collected, washed with PBS, stored at -20°C for 30 min, and cut into 2 to 3-mm-thick slices. The tissue sections were submerged in 2% TTC solution for 20 min at 37°C in the dark, and the stained sections were fixed with 4% paraformaldehyde for 15 min.

2.4 Immunofluorescence

Fresh heart tissues were fixed in 4% paraformaldehyde, dehydrated in a gradient ethanol series and embedded in paraffin. The sections were subsequently incubated with 0.3% hydrogen peroxide in PBS to block endogenous peroxidase activity. Thereafter, the sections were blocked with 5% goat serum in PBS for 2 h and incubated overnight at 4°C with a rabbit polyclonal anti-TRIM9 antibody (1:200, Proteintech). After three washes with PBS, the tissues were incubated with FITC-conjugated anti-rabbit immunoglobulin (IgG) for an additional 2 h at room temperature, washed with TBST for 3×5 min, incubated with 4,6-diamino-2-phenylindole (DAPI, 1 µg/mL, Sigma, USA) for nuclear staining and sealed. The tissues were observed and photographed using a fluorescence microscope (Olympus, Japan).

2.5 DAPI fluorescence staining

Apoptotic cells were analyzed by DAPI staining, and stronger fluorescence intensity of apoptotic cells than normal cells or condensed ruptured nuclei were observed due to nuclear fragmentation and chromatin condensation. The cells in each group were treated with DAPI and placed at room temperature for 2 h in the dark, and nuclear morphology was observed and recorded under fluorescence microscope (Olympus, Japan).

2.6 Cell culture and treatment

H9c2 rat cardiomyocytes were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in low-glucose DMEM/F12 containing 10% fetal bovine serum and antibiotics (1% penicillin and streptomycin) in an incubator at 37°C and 5% CO₂. The reagents were purchased from Gibco.

2.7 H9c2 cell oxygen and glucose deprivation (OGD) model

Cardiomyocytes were cultured in low-glucose medium without serum in an incubator containing 1% O₂, 94% N₂ and 5% CO₂ for 2 h to simulate ischemia and then cultured in complete medium for 2 h in an incubator at 5% CO₂ and 37°C to simulate reperfusion.

2.8 Cell transfection

Small interfering RNA (siRNA) targeting TRIM9 and negative control siRNA were purchased from Gene Pharma (Shanghai, China). H9c2 cells in plates were transfected with the HA-UB plasmid, and siRNA and plasmid DNA were added according to the instructions provided by the manufacturer of Lipofectamine 3000 (Thermo). The transfected cells were used for other experiments according to the experimental requirements.

2.9 Western blot analysis

Cell lysis products were extracted using radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor PMSF, the cell supernatant was collected after centrifugation at 4°C and 12,000 rpm for 30 min, and the protein content was determined using a Beyotime protein quantification kit. The protein samples were separated by electrophoresis using different concentrations of sodium dodecyl sulfate polyacrylamide gels and then transferred to PVDF membranes. Subsequently, the membranes were blocked with 5% skimmed milk for 3 h at room temperature and incubated overnight at 4°C with the primary antibody (1:1000 dilution). The membrane was washed with TBST, and the secondary antibody (1:2000 dilution) was added and incubated at room temperature for 2 h. The bands were visualized using enhanced chemiluminescence reagents and quantified using ImageJ software.

2.10 Immunoprecipitation

An immunoprecipitation (IP) kit (Thermo, 88804) was used to assess the interaction between different proteins. Protein was extracted from H9c2 cells and incubated with primary antibody (5 µg) or an equivalent amount of normal mouse IgG (Santa Cruz Biotechnology, SC-2025) or normal rabbit IgG (Cell Signaling Technology, 2729) at 4°C overnight. After extensive washing, the supernatant was removed, and 10 µl neutralizing solution was added. If the primary antibody and the secondary antibody were rabbit antibodies, the samples were prepared after incubation at room temperature for 10 min. If the primary antibody and the secondary antibody were different, the samples were heated to 95°C for 10 min, and then the samples were used for Western blotting.

2.11 mRFP-GFP-LC3

H9c2 cells were transfected with an mRFP-GFP-LC3 lentivirus (Shanghai Hanhengsheng Material Science and Technology Co., Ltd., China) according to the manufacturer's instructions. mRFP was used to label LC3, and cells expressed both red (mRFP) and green (GFP) fluorescence at neutral pH. Since GFP is sensitive to acidic conditions, only red fluorescence was detected when GFP was quenched after fusion of autophagosomes and lysosomes. Thus, a decrease in the GFP signal may indicate the formation of autophagolysosomes by the fusion of lysosomes and autophagosomes. Yellow (green + red) puncta represent autophagosomes. The ratio of the number of red puncta to the number of yellow puncta was used as an indicator of autophagic flux.

2.12 CCK-8

Proliferation and viability of CCK8 cells were determined H9c2 cells (100 µl/well) were planted in 96-well plates, and modeling was started after cells adhered to the wall and grew to an appropriate density. After modeling, 10 µl CCK8 solution was added to each well and placed in an incubator for incubation for 1-4h. The absorbance at 450nm was measured with a microplate reader.

2.13 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0.1 software, and all data are expressed as the mean $\pm$ standard deviation (SD). Student's t-test was used to analyze the differences between two groups, and one-way ANOVA and Tukey's post hoc test were used for multiple group comparisons. For nonnormally distributed data, the Wilcoxon signed rank test for paired samples was used to compare the differences between the two groups. A value of $p < 0.05$ indicated statistical significance.

3. Results

3.1 Cardiac TRIM9 expression is increased after MIRI

A report from Shi [25] et al. have shown that TRIM9 in mice has a protective effect against ischemic stroke. However, the role of TRIM9 in MIRI remains unclear. Previous studies have suggested that the early time point of reperfusion is 20min–3h [26]. Therefore, in order to determine the function of TRIM9 in the early myocardial ischemia reperfusion, the left anterior descending coronary artery of SD rats was ligated with a prolene suture. After 45 min, the reperfusion was performed by cutting the line for 20 min. Then we used TTC staining to assess the infarct size in the hearts from normal rats and rats subjected to myocardial ischemia-reperfusion. The myocardial tissue was white after 45 min of ischemia and 20min of reperfusion (Figure 1A), and the analysis of ECG of the heart showed that ST segment was elevated (Figure 1B). Moreover, using Western blot, our result showed that the expression level of TRIM9 the MIRI group was increased compared with the sham group (Figure 1C-1D). To further verify these results, immunofluorescence staining was used to detect the expression of TRIM9 in the sham and MIRI group, and we found that TRIM9 expression was significantly upregulated in the MIRI group compared with that in the sham group (Figure 1E). These results indicate that TRIM9 expression is up-regulated in MIRI.

3.2 TRIM9 expression is increased in the in vitro model of MIRI

Next, the *in vitro* model of myocardial ischemia and reperfusion was established. We extracted proteins from myocardial cells and performed Western blot. and found that the expression of TRIM9 was also increased (Figure 2A-2B). In line with these results, we performed immunofluorescence staining of myocardial cells after 2 h of ischemia and reperfusion for 2 h and observed that TRIM9 expression was up-regulated in the oxygen and glucose deprivation condition compared to that in the normal oxygen and glucose case (Figure 2C). In order to rule out the effect of cell type on TRIM9 expression, we used human-derived myocyte cell line (AC16), and then performed the same experiment. Consistent with those in H9C2 cell line, TRIM9 expression was also elevated (Figure 4D-4F). Therefore, our results suggest that ischemia-reperfusion can up-regulates the expression of TRIM9 both *in vivo* and *in vitro*.

3.3 Knockdown of TRIM9 can regulate autophagy and autophagic flow

Perera et al. reported that TRIM55 was closely related to the expression of the autophagy-related molecules SQSTM1/p62 and LC3 during the development of striated muscle [27]. Thus, to identify whether TRIM9 is also related to autophagy in myocardial ischemia-reperfusion conditions. The transfection with siRNAs targeting three different domains of the TRIM9 gene was executed in H9c2. By Western blot analysis, the results showed that the transfection efficiency of the three different TRIM9 siRNAs was similar. At the same time, we found that LC3II expression was up-regulated after TRIM9 siRNA transfection, indicating that autophagy occurred after the knockdown of TRIM9 (Figure 3A-3C). Besides, we determined the SQSTM1/p62 expression and found that p62 expression was also up-regulated (Figure 3D-3E), indicating that autophagic flux was disrupted in MIRI. To further demonstrate these results, we transfected H9c2 cells with a lentivirus harbouring tandem fluorescent mRFP-GFP-LC3. After decreasing TRIM9 expression, there was more yellow puncta, indicating defective autophagic flux. (Figure 3F). Combined with the above results, our data demonstrate that TRIM9 enhances autophagic flux in MIRI.

3.4 TRIM9 is required for MIRI

To determine the effect of down-regulation of TRIM9 in MIRI, we transfected H9c2 cells with siRNA targeting the TRIM9 gene, and before extracted the protein, two of these four groups were exposed to OGD like previous described. Using western blot, we found that the expressions of p62 and LC3II were up-regulated after the knockdown of TRIM9, indicating that autophagic flux was destroyed (Figure 4A-4C). Next, we built the same model in the same way, and the cell viability and proliferation was measured by CCK8 method, we found that the condition of H9c2 was even worse after TRIM9 knocked down compared with simple ischemia reperfusion group (Figure 4D). Taken together, these observations demonstrate that TRIM9 enhances autophagic flux and aggravate myocardial ischemia reperfusion injury.

3.5 TRIM9 regulates autophagy via ubiquitination of Beclin1

In order to deeply understand the function of TRIM9, we used the antibody against TRIM9 and performed immunoprecipitation, then made the mass spectrometry and bioinformatics analysis. We found that TRIM9 was related to autophagy, spliceosomes, RNA degradation and other pathways (Figure 5A). Our previous experiments revealed that TRIM9 was indeed associated with autophagy. Additionally, Beclin1 plays a central role in autophagy and triggers the expression of multiple proteins involved in autophagosome formation [28]. Therefore, to verify the relationship between TRIM9 and Beclin1, we performed coimmunoprecipitation (co-IP). The results confirmed that Beclin1 was present in the samples pulled down with a TRIM9 antibody but not in the samples pulled down with IgG (Figure 5B). This indicated that an interaction occurs between TRIM9 and Beclin1.

To further understand the regulatory relationship between TRIM9 and Beclin1, H9c2 cardiomyocytes were transfected with siRNA targeting the TRIM9 gene as described above. Proteins were extracted 48 h later, and the change in Beclin1 expression at the translational level was assessed by Western blotting, Beclin1 expression was upregulated (Figure 5C-5D). As the downstream of TRIM9, Beclin1, how did it regulate

autophagy. Hence, we added the siRNA targeting the Beclin1 gene to cardiomyocyte cell, after 48h, we extracted the protein and did SDS-page, which found that LC3II and P62 were down-regulated (Figure 5E-5H). Therefore, we added UB with HA label in cardiomyocytes, after 48h, coimmunoprecipitation was performed, and the results confirmed that Beclin1 ubiquitination was reduced by knockdown of TRIM9 expression in H9c2 rat cardiomyocytes (Figure 5I). These results indicate that TRIM9 may regulate Beclin1 degradation in MIRI to induce autophagy but that autophagic flow is disrupted.

4. Discussion

MIRI accompanied by clinical complications like reperfusion arrhythmias, lethal reperfusion, myocardial stunning and no-reflow [29], and it is a common clinical disease which needs to be solved. The purpose of our study was to investigate the role of the ubiquitin ligase TRIM9 in early cardiac ischemia-reperfusion. We found that the expression of TRIM9 was up-regulate in MIRI, and acts as a negative regulator of the translation of Beclin1, a critical autophagy protein. Moreover, we have showed that knocked down Beclin1 decreased the expression of LC3II and p62, and reduced autophagy. More importantly, after TRIM9 knockdown in cardiomyocytes, autophagic flux was disrupted through upregulation of Beclin1 expression and inhibiting Beclin1 ubiquitination.

A recently study provides compelling evidence that the coupling of ubiquitination to proteasomal degradation becomes inadequate during IRI and that such inadequacy contributes to MIRI [30], and as a E3 ubiquitin ligases, TRIM9 is a newly identified RING-type E3 ubiquitin protein ligase that was first described to be specifically expressed in the embryonic and adult nervous systems [31]. However, more researches on this ubiquitin-protein ligase were needed to gain more insights into its roles in other tissues and diseases. For example, in cancer, previous studies have shown that methylated TRIM9, which inhibits the expression of TRIM9 mRNA, can be used as a blood biomarker for breast cancer in patients [32]. In addition, overexpression of TRIM9 can promote uterine fibroid cell proliferation and inhibit apoptosis through the NF- κ B signaling pathway [33]. Regarding the nervous system, in patients with dementia and Lewy bodies, the expression of TRIM9 in the brain is decreased. More recent studies have shown that TRIM9 and TRIM67 may regulate the structure and function of neurons by interacting with a large number of proteins and potential substrates [34]. These interactions have opened many new research directions related to TRIM9, such as its ability to regulate actin, simulate membrane remodeling and regulate the morphology of protrusions. However, there have been no studies on the role of TRIM9 in MIRI. Our results not only demonstrated that TRIM9 was up-regulated in MIRI, but also might a protected gene for myocardial. It also elucidated that the up-regulation of TRIM9 in MIRI is for self-protection.

Previous studies have shown that autophagy and associated cell death are increased during myocardial infarction/reperfusion [35, 36]. And autophagy is the most prominent mechanism that results in the death of cardiomyocytes in end-stage heart failure [37]. Beclin1 is central to autophagy and is involved in a variety of other pathways. Down-regulation of Beclin1 expression reduces myocardial ischemia-reperfusion-induced autophagy and improves cell survival [38]; however, other studies have revealed that Beclin1 decreases the infarct size after myocardial ischemia-reperfusion [39]. It is worth noting that in our

study, the expression of TRIM9 in the hearts of mice subjected to myocardial ischemia-reperfusion was significantly upregulated and the *in vitro* studies revealed that although autophagy was increased after TRIM9 knockdown, autophagic flow was impaired. Accumulating evidences suggest that the inhibition of autophagic cell death protects cardiomyocytes during ischemia/reperfusion [40]. Hence, disruption of autophagic flow is not beneficial for the ischemic myocardium or recovery of the ischemic myocardium after reperfusion. Therefore, we hypothesized that upregulation of TRIM9 expression during myocardial ischemia-reperfusion may protect the heart or myocardial cells from MIRI through regulation of the core autophagy-related protein Beclin1 and alleviation of autophagic flow impairment. We found that the expression of Beclin1 was increased at the translational level after TRIM9 knockdown and that the ubiquitination of Beclin1 was inhibited, confirming this hypothesis.

Zeng [41] et al. found that upregulation of TRIM9 expression seems to be critical to resolving inflammation and minimizing the area of damage around the cerebral infarct. Therefore, we speculated that TRIM9 expression is also upregulated during myocardial ischemia-reperfusion, regardless of whether it has a potential regulatory role in the early stage of reperfusion. And we focused on one type of cell death, autophagy. Moreover, through *in vivo* and *in vitro* studies, we confirmed that TRIM9 was up-regulated in the early stage of reperfusion after cardiac ischemia, and TRIM9 can regulate autophagy in cardiomyocytes via regulating Beclin1 expression. This finding will help to identify potential molecular biomarkers and therapeutic targets related to TRIM9-mediated regulation of autophagy in cardiomyocytes and MIRI. Thus, whether the TRIM9-Beclin1 network regulates autophagy in MIRI is a very interesting and potential question for future studies. Whether autophagy can be modulated by targeting TRIM9 and Beclin1 may also need to be confirmed in the future.

5. Conclusion

In summary, our present study illustrated that a pivotal E3 ubiquitin ligase TRIM9 expression is up-regulated in MIRI *in vivo* and *in vitro* model. The inhibition of TRIM9 expression further increased the protein levels of LC3II and p62, while they were elevated in the process of MIRI, suggesting the disruption of autophagic flow. Mechanistically, TRIM9 down-regulated the expression of Beclin1, a crucial protein in autophagy, and promoted the ubiquitination of Beclin1, followed by the decreased expression of LC3II and p62. Which revealed that TRIM9 can protect against MIRI and might be a novel and potential therapeutic target for cardiovascular disease.

Declarations

Conflict of interest

All authors declare no conflicts of interest.

Ethics approval and consent to participate

The procedure used in animal experiment have an approval of Kunming Medical University (Permitted number: LA2020312)

Authors' contributions

Limeiting Wang profermed this study and acquired data; Limeiting Wang wrote the manuscript; Nan Wang and Pen Rao were responsible for part of animal experiments; Xiang Liu, Changyan Li, Rumin Lv and Wenting Song quantified experimental datas; Overall, Lin Sun and Di Lu conceived and designed the study.

Acknowledgements

We especially appreciate Dr.Jun Tao and Ping Yang for his support of the experiment.

Data availability statement

All data generated or analyzed during this study are included in this article.

Fund

This work was supported in part by National Natural Science Foundation of China (grant No. 31760292; grant No. 81760087; grant No.81560050), and Department of Science and Technology of Yunnan Province (grant No.2019ZF011-2; grant No.2017FA035; grant No. 2017FE467 (-008)).and the Graduate Innovation Fund of Kunming Medical University (NO. 2021S019).

References

1. Steg PG, Green law N. Prospective Observational Longitudinal Registry of Patients with Stable Coronary Artery Disease (CLARIFY) Investigators. Prevalence of anginal symptoms and myocardial ischemia and their effect on clinical outcomes in outpatients with stable coronary artery disease: data from the International Observational CLARIFY Registry. JAMA Intern Med. 2014 Oct;174(10):1651-1659.
2. Chen G, Xu C, Gillette TG. Cardiomyocyte-derived small extracellular vesicles can signal eNOS activation in cardiac microvascular endothelial cells to protect against Ischemia/Reperfusion injury. Theranostics.2020 Sep 23;10(25):11754-11774.
3. Hause loy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. J Clin Invest. 2013 Jan;123(1):92-100.
4. Saha S, Panigrahi DP, Patil S. Autophagy in health and disease: A comprehensive review. Biomed Pharmacother. 2018 Aug; 104:485-495.
5. Wright CF, Oswald BW, Dellis S. Vaccinia virus late transcription is activated in vitro by cellular heterogeneous nuclear ribonucleoproteins. J Biol Chem. 2001 Nov 2;276(44):40680-40686.

6. Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin1. *Nature*.1999;402:672–676.
7. Aghaei M, Motallebnezhad M, Ghorghanlu S. Targeting autophagy in cardiac ischemia/reperfusion injury: A novel therapeutic strategy. *J Cell Physiol*. 2019 Aug;234(10):16768-16778.
8. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin1. *Nature*. 1999 Dec 9;402(6762):672-676.
9. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B. Bcl-2 antiapoptotic proteins inhibit Beclin1-dependent autophagy. *Cell*. 2005 Sep 23;122(6):927-39.
10. Matsunaga K, Saitoh T, Tabata K, Omori H, Satoh T, Kurotori N, Maejima I, Shirahama-Noda K, Ichimura T, Isobe T, Akira S, Noda T, Yoshimori T. Two Beclin1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat Cell Biol*. 2009 Apr;11(4):385-96.
11. Kihara A, Kabeya Y, Ohsumi Y, Yoshimori T. Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep*. 2001 Apr;2(4):330-335.
12. Kwon YT, Ciechanover A. The Ubiquitin Code in the Ubiquitin-Proteasome System and Autophagy. *Trends Biochem Sci*. 2017 Nov;42(11):873-886.
13. Ohsumi Y. Molecular dissection of autophagy: two ubiquitin-like systems. *Nat Rev Mol Cell Biol*. 2001 Mar;2(3):211-216.
14. Ciechanover A, Kwon YT. Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies. *Exp Mol Med*. 2015 Mar 13;47(3): e147.
15. Ciechanover A. The unravelling of the ubiquitin system. *Nat Rev Mol Cell Biol*. 2015 May;16(5):322-324.
16. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem*. 1998; 67:425-479.
17. Wang X, Yang P, Jiang Y, Xu Y, Wang N, Rao P, Yang L, Sun L, Lu D. UBE2D3 contributes to myocardial ischemia-reperfusion injury by regulating autophagy in dependence of p62/SQSTM1. *Cell Signal*. 2021 Nov; 87:110118.
18. Kobayashi M, Higa JK, Matsui T. The role of ubiquitin in cardiac ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2019 Mar 1;316(3):H583-H585.
19. Tanji K, Kamitani T, Mori F. TRIM9, a novel brain-specific E3 ubiquitin ligase, is repressed in the brain of Parkinson's disease and dementia with Lewy bodies. *Neurobiol Dis*. 2010 May;38(2):210-218.
20. Reymond A, Meroni G, Fantozzi A, Merla G, Cairo S, Luzi L, Riganelli D, Zanaria E, Messali S, Cainarca S, Guffanti A, Minucci S, Pelicci PG, Ballabio A. The tripartite motif family identifies cell compartments. *EMBO J*. 2001 May 1;20(9):2140-51.) (Venuto S, Merla G. E3 Ubiquitin Ligase TRIM Proteins, Cell Cycle and Mitosis. *Cells*. 2019 May 27;8(5):510.
21. Kimura T, Jain A, Choi SW, Mandell MA, Schroder K, Johansen T, Deretic V. TRIM-mediated precision autophagy targets cytoplasmic regulators of innate immunity. *J Cell Biol*. 2015 Sep 14;210(6):973-989.

22. Mandell MA, Jain A, Arko-Mensah J, Chauhan S, Kimura T, Dinkins C, Silvestri G, Münch J, Kirchhoff F, Simonsen A, Wei Y, Levine B, Johansen T, Deretic V. TRIM proteins regulate autophagy and can target autophagic substrates by direct recognition. *Dev Cell*. 2014 Aug 25;30(4):394-409.
23. Han T, Guo M, Gan M, Yu B, Tian X, Wang JB. TRIM59 regulates autophagy through modulating both the transcription and the ubiquitination of Beclin1. *Autophagy*. 2018;14(12):2035-2048.
24. Reichert K, Colantuono B, McCormack I, Rodrigues F, Pavlov V, Abid MR. Murine Left Anterior Descending (LAD) Coronary Artery Ligation: An Improved and Simplified Model for Myocardial Infarction. *J Vis Exp*. 2017 Apr 2;(122):55353.
25. Shi M, Cho H, Inn KS, Yang A, Zhao Z, Liang Q, Versteeg GA, Amini-Bavil-Olyaei S, Wong LY, Zlokovic BV, Park HS, García-Sastre A, Jung JU. Negative regulation of NF- κ B activity by brain-specific TRIPartite Motif protein 9. *Nat Commun*. 2014 Sep 5; 5:4820.
26. Davidson SM, Ferdinandy P, Andreadou I, Bøtker HE, Heusch G, Ibáñez B, Ovize M, Schulz R, Yellon DM, Hausenloy DJ, Garcia-Dorado D; CARDIOPROTECTION COST Action (CA16225). Multitarget Strategies to Reduce Myocardial Ischemia/Reperfusion Injury: JACC Review Topic of the Week. *J Am Coll Cardiol*. 2019 Jan 8;73(1):89-99.
27. Perera S, Holt MR, Mankoo BS, Gautel M. Developmental regulation of MURF ubiquitin ligases and autophagy proteins nbr1, p62/SQSTM1 and LC3 during cardiac myofibril assembly and turnover. *Dev Biol*. 2011 Mar 1;351(1):46-61.
28. Jung YY, Lee YK, Koo JS. The potential of Beclin1 as a therapeutic target for the treatment of breast cancer. *Expert Opin Ther Targets*. 2016;20(2):167-178.
29. González-Montero J, Brito R, Gajardo AI, Rodrigo R. Myocardial reperfusion injury and oxidative stress: Therapeutic opportunities. *World J Cardiol*. 2018 Sep 26;10(9):74-86.
30. Hu C, Tian Y, Xu H, Pan B, Terpstra EM, Wu P, Wang H, Li F, Liu J, Wang X. Inadequate ubiquitination-proteasome coupling contributes to myocardial ischemia-reperfusion injury. *J Clin Invest*. 2018 Dec 3;128(12):5294-5306.
31. Berti C, Messali S, Ballabio A, Reymond A, Meroni G. TRIM9 is specifically expressed in the embryonic and adult nervous system. *Mech Dev*. 2002 May;113(2):159-62.
32. Mishima C, Kagara N, Matsui S, Tanei T, Naoi Y, Shimoda M, Shimomura A, Shimazu K, Kim SJ, Noguchi S. Promoter methylation of TRIM9 as a marker for detection of circulating tumor DNA in breast cancer patients. *Springerplus*. 2015 Oct 22; 4:635.
33. Yang F, Liu H, Yu Y, Xu L. TRIM9 overexpression promotes uterine leiomyoma cell proliferation and inhibits cell apoptosis via NF- κ B signaling pathway. *Life Sci*. 2020 Sep 15; 257:118101.
34. Menon S, Goldfarb D, Ho CT, Cloer EW, Boyer NP, Hardie C, Bock AJ, Johnson EC, Anil J, Major MB, Gup-ton SL. The TRIM9/TRIM67 neuronal interactome reveals novel activators of morphogenesis. *Mol Biol Cell*. 2021 Feb 15;32(4):314-330.
35. Shi B, Ma M, Zheng Y, Pan Y, Lin X. mTOR and Beclin1: Two key autophagy related molecules and their roles in myocardial ischemia/reperfusion injury. *J Cell Physiol*. 2019 Aug;234(8):12562-12568.

36. Dong Y, Chen H, Gao J, Liu Y, Li J, Wang J. Molecular machinery and interplay of apoptosis and autophagy in coronary heart disease. *J Mol Cell Cardiol.* 2019 Nov; 136:27-41.
37. Kostin S, Pool L, Elsässer A, Hein S, Drexler HC, Arnon E, Hayakawa Y, Zimmermann R, Bauer E, Klövekorn WP, Schaper J. Myocytes die by multiple mechanisms in failing human hearts. *Circ Res.* 2003 Apr 18;92(7):715-724.
38. Nah J, Zablocki D, Sadoshima J. Autosis: A New Target to Prevent Cell Death. *JACC Basic Transl Sci.* 2020 Aug 24;5(8):857-869.
39. Sala-Mercado JA, Wider J, Undyala VV, Jahania S, Yoo W, Mentzer RM Jr, Gottlieb RA, Przyklenk K. Profound cardioprotection with chloramphenicol succinate in the swine model of myocardial ischemia-reperfusion injury. *Circulation.* 2010 Sep 14;122(11 Suppl): S179-84.
40. Latchman DS, Stephanou A. Urocortin inhibits Beclin1-mediated autophagic cell death in cardiac myocytes exposed to ischemia/reperfusion injury. *J Mol Cell Cardiol.* 2006 Jun;40(6):846-852.
41. Zeng J, Wang Y, Luo Z, Chang LC, Yoo JS, Yan H, Choi Y, Xie X, Deverman BE, Gradinaru V, Gupton SL, Zlokovic BV, Zhao Z, Jung JU. TRIM9-Mediated Resolution of Neuroinflammation Confers Neuroprotection upon Ischemic Stroke in Mice. *Cell Rep.* 2019 Apr 9;27(2):549-560.e6.

Figures

Figure 1

Cardiac TRIM9 expression increased after myocardial IR injury. (A) The myocardial infarct area in SD rats was determined by TTC staining after myocardial ischemia-reperfusion, the red color represent survival area, whereas the white area indicates the infarcted size. (B) The ECG changes of rats with sham or I/R were detected by two-lead ECG. (C) The expression of TRIM9 in SD rats after 45 min of ischemia and 20 min of reperfusion. Cardiac tissue was extracted and then performed Western blotting to detect the expression of TRIM9. (D) Datas of (C) are presented as means $\pm$ SD from three independent experiments. (F) Immunofluorescence analysis of TRIM9 expression in heart tissue after ischemia-reperfusion (blue fluorescence: nuclei; green fluorescence: TRIM9). **P value < 0.01, *P value < 0.05, ns: no signal.

Figure 2

TRIM9 expression is increased in the MIRI model *in vitro*. (A) H9c2 exposed to OGD (I2h/R2h) and western blotting was used to detect the expression of TRIM9. (B) Datas of (A) are presented as means $\pm$ SD from three independent experiments. (C) Immunofluorescence analysis of TRIM9 expression in the rat myocardium cell H9c2 after I2h/R2h, (blue fluorescence: nuclei; red fluorescence: TRIM9). (D) AC16 exposed to OGD (I2h/R2h) and western blotting was used to detect the expression of TRIM9. (E) Datas of (D) are presented as means $\pm$ SD from three independent experiments. (F) Immunofluorescence analysis of TRIM9 expression in the human myocardium cell AC16 after I2h/R2h, (blue fluorescence: nuclei; red fluorescence: TRIM9). **P value < 0.01, *P value < 0.05, ns: no signal.

Figure 3

Knockdown of TRIM9 can regulate autophagy and autophagic flow. (A) The expression of the autophagy initiation protein LC3II was upregulated after TRIM9 expression was downregulated. (B, C) Datas of (A) are presented as means $\pm$ SD from three independent experiments. (D) P62 expression was also upregulated after TRIM9 expression was downregulated. (E) Datas of (D) are presented as means $\pm$ SD from three independent experiments. (F) mRFP-GFP-LC3 was used to evaluate autophagic flow. After H9c2 cells were transfected with TRIM9 siRNA and mRFP-GFP-LC3, there was more yellow fluorescence than red fluorescence in the merged images. (green fluorescence: autophagosome; red fluorescence: lysosome; yellow fluorescence: autolysosome.) **P value < 0.01, *P value < 0.05, ns: no signal. NC: negative control.

Figure 4

TRIM9 is required for MIRI. (A) LC3II and P62 expression were upregulated after TRIM9 expression was downregulated after ischemia reperfusion. (B, C) Datas of (A) are presented as means $\pm$ SD from three independent experiments. (D) CCK8 tested the cell viability of NC, NC+IR, siTRIM9 and siTRIM9+IR (12 h/R2 h) in H9c2 myocardial cells. **P value < 0.01 , *P value < 0.05 , ns: no signal.

Figure 5

TRIM9 regulates autophagy via ubiquitination of Beclin1. (A) Bubble diagram of the mass spectrometry results, and the autophagy pathway was detected. (B) The result of co-IP found that Beclin1 was detected in the samples pulled down with a TRIM9 antibody but not in the samples pulled down with IgG. (C) Beclin1 expression was increased after TRIM9 levels were reduced. (D) Datas of (C) are presented as means $\pm$ SD from three independent experiments. (E) LC3II and P62 expression were downregulated when Beclin1 knocked down. (F-H) Datas of (E) are presented as means $\pm$ SD from three independent experiments. (I) HA-UB was detected in the samples pulled down with a Beclin1 antibody in both the HA-UB plasmid and TRIM9 siRNA-transfected groups, and downregulation of TRIM9 expression affected the ubiquitination of Beclin1. **P value < 0.01, *P value < 0.05, ns: no signal. NC: negative control.

Figure 6

TRIM9 regulates autophagy through ubiquitin modified Beclin1 to MIR1. Knocked down TRIM9 would up-regulated the expression level of Beclin1, and both LC3II and p62, so that autophagosome and lysosome could not fuse normally, and aggravated the damaged cardiomyocytes by ischemia reperfusion injury.