**Original Article**

**SYVN1/GPX5 axis affects ischemia/reperfusion induced apoptosis of AC16 cells by regulating ROS generation**

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**Abstract:** Ischemia/reperfusion (I/R) induced injury is a major cause of coronary heart disease (CHD). Increased production of reactive oxygen species (ROS) can lead to an I/R injury in CHD, and the ROS level can be regulated by Glutathione peroxidase (GPX) enzyme family. In this study, we investigated the role and underlying molecular mechanism of GPX5 in I/R-induced AC16 cells. We found that the serum level of GPX5 was down-regulated in patients with CHD and I/R-induced AC16 cells. Overexpression of GPX5 inhibited I/R-induced apoptosis by suppressing the production of ROS. On the other hand, knock-down of GPX5 promoted apoptosis in AC16 cells by up-regulating the level of ROS. Furthermore, we found that GPX5 was regulated by synovial apoptosis inhibitor 1 (SYVN1)-mediated ubiquitination in AC16 cells. In I/R-induced AC16 cells, the expression of SYVN1 was up-regulated, and SYVN1 knock-down decreased the ROS levels and apoptotic rate but increased GPX5 levels. Moreover, GPX5 knockdown promoted ROS production and apoptosis, while its effects were attenuated by SYVN1 knockdown. Furthermore, SYVN1 was up-regulated while GPX5 was down-regulated in the myocardial tissue of I/R-injured rats. Taken together, our data demonstrate that GPX5 inhibits I/R-induced apoptosis of AC16 cells by down-regulating ROS level, and its stabilization is regulated by SYVN1-mediated ubiquitination.

**Keywords:** Coronary heart disease, ischemia/reperfusion, GPX5, SYVN1, ROS, apoptosis

**Introduction**

Ischemia/reperfusion (I/R) induced myocardial damage is a serious problem in patients with coronary heart disease (CHD) [1]. Multiple factors, such as the increased expression of reactive oxygen species (ROS), play important roles in the pathogenesis of I/R injury [2, 3]. ROS, for example, can activate cell death signaling pathways: apoptosis and necroptosis [4], leading to the programmed cell death of cardiomyocytes after I/R injury [5, 6]. Inhibition or scavenging of ROS production provides protection against I/R-induced heart injury [7, 8].

Glutathione peroxidases (GPXs) function as a key enzyme family for directly regulating ROS and play a vital role in the antioxidant defense [9]. GPX family has eight members, and these enzymes are expressed in different tissues with different specificity. They protect the cells from oxidative damage by reducing free hydrogen peroxide and lipid hydroperoxides [10]. GPXs have been reported to be involved in the pathogenesis of many diseases, including non-small cell lung cancer (NSCLC), diabetes, aging and atherosclerosis [9, 11, 12].

GPX5, a member of the GPX family, regulated by androgen, is mainly expressed in the epididymis. Recent studies, however, show that GPX5 is also expressed in human bronchial epithelial cells, NSCLC cells, and melanoma cells and tissues [11, 13-16]. GPX5 expression in these cells correlates with the overall survival of patients. Although the role of GPX5 as an antioxidant has been well studied [17], its role in regulating ROS expression in CHD, especially in IR-induced injury, remains unknown.

The ubiquitin-proteasome system (UPS) is an important pathway to control protein quality in cardiovascular physiology and pathology [18]. In the UPS, several E3-ubiquitin ligases are involved in the regulation of I/R injury, such as TRIM6 [19], MuRF1 [20], Nrdp1 [21], and MAFb
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[22]. Given the importance of E3 ubiquitin-protein ligases in I/R injury, we hypothesized that E3 ubiquitin-protein ligases may regulate GPX5 in I/R-induced injury.

In this study, we found that GPX5 level was down-regulated in both CHD patients serum and I/R-induced AC16 cardiomyocytes. Moreover, we demonstrated that GPX5 inhibited I/R-induced myocardial apoptosis by reducing the production of ROS. Furthermore, through computer predication and experimental screening, we identified the Synovial apoptosis inhibitor 1 (SYVN1), an E3 ubiquitin-protein ligase, to be the regulator of GPX5 protein in I/R-induced injury. Overall, this study reveals the important role of SYVN1/GPX5 axis in I/R-induced apoptosis in AC16 cardiomyocytes.

Material and methods

ELISA analysis of serum GPX5 protein

The serum samples were collected from 20 patients with stable CHD and 20 patients with unstable CHD in the Seventh People’s Hospital of Shanghai University of Traditional Chinese Medicine. Serum samples from 20 healthy people were used as negative controls. This study was initiated under the approval by the ethical community of the Seventh People’s Hospital of Shanghai University of Traditional Chinese Medicine. The serum GPX5 protein level was measured by ELISA kit (Bio-swap, Shanghai, China).

Ischemia/reperfusion (I/R) induced AC16 cell model

AC16 cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% Penicillin-Streptomycin and 10 mM glucose. For the in vitro I/R injury model, AC16 cells were cultured in 5% CO₂ and 1% O₂ at 37°C for 6 h, and then cultured in 5% CO₂ and 95% O₂ at 37°C for 12 h.

RT-qpcr

Total RNA was isolated from AC16 cells using Trizol Reagent (Invitrogen, Carlsbad, USA). The isolated RNA was then reverse transcribed into cDNA. The real-time qPCR was performed with the SYBR® Green kit (Thermo Fisher Scientific, Waltham, USA) in ABI 7300 Real-Time PCR System (ABI, Foster City, USA). GAPDH was used as an internal control. The mRNA levels of each sample were evaluated using the ΔΔCT method. The primer sequences are listed below: GPX5-forward: 5’-CCCCAGGAGGAGGATGAGG-3’, reverse: 5’TGGAGGAAAGGCAAACACGAC-3’; GAPDH-forward: 5’-AATCCCATCACCATTTC-3’, reverse: 5’-AGCTTGTGTCATACCTTC-3’; MARCH8-forward: 5’-TTCCCTCTTTTACCTTTGA-TC-3’, reverse: 5’-GACATTAGCTACCACTATTCTAC-3’; SYVN1-forward: 5’-AGCTGGAATCTCTGTGGT-3’, reverse: 5’-AGAGGAAGGCTGAACCTG-3’.

Western blotting

For western blot analysis, whole-cell lysates were prepared. Total proteins were collected by centrifugation and then quantified by the BCA method (Thermo Fisher Scientific, Waltham, USA). To run SDS-PAGE, equal amounts of total proteins were loaded onto 10% SDS gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked in 5% skim milk at 4°C overnight. The membranes were washed and then incubated at room temperature for 2 h with primary antibodies against GPX5 (1:1,000; Santa Cruz Technology, Santa Cruz, CA, USA), SYVN1 (1:1,000; Abcam, Cambridge, MA, USA), MARCH8 (1:2,000; Abcam), STUB1 (1:6,000; Abcam), UBE4A (1:1,000; Abcam), UBE4B (1:1,000; Abcam), Cleaved Caspase3 (1:500; Abcam) or GAPDH (1:2,000; CST, Beverly, MA, USA). After washing, the membranes were probed with 1000-fold diluted secondary antibodies (Beyotime, Shanghai, China). Finally, the proteins were detected by an ECL detection kit (Millipore, Billerica, MA, USA) and visualized in the Tanon-5200 system (Tanon, Shanghai, China).

Lentiviruses generation and cell transduction

The lentiviral vectors used in the study were constructed commercially (Genomeditech, Shanghai, China). For overexpression studies, the target genes were cloned into pLVX-Puro vector (Clontech, Palo Alto, USA). For knockdown experiments, the siRNAs were inserted into the PLKO.1 vector (Addgene, WaterTown, USA). The sequences of siRNAs were listed as follows: siGPX5-1: 5’-GCACATCCTCTTTGCAAC-3’; siGPX5-2: 5’-CTATGAGTCTAAGTGTTG-3’; siGPX5-3: 5’-GCCACCGTAAAGGCTGTCAT-3’; siSYVN1-1: 5’-GCAGCTGAGTGGTCGTTT-3’; siSYVN1-
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To generate lentiviruses, 293T cells were co-transfected with the above lentiviral vectors and packaging plasmids psPAX2 (Addgene, WaterTown, USA) and pMD2G (Addgene, WaterTown, USA). After 8 h transfection, the cell media were replaced with fresh media. After 72 h incubation, the supernatants containing lentiviruses were harvested and then filtered through 0.45 μm filters (Millipore, Billerica, MA, USA).

For cell transduction, 5×10⁵ AC16 cells were seeded into the each well of a 6-well plate. The cells were transduced with lentiviruses at a multiplicity of infection of 10 for 12 h at 37°C. Then these cells were cultured with fresh medium for another 12 h.

Apoptosis analysis

After 48 h of treatment, the cells were collected and washed three times with cold PBS buffer. Then the cells were labeled with Annexin V-FITC and PI using an apoptosis detection kit (Beyotime, Shanghai, China) at room temperature for 20 min. Apoptotic cells were detected by BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, USA).

Reactive oxygen species (ROS) assay

To evaluate the intracellular levels of ROS production, the AC16 cells were stained with 10 μM DCFH-DA probe (Beyotime, Shanghai, China) for 20 min at 37°C. After three washes with serum-free media, the cells were analyzed on BD Accuri™ C6 flow cytometer.

Co-immunoprecipitation and ubiquitination analyses

Cell lysates were prepared in RIPA buffer. For co-immunoprecipitation, equal amounts of total proteins were incubated with respective antibodies at 4°C overnight. Proteins were then immunoprecipitated by Protein A/G-Agarose at 4°C for 2 h. Then the A/G Plus-Agarose beads were washed 3 times by lysis buffer and subsequently boiled with 2× loading buffer for 5 min. After centrifugation at 1000 rpm for 1 min, the precipitated proteins were collected and western blot analysis was carried out. IgG from normal rabbit was used as a negative control. For ubiquitination analysis with GPX5, an anti-GPX5 antibody was used to pull-down the immunocomplex of GPX5. An anti-ubiquitin antibody (1:2,000; Abcam, Cambridge, MA, USA) was used in the subsequent immunoblot analysis.

I/R-induced rat model

Twelve healthy Sprague-Dawley rats (250 ± 20 g) were obtained from The Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine. These rats were randomly divided into two groups: Control and I/R groups. In I/R group, rats received I/R treatment following the workflow described by (Tang et al., 2017). In brief, rat’s coronary artery was clamped with a plastic tube for 60 min, and then the coronary artery was restored by releasing the clamp. 24 h later, rats were sacrificed and cardiac tissue was collected. The animal experiments were approved by the ethical community of The Seventh People’s Hospital of Shanghai University of Traditional Chinese Medicine (license number: 2019-AR-008).

Dihydroethidium staining

Cardiac tissue was fixed in 4% paraformaldehyde for 24 h, and then dehydrated with 15% and 30% sucrose solution. After being embedded in OCT compound in liquid nitrogen, the tissue sample was cut into 8 μm frozen sections. To detect the ROS level, the sections were stained with 10 μM DHE for 30 min at 37°C, and then stained with DAPI for 5 min. Fluorescence intensity was assessed using a fluorescence microscopy (Olympus, Japan).

Tunel assay

Cardiac tissue was fixed in 10% formalin for 48 h, and then dehydrated in graded ethanol. After being embedded in paraffin, the tissue was cut into 4 μm sections. The section was incubated with 50 μl Tunel reaction mixture for 1 h at 37°C, and then incubated with 50 μl POD for 30 min at 37°C. Finally, the section was stained with DAB and counterstained with hematoxylin. The numbers of Tunel-positive cells in five random fields were counted using a microscope.

Immunohistochemistry

Immunohistochemical staining was performed with diaminobenzidine (DAB) as the chromogen for SYVN1 and GPX5. In brief, the sections were
incubated with antibodies against SYVN1 (Abcam) and GPX5 (Santa Cruz) for 30 min, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for another 30 min. After being washed by TBS, the reaction was developed using DAB substrate for 5 min. These sections were counterstained with Mayer’s hematoxylin.

Statistical analysis

All analyses were performed by GraphPad Prism 7.0 software (GraphPad Software, USA). The data were expressed as mean value ± SD. Statistical differences were determined by using the Student’s t test or ANOVA analysis.

Results

The GPX5 level is down-regulated in CHD patients serum and I/R-induced AC16 cells

Although GPX5 as an antioxidant has been well studied [17], the role of GPX5 in I/R damage remains unknown. Here, we compared the serum GPX5 level in patients with CHD and healthy people were measured by ELISA. **P<0.01 and ****P<0.0001 vs control; ####P<0.0001 vs stable_CHD. (B, C) GPX5 mRNA (B) and protein (C) levels were measured in I/R-induced AC16 cells. *P<0.05, **P<0.01, and ***P<0.001 vs 0 h.

GPX5 knock-down promotes apoptosis by up-regulating ROS level in AC16 cells

To further evaluate the effect of GPX5, we knocked down GPX5 expression in AC16 cells by siRNA. As shown in Figure S1, all GPX5 siRNAs markedly reduced the mRNA and protein levels of GPX5, and siGPX5-1 and siGPX5-2 were selected for subsequent experiments. We then investigated the effects of GPX5 knockdown on apoptosis, ROS level, and cleaved caspase3 protein. Our data showed that GPX5 siRNA up-regulated ROS level and apoptotic rate (Figure 3). ROS inhibitor N-Acetyl-L-Cysteine (NAC) was also used. We found that NAC abolished GPX5 siRNA-mediated ROS generation and apoptosis (Figure 3). Taken together, these data suggest that GPX5 knockdown promotes apoptosis by up-regulating the ROS level in AC16 cells.
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GPX5 is regulated by SYVN1-mediated ubiquitination in AC16 cells

To investigate whether ubiquitin-proteasome system regulates GPX5 expression, we identified five potential ubiquitin-protein ligases by searching through the protein ubiquitination database (http://ubibrowser.ncpsb.org/ubibrowser/), including Synovial apoptosis inhibitor 1 (SYVN1), membrane-associated RING-CH 8 ubiquitinates (MARCH8), STIP1 homology and U-Box containing protein 1 (STUB1), ubiquitin conjugation factor E4A (UBE4A), and E4B (UBE4B). Co-immunoprecipitation (Co-IP) assay was performed. We found that only SYVN1 and MARCH8 interacted with GPX5 (Figure 4A). We then overexpressed MARCH8 and SYVN1 in AC16 cells and detected the expression level of GPX5. As shown in Figure 4B, MARCH8 overexpression had no effect on GPX5 mRNA and protein levels. Interestingly, SYVN1 overexpression decreased the protein level of GPX5, but didn’t affect its mRNA level (Figure 4C). Further Co-IP assay confirmed the interaction of GPX5 and SYVN1 (Figure 4D). Moreover, by using protease inhibitor (MG132), we found that SYVN1 overexpression-mediated decrease of GPX5 protein was reversed by MG132 (Figure 4E). Finally, we knocked down SYVN1 in AC16 cells by siRNA and detected the ubiquitination of GPX5. We found that SYVN1 knock-down decreased the level of GPX5 ubiquitination (Figures S2, 4F). Overall, these results indicate that GPX5 is regulated by SYVN1-mediated ubiquitination in AC16 cells.

SYVN1 knock-down decreases ROS level and apoptotic rate, while increases GPX5 protein in I/R-induced AC16 cells

The expression level of SYVN1 in I/R-induced AC16 cells was then investigated. As shown in Figure 5, SYVN1 mRNA and protein levels were decreased the protein level of GPX5, but didn’t affect its mRNA level (Figure 4C). Further Co-IP assay confirmed the interaction of GPX5 and SYVN1 (Figure 4D). Moreover, by using protease inhibitor (MG132), we found that SYVN1 overexpression-mediated decreases of GPX5 protein was reversed by MG132 (Figure 4E). Finally, we knocked down SYVN1 in AC16 cells by siRNA and detected the ubiquitination of GPX5. We found that SYVN1 knock-down decreased the level of GPX5 ubiquitination (Figures S2, 4F). Overall, these results indicate that GPX5 is regulated by SYVN1-mediated ubiquitination in AC16 cells.

SYVN1 knock-down decreases ROS level and apoptotic rate, while increases GPX5 protein in I/R-induced AC16 cells

The expression level of SYVN1 in I/R-induced AC16 cells was then investigated. As shown in Figure 5, SYVN1 mRNA and protein levels were
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Figure 3. GPX5 knock-down promoted apoptosis of AC16 cells by up-regulating the ROS level. A. The ROS level was detected by the DCFH-DA assay. B. The apoptotic cells were detected using a flow cytometer. ****P<0.0001 vs siNC; ####P<0.0001 vs siGPX5+vehicle.

up-regulated in a time-dependent manner. To explore the role of SYVN1 in I/R injury, we knocked down SYVN1 in I/R-induced AC16 cells and measured the ROS level and apoptotic rate. As shown in Figure S2, all SYVN1 siRNAs markedly reduced the mRNA and protein levels of SYVN1. siSYVN1-1 and siSYVN1-2 were selected for subsequent experiments. Figure 6A and 6B showed that SYVN1 knock-down alleviated I/R-induced ROS generation and
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Apoptosis. Moreover, the protein levels of GPX5 and cleaved caspase3 were also measured. We found that GPX5 protein was decreased while cleaved caspase3 was increased in I/R-induced...
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AC16 cells; however, SYVN1 knock-down partly abolished I/R-mediated regulation of these two proteins (Figure 6C).

SYVN1 knockdown attenuated GPX5 knock-down-induced apoptosis and ROS generation

To further investigate if SYVN1 affects apoptosis and ROS level in AC16 cells by regulating GPX5, we co-transduced siSYVN1 and siGPX5 into AC16 cells. Results showed that ROS level, apoptotic rate, and protein level of cleaved-caspase3 in AC16 cells were up-regulated by GPX5 knockdown, while they were down-regulated by SYVN1 knockdown (Figure 7). Moreover, the effects of GPX5 knockdown were attenuated by SYVN1 knockdown (Figure 7).

The expression of SYVN1 and GPX5, apoptotic rate and ROS level in the myocardial tissue of I/R-injured rats

We detected the expression levels of SYVN1 and GPX5, apoptotic rate, and ROS level in the myocardial tissue of I/R-injured rats. The results of immunohistochemistry and western blot showed that I/R-injured rats had increased SYVN1 and decreased GPX5 (Figure 8A, 8B). TUNEL assay showed that I/R-injured rats had up-regulated myocardial apoptosis (Figure 8C). Dihydroethidium staining showed that I/R-injured rats had elevated ROS level (Figure 8D).

SYVN1 knockdown inhibited I/R-induced ROS generation and apoptosis by regulating PI3K/Akt pathway

The activation of PI3K/Akt pathway can markedly attenuate myocardial I/R injury [23]. Therefore, we also investigated whether PI3K/Akt pathway was involved in the regulation of SYVN1 on cardiomyocyte apoptosis. We treated I/R-injured AC16 cells with lentivirus siSYVN1 and PI3K inhibitor LY294002. As shown in Figure S3, the activity of PI3K/Akt pathway was inhibited by I/R treatment. SYVN1 knockdown attenuated I/R-inhibited activation of PI3K/Akt pathway, while its effect was abolished by PI3K inhibitor LY294002 (Figure S3A). Moreover, SYVN1 knockdown attenuated I/R-induced cardiomyocyte apoptosis, while its effect was attenuated by PI3K inhibitor LY294002 (Figure S3B). Taken together, these data suggest that SYVN1 promotes cardiomyocyte apoptosis by inhibiting the activation of PI3K/Akt pathway.

Discussion

In this study, we found that the serum GPX5 level was down-regulated in patients with CHD. Using an in vitro I/R-induced AC16 cardiomyocytes model, we confirmed this phenomenon. Our subsequent experiments demonstrated that GPX5 inhibited apoptosis through down-regulating ROS levels in I/R-induced AC16 cells. Moreover, GPX5 was regulated by the SYVN1-mediated ubiquitination. Thus, SYVN1-mediated ubiquitination of GPX5 promoted I/R-induced apoptosis of AC16 cells by regulating ROS generation.

GPX5 belongs to the GPX family, a key enzyme family that regulates ROS in the antioxidant defense system [9]. Previous studies have shown that GPX5 is androgen-regulated and is specifically expressed in the epididymis. Recent studies, however, show that GPX5 is also expressed in many other cells and tissues, such as human bronchial epithelial cells, NSCLC cells, and melanoma cells [11, 13-16]. Furthermore, GPX5 has been suggested as a diagnostic biomarker for metastatic melanoma [14] and NSCLC [11]. In this study, we found that GPX5 was expressed in cardiomyocytes and could be detected in the serum. Interestingly, we found that GPX5 expression was inversely correlated with the severity of CHD.
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This raises the possibility that GPX5 expression could be used as a potential diagnostic biomarker for early detection of myocardial I/R injury. However, it's possible that the source of
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Figure 7. SYVN1 knockdown attenuated GPX5 knockdown-induced apoptosis and ROS generation. AC16 cells were treated with lentivirus siSYVN1 and siGPX5. A. The ROS level was detected by the DCFH-DA assay. B. The apoptotic cells were detected using a flow cytometer. C. The protein levels of SYVN1, GPX5, and cleaved caspase3 were measured by western blotting. *P<0.05, **P<0.01, and ***P<0.001 vs siNC; ##P<0.01 vs siGPX5.

serum GPX5 is diversified due to its intercellular expression, which will decrease the clinical value of serum GPX5. Further studies are needed to analyze the source of serum GPX5.

The antioxidant function of GPX5 has been well studied [17]; however, the role of GPX5 in regulating ROS production in CHD, especially in IR-induced myocardial injury, remains unknown. To our knowledge, our study is the first to demonstrate the role of GPX5 in myocardial I/R injury. Other members of the GPX family, such as GPX1 and GPX4, are also involved in the I/R injury through a similar mechanism [24-26]. GPX1, for example, protects the heart from ischemic injury by promoting ROS scavenging [25]. However, our results indicated that GPX1 and GPX5 were regulated by different ubiquitination pathways. TRIM33 regulates GPX1 ubiquitination and proteasome-dependent degradation to regulate ROS production in I/R injury [24], whereas we found that SYVN1-mediated ubiquitination regulated GPX5 expression.

**Figure 8.** The expression of SYVN1 and GPX5, apoptotic rate and ROS level in the myocardial tissue of I/R-injured rats. A. SYVN1 and GPX5 were stained with Immunohistochemistry. B. SYVN1 and GPX5 expression was detected by western blotting. C. Apoptotic cardiomyocytes were detected by Tunel assay. D. ROS was detected with Dihydroethidium staining. **P<0.01 and ***P<0.001 vs Control.
Ubiquitination is an important pathway that controls protein quality involved in cardiovascular physiology and pathology [18]. In the ubiquitination system, several E3-ubiquitin ligases are involved in I/R injury, such as TRIM6 [19], MuRF1 [20], Nrdp1 [21], and MAFBx [22]. Here, we found that SYVN1-mediated ubiquitination played a pivotal role in myocardial I/R injury. SYVN1 (Hydroxymethyl glutaryl-coenzyme A reductase degradation protein 1, or Hrd1), is an E3-ubiquitin ligase located in the endoplasmic reticulum (ER) [27, 28]. SYVN1 has been reported to be a critical component in preserving heart function in response to ER stress in cardiomyocytes [29]. But our study is the first to report that SYVN1-mediated ubiquitination of GPX5 plays a pivotal role in I/R injury. Our data show that knock-down of SYVN1 decreased ROS level and apoptotic rate while increased GPX5 protein level in I/R-induced AC16 cells. Although these findings still need to be evaluated in animal models and in clinical trials, targeting SYVN1 and GPX5 may be useful in treating myocardial I/R injury in the future.

It’s worth noting that our data also found that SYVN1 knockdown inhibited I/R-induced cardiomyocyte apoptosis by activating PI3K/Akt pathway. Previous studies have revealed that the activation of PI3K/Akt pathway can remarkably attenuate myocardial I/R injury [23]. Therefore, SYVN1 may also function in myocardial I/R injury by regulating the activation of PI3K/ Akt pathway.

In conclusion, in this study, we found that GPX5 expression is inversely correlated with the severity of CHD and that SYVN1-mediated ubiquitination of GPX5 promotes I/R-induced apoptosis by regulating ROS generation. Our findings not only demonstrate the role of SYVN1/GPX5 in I/R-induced myocardial injury but also provide a potential diagnostic biomarker as well as new insights in the treatment of myocardial I/R injury.

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Disclosure of conflict of interest

None.

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Figure S1. The effects of oeGPX5 and siGPX5 on the expression levels of GPX5 in AC16 cells. GPX5 mRNA (A) and protein (B) levels were measured by RT-qPCR and western blotting, respectively. **P<0.01 and ****P<0.0001 vs vector; ##P<0.01 and ###P<0.001 vs siNC.

Figure S2. The effects of oeSYVN1 and siSYVN1 on the expression levels of SYVN1 in AC16 cells. SYVN1 mRNA (A) and protein (B) levels were measured by RT-qPCR and western blotting, respectively. **P<0.01 and ****P<0.0001 vs vector; ##P<0.01 and ###P<0.001 vs siNC.
Figure S3. SYVN1 knockdown inhibited I/R-induced apoptosis by activating PI3K/akt pathway. I/R-induced AC16 cells were treated with lentivirus siSYVN1 and PI3K inhibitor LY294002. A. Protein levels of PI3K, p-PI3K, AKT, p-AKT were detected by western blotting. B. Apoptotic cells were detected by a flow cytometer. *P<0.05, **P<0.01, and ***P<0.001 vs Control; #P<0.05 and ##P<0.01 vs I/R+siNC; &&P<0.01 vs I/R+siSYVN1.