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The regulatory role of Sigmar1 in CVB3-induced myocarditis

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Abstract. Sigma-1 receptor (Sigmar1) is associated with endoplasmic reticulum (ER) stress. This study investigated whether Sigmar1 affect myocarditis progression *via* Coxsackievirus B3 (CVB3)-caused ER stress. Neonatal mouse cardiomyocytes (NMCs) were transfected with Sigmar1 and/or stress-induced phosphoprotein 1 homology and U-box containing protein 1 (STUB1) overexpression plasmids and exposed to CVB3 for 2 h. E3 ubiquitin ligases binding with Sigmar1 were predicted by Unibrowser. STUB1-Sigmar1 interaction was measured by Co-IP and/or ubiquitination analyses. Lactate dehydrogenase (LDH) release and apoptosis were detected by LDH assay kits and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) assay. Expressions of Sigmar1, STUB1, glucose regulated protein 78 (GRP78), cysteinyl aspartate specific proteinase 12 (Caspase-12), and C/EBP homologous protein (CHOP) were determined *via* quantitative real-time polymerase chain reaction (qRT-PCR)/Western blot. After CVB3 infection, STUB1 level was reduced, while Sigmar1 level was enhanced at 12 h and decreased at 48 h. CVB3 increased apoptosis, LDH release, and expressions of GRP78, Caspase-12 and CHOP of NMCs, which were reversed by Sigmar1 overexpression and promoted by STUB1 overexpression. STUB1 degraded Sigmar1 by ubiquitination. STUB1 overexpression and Sigmar1 overexpression mutually counteracted their effects on CVB3-affected NMCs. In conclusion, STUB1-mediated Sigmar1 degradation promotes ER stress in CVB3-induced *in-vitro* myocarditis.

Key words: Sigmar1 — Endoplasmic reticulum stress — Myocarditis — STUB1 — Ubiquitination

Introduction

Myocarditis is a nonspecific inflammatory disease with viral infection and the consequent immunoreaction as culprits (Kobak et al. 2021). Studies have shown that myocarditis is a serious threat to human health and is one of the major causes of sudden cardiac mortality and heart failure in adolescents (Magnani et al. 2006; Martin-Sanchez et al. 2020). However, delineating the pathological mechanism of myocarditis is necessary to achieve effective treatment modality.

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As an organelle in eukaryotic cells, endoplasmic reticulum (ER) is vital for protein processing and synthesis (Zhang et al. 2017). ER stress indicates the disruption of ER homeostasis and the disturbance of function. Evidence has revealed that Coxsackievirus B3 (CVB3) could stimulate the ER stress of cardiomyocytes, and inhibition of ER stress could relieve the CVB3-induced myocarditis (Zhang et al. 2017; Shi et al. 2021). Nonetheless, the precise mechanism of CVB3 on ER stress of cardiomyocytes is not fully elucidated.

Sigma-1 receptor (Sigmar1) is an intracellular receptor that is expressed in ER as the major molecular chaperone, distributing on mitochondria-associated ER membrane structures, and regulating the mitochondrial metabolism (Robb-Gaspers et al. 1998; Hayashi and Su 2007; Mori et al. 2013; Tagashira et al. 2014; Bernard-Marissal et al. 2015). Sigmar1 is a kind of protein existing in cardiomyocytes

and could regulate ER stress (Bernard-Marissal et al. 2015; Abdullah et al. 2020). Knockdown of Sigmar1 has been proved to disrupt ER-mitochondrial contacts, which is accompanied by activation of ER stress (Hayashi and Su 2007; Bernard-Marissal et al. 2015). Therefore, we speculated that Sigmar1 might reduce ER stress to mitigate the progression of CVB3-induced myocarditis.

Previous studies suggested that ubiquitination is intimately associated with the development of various diseases, including infection, tumors, etc. (Gwon et al. 2021; Tsuchida and Nakayama 2021; Yu et al. 2021). Stress-induced phosphoprotein 1 (STIP1) homology and U-box containing protein 1 (STUB1) are modifying enzymes most likely to affect the ubiquitination of Sigmar1, as predicted by bioinformatics analysis (Unibrowser, http://ubibrowser.ncpsb.org/). STUB1 is a member of the E3 ligase, which is involved in various diseases (Wang Z et al. 2021). Reportedly, STUB1 could mediate the degradation of retinoic acid inducible gene 1 (RIG-1) to relieve antiviral innate immune responses (Zhou et al. 2018). STUB1 also represses the ubiquitination of human epidermal growth factor receptor-2 to regulate the progression of breast cancer cells (Luan et al. 2021). STUB1 could induce ubiquitination and degradation of brain and muscle Arnt-like protein-1 (BMAL1) to inhibit cell senescence (Ullah et al. 2020). Thus, we surmised that STUB1 might regulate the Sigmar1 ubiquitination to regulation the progression of myocarditis. However, the role of STUB1-affected Sigmar1 in myocarditis still requires further illumination.

Our study aimed to explore the mechanism of Sigma-1 regulating the CVB-induced myocarditis *via* ER stress. Besides, the present study also investigated whether STUB1 could activate ER stress to aggravate CVB3-induced myocarditis *via* Sigmar1 ubiquitination, which provided a new direction to the treatment of CVB3 virus-induced myocarditis.

Materials and Methods

Ethics statement

All animal experiments in our study were approved by the ethics committee of Zhejiang Baiyue Biotech Co., Ltd for experimental animals' welfare (No. ZJBYLA-IACUC-20240205) and carried out according to the guidelines of the China Council on Animal Care and Use.

Neonatal mouse cardiomyocytes (NMCs) extraction and culture

Balb/c mice (30 males and 30 females) purchased from Cyagen (Suzhou, China) were housed at 20–26°C in 60% humidity, and fed on a 12-h light-dark cycle. Then the mice were mated and the neonatal mice were obtained. To gain the NMCs, the 1/2-day-old neonatal mice were anaesthetized using 0.3% pentobarbital sodium (50 mg/kg, P3761, Haoran Biological Technology, Shanghai, China) (Wang et al. 2019), and sacrificed by cervical dislocation. After that, the hearts were cut into small pieces and cultured in the Dulbecco's Modified Eagle Medium (DMEM, 11995, Solarbio, Beijing, China) with 0.0125% Trypsin-EDTA solution (T1300, Solarbio) and 20 mM 2,3-butanedione 2-monoxime (BDM, 57-71-6, Merck, Darmstadt, Germany) overnight at 4°C. Then 1.5 mg/ml dissolved Collagenase II (C8150, Solarbio) and a medium containing 20 mM BDM were added into the DMEM, followed by 20-min incubation at 37°C and 5-min centrifugation at 100×*g*. The collected cells were resuspended at plating medium and cultured at 37°C with 5% CO_2 and 21% O_2. After dilution, the supernatant was transferred into a cell culture dish that had been pre-coated by 1% gelatin (G8061, Solarbio). 1 day later, the cells were incubated in maintenance medium for 24 h, and stored in DMEM with 10% fetal bovine serum (FBS, S9030, Solarbio) at 37°C with 5% CO_2 in a humidified chamber (Wu et al. 2020).

Cell transfection

The sequences of Sigmar1 and STUB1 were inserted into the pcDNA3.1 vectors (BR039, Fenghui Biotechnology, Changsha, China) to obtain overexpression plasmids of Sigmar1 and STUB1, with pcDNA3.1 (empty vector) as negative control (NC). Besides, the small interfering RNA (siRNA) of STUB1 (forward, 5'-UCUUCUUCGCGAUUCGAAGAG-3'; reverse, 5'-CUUCGAAUCGCGAAGAAGAAG-3') and siRNA negative control (siNC) were provided by GenePharma (Shanghai, China). When cell confluence reached 80% in a 96-well plate, transfection was performed. Lipofectamine™ 3000 reagent was diluted by Opti-MEM™ medium (31985062, Thermo Fisher Scientific). Then siRNA and plasmid vectors were diluted in Opti-MEM™ medium, followed by adding into each tube that contained diluted Lipofectamine™ 3000 reagent. After that, 10-min incubation at room temperature was carried out. Finally, siRNA-lipid complexes or plasmid vectors-lipid complexes were cultivated with CVB3-induced NMCs for 48 h at 37°C.

CVB3 infection

For CVB3 infection, NMCs (1×10^5 cells/well) were inoculated in 6-well plates for 24-h incubation, and then exposed to CVB3 virus (multiplicity of infection of 10, VR-30, American type culture collection, Gaithersburg, MD, USA) for 2 h at 37°C. The infected NMCs were observed by the

IXplore Pro inverted microscope (Olympus, Tokyo, Japan, 100×magnification) at 0 h, 12 h and 24 h (Wu et al. 2020).

Bioinformatics analysis

The E3 ubiquitinases that could bind with Sigmar1 were predicted by Unibrowser (http://ubibrowser.ncpsb.org/).

Co-immunoprecipitation (Co-IP) and ubiquitination analyses

Initially, NMCs (1×10^5 cells/ ml) transfected with/without STUB1 overexpression plasmids received treatment with/without 10 μM MG132 (the inhibitor of proteasome, IM0310, Solarbio) that was dissolved in DMSO (D8371, Solarbio). Then, the Co-IP kit (abs955, Absin, Shanghai, China) was employed to perform the Co-IP. Briefly, 500 μl cell lysate was incubated with 5 μg anti-Sigmar1 (15168-1-AP, Thermo Fisher Scientific)/control IgG (ab205719, Abcam, Cambridge, UK) for 1 h at 4°C, and with 5 μl protein G and 5 μl protein A for 3 h, followed by 1-min centrifugation at 12000×*g*. The protein complexes underwent elution in 0.5 ml wash buffer, followed by Western blot assay. For ubiquitination analysis of Sigmar1 in siSTUB1-transfected NMCs, the immune-complex of Sigmar1 was pulled down by the anti-Sigmar1 (PA5-30372, Thermo Fisher Scientific) and anti-STUB1 (PA5-29024, Thermo Fisher Scientific) antibodies. Anti-ubiquitin (ab140601, Abcam) was performed in Western blot to detect the Sigmar1 ubiquitination.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

After transfection and CVB3 infection, total RNA of NMCs extracted using RNeasy mini kit (74104, Qiagen, Hilden, Germany) was synthesized into complementary DNA (cDNA) with PrimeScript™ RT reagent kit (RR037A, TaKaRa, Beijing, China). QRT-PCR was carried out on an ABI 7500 System (ABI 7500, Thermo Fisher Scientific) using 2×SYBR Green qPCR Master Mix (B21203, Bimake, TX, USA), and amplified for 40 cycles at 95°C (15 s), 55°C (30 s) and 72°C (30 s). Sigmar1 and STUB1 levels were normalized by the internal loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the STUB1 and Sigmar1 expressions were estimated by $2^{-\Delta\Delta Ct}$ method (Zhang J et al. 2021). The sequences of primers (Genechem, Shanghai, China) were shown below. STUB1: forward, 5'-CAGGGCAATCGTCTGTTCGT-3'; reverse, 5'-CCGGGTCTAGGGACCAAGG-3'. Sigmar1: forward, 5'-CCATTCGGGACGATACTGGG-3'; reverse, 5'-CTGGGTGCTGGGTAGAAGAC-3'. GAPDH: forward, 5'-AATGGGCAGCCGTTAGGAAA-3'; reverse, 5'-GCCCAATACGACCAAATCAGAG-3'.

Lactate dehydrogenase (LDH) assay

LDH assay kit (C0016, Beyotime, Shanghai, China) was employed to determine the release of LDH and assess the degree of cardiomyocytes injury. Concretely, after transfection, 100 μl supernatant of CVB3-induced NMCs (1×10^4 cells/well) was added to a 96-well plate, and reacted with 60 μl LDH Reaction Mixtures for 30 min in the dark at 25°C. The absorbance at 490 nm was detected *via* Infinite M200 microplate reader (30190085, Männedorf, Switzerland).

TdT-mediated dUTP nick end labeling (TUNEL) assay

TUNEL apoptosis assay kit (11684795910, Wolcavi, Beijing, China) was exploited to detect the apoptosis. Firstly, cells (3×10^4/well) were maintained on the gelatin-coated slides (SLD01-BX, SouthernBiotech, Birmingham, AL, USA), fixed by 4% paraformaldehyde (P1110, Solarbio) for 1 h, and permeabilized with 0.1% Triton X-100 (P1080, Solarbio) for 10 min on ice. Thereafter, cells were immersed in TUNEL reaction solution (50 μl) at 37°C for 1 h in the dark, and sealed by one drop of anti-fade mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (P0131, Beyotime). A fluorescence microscope (BX63, Olympus, Japan, 200×magnification) was utilized to observe the cells (Wu et al. 2020).

Western blot analysis

Total protein of NMCs (4×10^5 cells/ml) was extracted by phosphatase inhibitors contained RIPA Lysis Buffer (V900854, Sigma-Aldrich, MO, USA), and underwent concentration determination by BCA kit (ZJ101, Epizyme, Shanghai, China). The proteins of lysates were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (P0670, Beyotime), and transferred to the Polyvinylidene Fluoride (PVDF) membranes (IPVH00010, Millipore, MA, USA). Then, the membranes were blocked with 5% non-fat milk (36120ES60, Yeasen, Shanghai, China) dissolved in Tris Buffered Saline with Tween-20 (TBST, T1085, Solarbio) for 2 h at 25°C. After that, each membrane was probed with primary antibodies (Abcam) for 24 h at 4°C: Glucose-regulated protein 78 (GRP78) antibody (1:1000, ab108615, 72 kDa), C/EBP-homologous protein (CHOP) antibody (5 μg/ml, ab11419, 31 kDa), Caspase-12 (0.5 μg/ml, ab235180, 38 kDa), Sigmar1 (1:1000, ab307548, 25 kDa) and GAPDH (1:1000, ab8245, 36 kDa), and with horseradish peroxidase (HRP)-labeled secondary antibodies mouse anti-rabbit IgG (1:1000, bs-0295M-HRP, Bioss, Beijing, China) and rabbit anti-mouse IgG (1:2000, ab6728, Abcam) for 2 h at 25°C. ECL reagent kit (FP300, ABP Biosciences, Rockville, MD, USA) was employed to detect protein blots on the Tanon 5200 imaging system (Shanghai, China), followed by quantitative analysis using ImageJ software (1.52s version, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data were analyzed by GraphPad Prism 8.0 statistical software (GraphPad Software Inc., San Diego, CA, USA). Mean ± standard deviation was applied to express measurement data. Two-group comparisons (independent *t*-tests) and multi-group comparisons (one-way ANOVA with Tukey's *post hoc* test) were carried out. Normality was tested using the Kolmogorov-Smirnov test. Statistical significance was deemed as $p < 0.05$.

Results

CVB3 changed the morphology of NMCs and Sigmar1 expression in the NMCs

After 2-h CVB3 infection, we observed the morphological changes of NMCs at 0 h, 12 h, and 24 h. As shown in Figure 1A, the morphology and the contraction frequency of NMCs were changed a little at 12 h after CVB3 infection. At 24 h after the infection, the NMCs became disengaged obviously, implying that CVB3 could induce the morphological changes of NMCs. The qRT-PCR results reflected that the Sigmar1 level in NMCs was promoted at 12 h after CVB3 infection (Fig. 1B; $p < 0.001$), but was inhibited at 48 h after CVB3 infection (Fig. 1B; $p < 0.001$).

Sigmar1 overexpression alleviated the CVB3-induced LDH release of NMCs

The transfection efficiency and level of Sigmar1 were assessed by qRT-PCR and Western blot. It was shown that after transfection with Sigmar1 overexpression plasmids, Sigmar1 expression was increased in the NMCs (Fig. 1C,D; $p < 0.01$), indicating successful transfection. LDH detection kit was adopted to test the LDH release of the CVB3-induced NMCs. According to Figure 2A, at 24 h after CVB3 infection, LDH release was enhanced remarkably in NMCs ($p < 0.001$), but then obviously reduced by the interference of Sigmar1 overexpression ($p < 0.001$), hinting a protective role of Sigmar1 overexpression in CVB3-induced NMCs.

Sigmar1 overexpression reversed CVB3-induced apoptosis of NMCs

TUNEL assay was conducted to measure the apoptosis of NMCs transfected with Sigmar1 overexpression plasmids

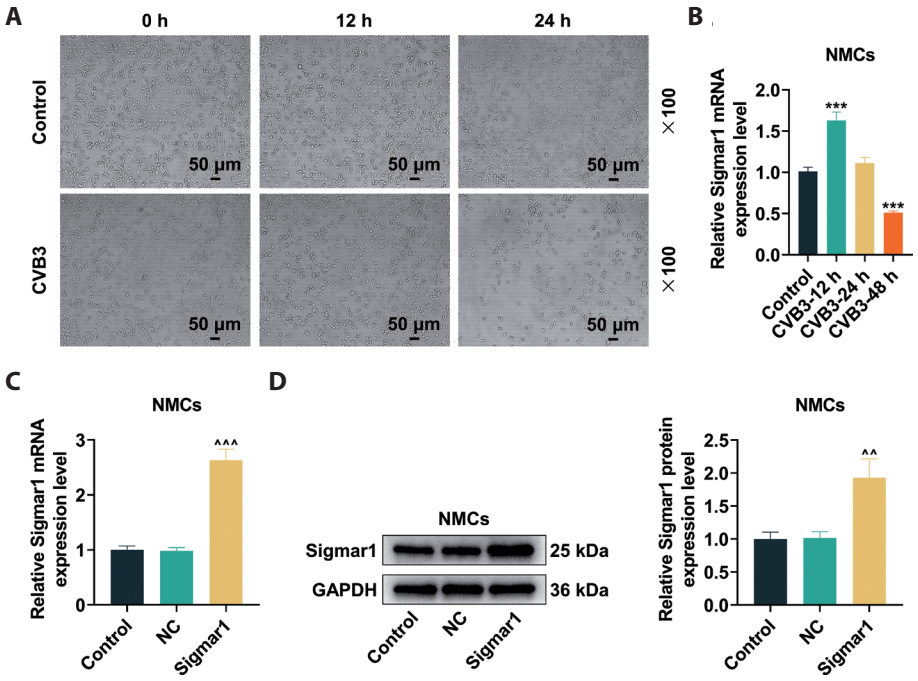

Figure 1. CVB3 changed the morphology of NMCs and Sigmar1 expression in the NMCs. **A.** After NMCs were infected by CVB3 for 2 h, the morphological changes of NMCs were observed under the IXplore Pro inverted microscope at 0 h, 12 h, and 24 h (scale: 50 μm; magnification: ×100). **B.** After 2-h CVB3 infection, the expression of Sigmar1 was measured by qRT-PCR at 12 h, 24 h, and 48 h. GAPDH was used as a loading control. After the transfection of Sigmar1 overexpression plasmids, Sigmar1 expression was detected by qRT-PCR (**C**) and Western blot (**D**). GAPDH was used as a loading control. *** $p < 0.001$ *vs.* Control; ^^ $p < 0.01$ *vs.* NC; ^^^ $p < 0.001$ *vs.* NC ($n = 3$ in each group). NC, negative control for Sigmar1 overexpression plasmid; CVB3, Coxsackievirus B3; NMCs, neonatal mouse cardiomyocytes; Sigmar1, Sigma-1 receptor; qRT-PCR, quantitative reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

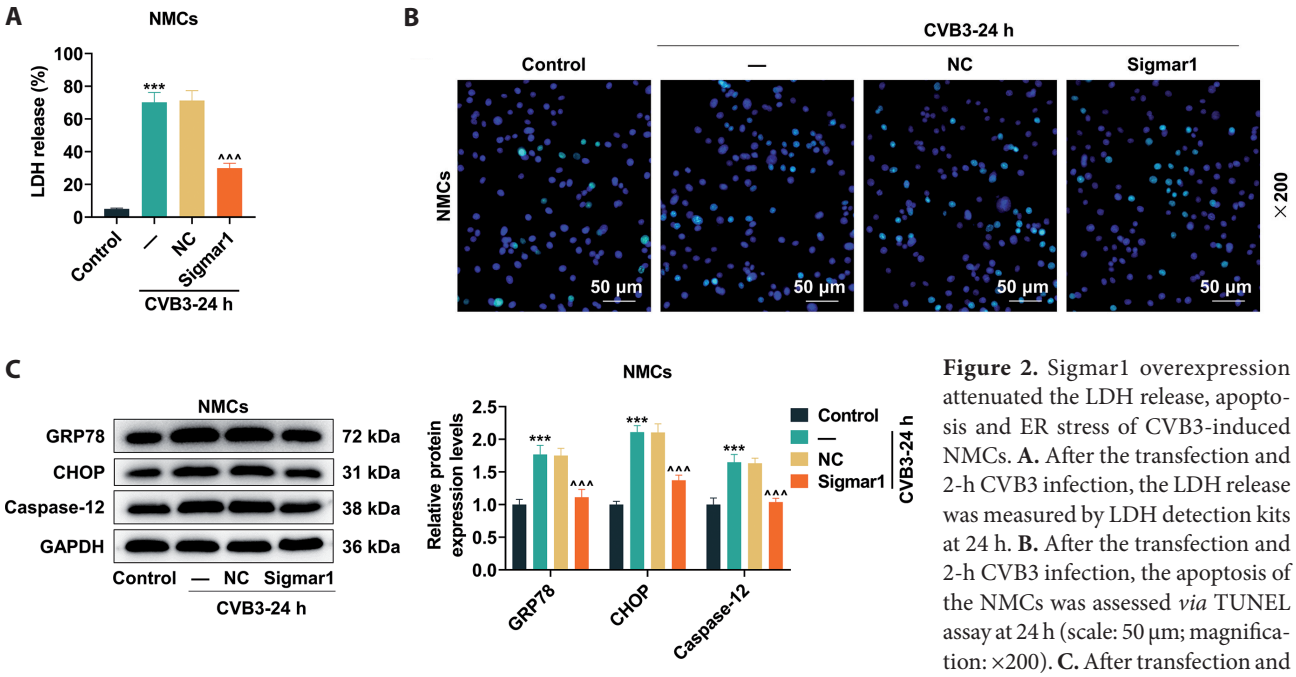

Figure 2. Sigmar1 overexpression attenuated the LDH release, apoptosis and ER stress of CVB3-induced NMCs. **A.** After the transfection and 2-h CVB3 infection, the LDH release was measured by LDH detection kits at 24 h. **B.** After the transfection and 2-h CVB3 infection, the apoptosis of the NMCs was assessed *via* TUNEL assay at 24 h (scale: 50 μm; magnification: ×200). **C.** After transfection and 2-h CVB3 infection, GRP78, CHOP and Caspase-12 levels were tested by Western blot at 24 h. GAPDH was used as a loading control. *** $p < 0.001$ *vs.* Control; ^^^ $p < 0.001$ *vs.* NC ($n = 3$ in each group). ER, endoplasmic reticulum; TUNEL, TdT-mediated dUTP nick end labeling; GRP78, glucose-regulated protein 78; CHOP, C/EBP-homologous protein. (For more abbreviations, see Fig. 1).

after CVB3 infection. Figure 2B revealed that the number of apoptotic NMCs was augmented at 24 h after CVB3 infection. Compared to transfection with NC, transfection with Sigmar1 overexpression plasmids resulted in suppressed apoptosis of CVB3-induced NMCs, denoting that Sigmar1 overexpression could reverse CVB3-induced apoptosis of NMCs.

Sigmar1 overexpression weakened the effects of CVB3 on ER stress in NMCs

The results displayed that GRP78, CHOP, and Caspase-12 levels were up-regulated in NMCs at 24 h after CVB3 infection (Fig. 2C; $p < 0.001$), confirming an active effect of CVB3 on ER stress in NMCs. Following Sigmar1 overexpression, levels of the three genes were decreased in CVB3-induced NMCs ($p < 0.001$), manifesting that Sigmar1 overexpression abrogated CVB3-induced ER stress in NMCs.

STUB1 could mediate degradation of Sigmar1 by ubiquitination

The E3 ubiquitin ligases that could bind with Sigmar1 were analyzed by Unibrowser (Fig. 3A). STUB1 was predicted as one of the most likely modifying enzymes to affect the ubiquitination of Sigmar1. As shown in Figure 3B, the Co-IP results showed that after the NMCs were treated with anti-Sigmar1 antibody, Sigmar1 and STUB1 protein bands were observed, confirming that Sigmar1 could interact with STUB1. Interestingly, after transfection with STUB1 overexpression plasmids (Fig. 3B), the Sigmar1 protein level of NMCs was obviously decreased, while such decrease was reversed by MG132 treatment, proving that STUB1 regulated the degradation of Sigmar1. The transfection efficiency and level of STUB1 were assessed by Western blot. It was shown that after transfection with siSTUB1, STUB1 expression was decreased in the NMCs (Fig. 3C; $p < 0.001$). The ubiquitination of Sigmar1 was assessed by Co-IP and ubiquitination analyses. In line with Figure 3D, the level of Sigmar1 ubiquitination was obviously reduced after the NMCs transfected with siSTUB1 were treated with anti-Sigmar1 antibody ($p < 0.01$). Besides, STUB1 protein expression level was decreased (Fig. 3D; $p < 0.001$), while Sigmar1 protein expression level was increased ($p < 0.05$) in NMCs transfected with siSTUB1 and treated with anti-Sigmar1 antibody. Thus, it was concluded that STUB1 could mediate degradation of Sigmar1 by ubiquitination.

CVB3 down-regulated STUB1 expression in NMCs

The qRT-PCR was conducted to measure STUB1 expression in NMCs at 12 h, 24 h, and 48 h after the infection of CVB3. Besides, we observed that the STUB1 level was reduced after CVB infection (Fig. 4A; $p < 0.001$), which may be due to the hysteresis of STUB1 protein expression.

STUB1 overexpression decreased Sigmar1 expression, and could counteract each other's effects on CVB-induced LDH release of NMCs with Sigmar1 overexpression

After the NMCs were transfected with STUB1 overexpression plasmids, the results of qRT-PCR and Western blot unraveled that STUB1 level was obviously enhanced (Fig. 4B,C; $p < 0.001$), and Sigmar1 level was decreased (Fig. 4B,C; $p < 0.001$). In CVB3-induced NMCs transfected with STUB1 and/or Sigmar1 overexpression plasmids, LDH release was measured by LDH detection kits. As displayed in Figure 5A, LDH level was elevated in CVB3-induced NMCs owing to transfection of STUB1 overexpression plasmids ($p < 0.01$), but was then lowered after the additional transfection of Sigmar1 overexpression plasmids ($p < 0.001$). Besides, in Figure 5A, the LDH release level was lower in CVB3-induced NMCs transfected with Sigmar1 overexpression plasmid than in the infected cells transfected with NC ($p < 0.001$); however, the lowered level was reversed by transfection with STUB1 overexpression plasmids ($p < 0.001$). These outcomes demonstrated that STUB1 and Sigmar1 could mutually reverse their effects on the LDH release of CVB3-induced NMCs.

STUB1 and Sigmar1 overexpression could mutually reverse their effects on apoptosis of CVB3-induced NMCs

The TUNEL results in Figure 5B unveiled that the apoptosis of CVB3-induced NMCs was promoted after transfection of

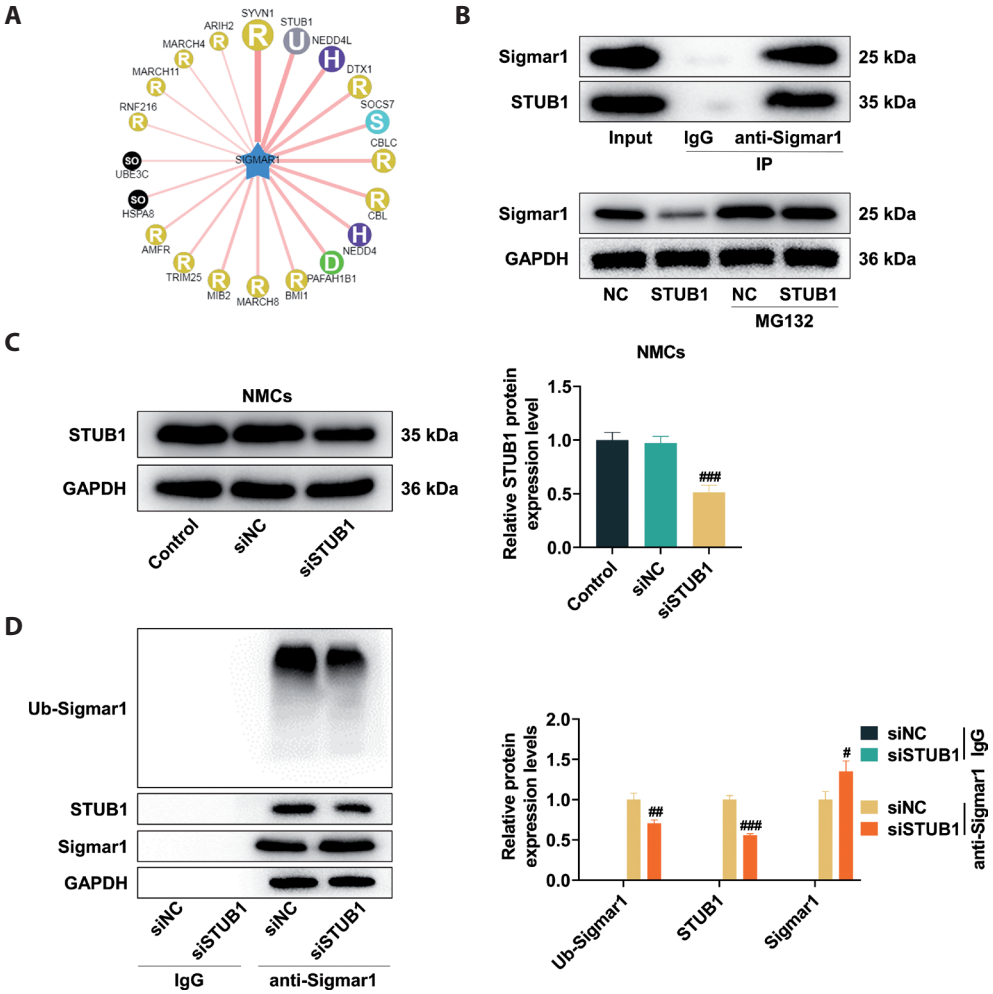

Figure 3. STUB 1 mediates the degradation of Sigmar1 through ubiquitination. **A.** E3 ubiquitin ligases that bound with Sigmar1 were predicted by Unibrowser. **B.** The interaction of Sigmar1 and STUB1 was assessed by Co-IP. **C.** After the transfection of siSTUB1, STUB1 expression was detected by Western blot. GAPDH was used as a loading control. **D.** Then the Co-IP and ubiquitination analyses were adopted to detect the effects of STUB1 on the ubiquitination of Sigmar1. GAPDH was used as a loading control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* siNC ($n = 3$ in each group). si-NC, negative control for si-STUB1; IgG, immunoglobulin G; Ub, ubiquitin; Co-IP, co-immunoprecipitation; STUB1, STIP1 homology and U-box containing protein 1. (For more abbreviations, see Fig. 1).

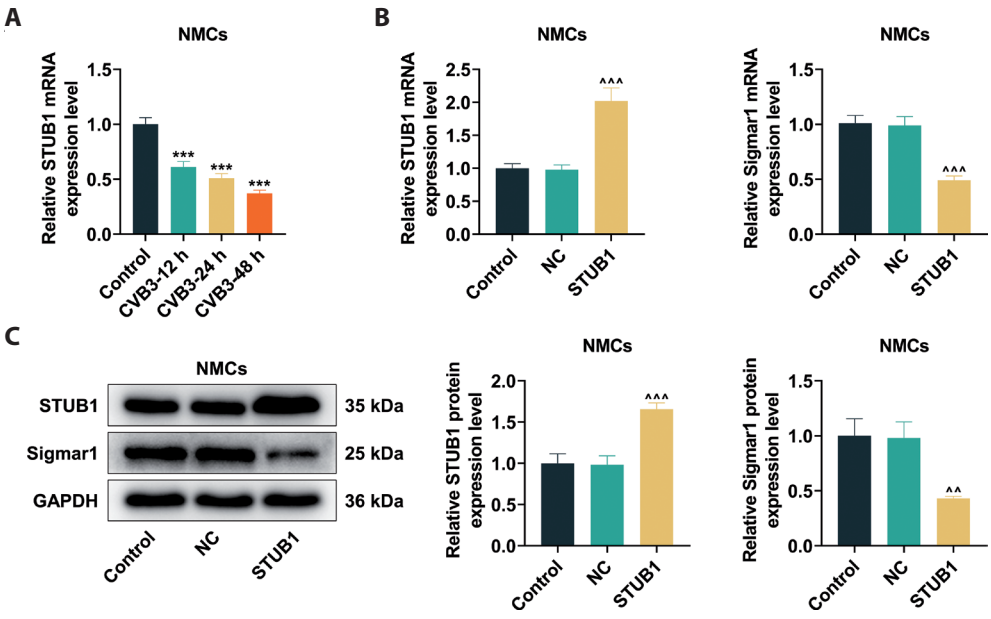

Figure 4. STUB1 overexpression inhibited the expression of Sigmar1. **A.** After 2-h CVB3 infection, the expression of STUB1 was measured by qRT-PCR at 12 h, 24 h, and 48 h. After NMCs were transfected with STUB1 and/or Sigmar1 overexpression plasmids, the expressions of STUB1 and Sigmar1 were detected by qRT-PCR (**B**) and Western blot (**C**). GAPDH was used as a loading control. *** $p < 0.001$ *vs.* Control; $^{\wedge\wedge}$ $p < 0.01$, $^{\wedge\wedge\wedge}$ $p < 0.001$ *vs.* NC ($n = 3$ in each group). si-NC, negative control for si-STUB1; STUB1, STIP1 homology and U-box containing protein 1. (For more abbreviations, see Fig. 1).

STUB1 overexpression plasmids yet dampened following transfection of Sigmar1 overexpression plasmids. Besides, the apoptosis was stronger in the infected NMCs transfected with Sigmar1 and STUB1 overexpression plasmids than in the cells transfected with Sigmar1 overexpression plasmids alone. Therefore, it was predicted that STUB1 and Sigmar1 could mutually reverse their effects on the apoptosis of NMCs.

STUB1 and Sigmar1 overexpression could mutually offset their effects on ER stress of CVB3-induced NMCs

It was shown that the GRP78, CHOP, and Caspase-12 levels were higher in the infected NMCs transfected with STUB1 overexpression plasmids than in the cells transfected with NC (Fig. 5C; $p < 0.05$). GRP78, CHOP, and Caspase-12 expressions were lower in the infected NMCs transfected with Sigmar1 and STUB1 overexpression plasmids than in the cells transfected with STUB1 overexpression plasmids alone (Fig. 5C; $p < 0.01$). Besides, compared with those in the infected NMCs transfected with Sigmar1 overexpression plasmids, the levels of the three genes were increased in the infected NMCs transfected with Sigmar1 and STUB1 overexpression plasmids ($p < 0.05$). Collectively, Sigmar1 and STUB1 could mutually reverse their effects on ER stress in CVB3-induced NMCs.

Discussion

Currently, the role of Sigmar1 in the progression of myocarditis still remains unknown. However, previous studies have demonstrated the regulatory effect of Sigmar1 on cardiomyopathy mice, and that Sigmar1 can protect cardiomyocytes from maladaptive ER stress (Alam et al. 2017; Abdullah et al. 2020). Of note, CVB3-induced myocarditis involves ER stress (Zha et al. 2015), activation of mitochondrial ROS (Liu et al. 2022), cell pyroptosis (Wang et al. 2024), cell apoptosis (Sun et al. 2023), etc. Hence, this study explored the mechanism of Sigmar1 regulating CVB-induced myocarditis *via* ER stress. Virus infections, including CVB3, have been identified as the primary contributor of myocarditis (Lasrado et al. 2021; Nie et al. 2022). Herein, NMCs from neonatal mice were exposed to 2-h CVB3 infection to establish myocarditis models *in vitro*, where Sigmar1 expression was elevated in CVB3-induced NMCs at 12 h post-infection and inhibited at 48 h after the infection. The initial up-regulation of Sigmar1 may be a response to the virus, while the subsequent down-regulation reflected cell damage and death from persistent infection. Therefore, we predicted that Sigmar1 could regulate the progression of myocarditis.

Then we further investigated the underlying mechanism of Sigmar1 on CVB3-induced NMCs. After NMCs were transfected with Sigmar1 overexpression plasmids and infected by

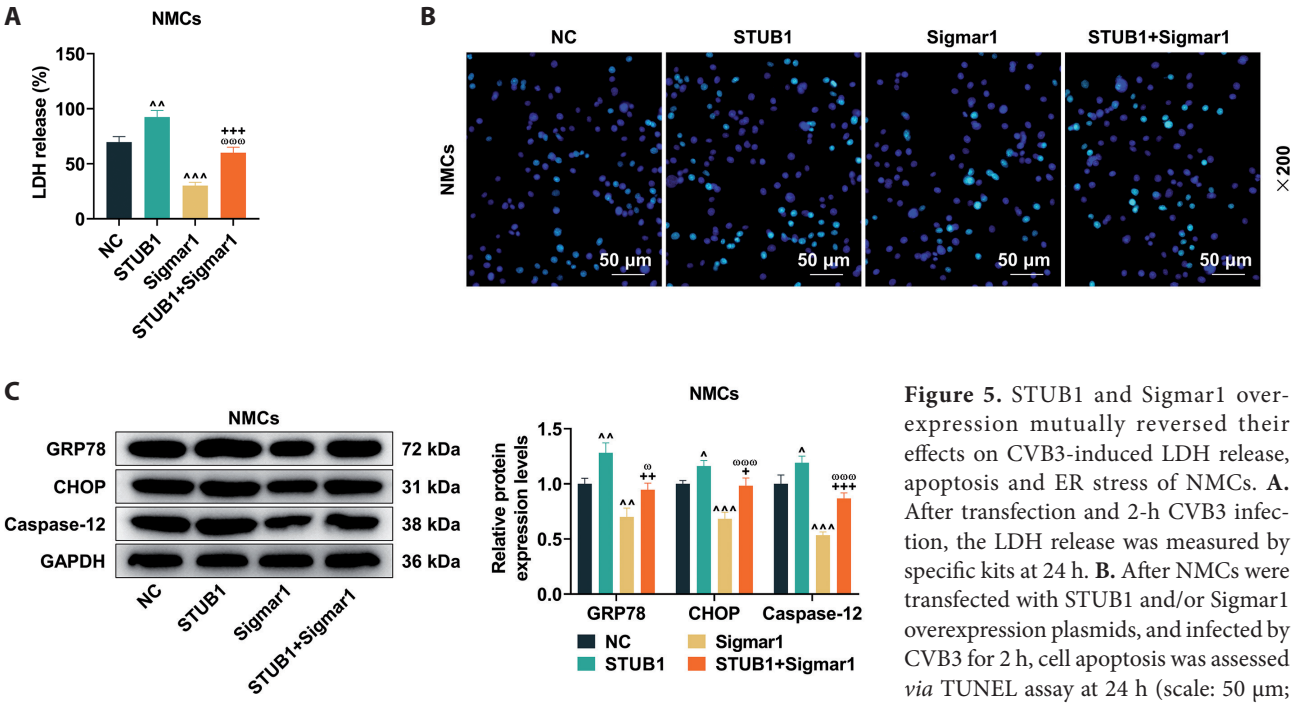

Figure 5. STUB1 and Sigmar1 overexpression mutually reversed their effects on CVB3-induced LDH release, apoptosis and ER stress of NMCs. **A.** After transfection and 2-h CVB3 infection, the LDH release was measured by specific kits at 24 h. **B.** After NMCs were transfected with STUB1 and/or Sigmar1 overexpression plasmids, and infected by CVB3 for 2 h, cell apoptosis was assessed *via* TUNEL assay at 24 h (scale: 50 μm; magnification: ×200). **C.** After transfection and 2-h CVB3 infection, the levels of GRP78, CHOP and Caspase-12 were measured *via* Western blot at 24 h. GAPDH was used as a loading control. $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$ *vs.* STUB1; $^{\wedge}$ $p < 0.05$, $^{\wedge\wedge}$ $p < 0.01$, $^{\wedge\wedge\wedge}$ $p < 0.001$ *vs.* NC; $^{\omega}$ $p < 0.05$, $^{\omega\omega\omega}$ $p < 0.001$ *vs.* Sigmar1 ($n = 3$ in each group). LDH, lactate dehydrogenase; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; CHOP, C/EBP-homologous protein; TUNEL, TdT-mediated dUTP nick end labeling; STUB1, STIP1 homology and U-box containing protein 1. (For more abbreviations, see Fig. 1).

CVB3, we assessed cell damage and apoptosis, and confirmed that Sigmar1 overexpression could relieve the damage caused by CVB3 virus. It has been evidenced that CVB3 stimulates ER stress of cardiomyocytes to promote the progression of CVB3-induced myocarditis (Zhang et al. 2017). Accordingly, we detected the expressions of ER stress-associated proteins (GRP78, CHOP, and Caspase-12) and proved the expressions of these proteins were reduced by Sigmar1 overexpression. Consistently, previous findings revealed that GRP78 level is up-regulated by ER stressors (Ibrahim et al. 2019), and Cleaved Caspase 12 and CHOP are considered as ER stress pathway-related proteins (Liu et al. 2015; Suzuki et al. 2017). Combined with previous studies, we concluded that CVB3-stimulated ER stress was suppressed by Sigmar1 overexpression in NMCs, manifesting that Sigmar1 can effectively alleviate CVB3-induced myocarditis *via* reducing ER stress.

Then we further probed the mechanism of Sigmar1 on the CVB3-induced myocarditis. Unibrowser predicted that STUB1 was one of the most likely modifying enzymes to affect the ubiquitination of Sigmar1. According to a previous study, STUB1 could promote the replication of bovine epidemic fever virus (BEFV) *via* degrading mitochondrial antiviral signaling protein (Zhang Y et al. 2021). STUB1 also can mediate the degradation of RIG-1 to relieve antiviral innate immune responses (Zhou et al. 2018). Moreover, STUB1 is associated with the inhibition of the tumor IFNγ signaling by destabilizing relative receptor complexes (Apriamashvili et al. 2022). Thus, we conjectured that STUB1 could aggravate myocarditis *via* regulating Sigmar1 expression. Then, we tested the interaction between STUB1 and Sigmar1, and the effects of STUB1 on the ubiquitination of Sigmar1. Our experimental results showed that STUB1 can interact with Sigmar1, and that STUB1 can mediate the ubiquitination of Sigmar1.

In order to further elucidate whether STUB1 impacts CVB3-induced NMCs *via* mediating the ubiquitination of Sigmar1, we detected the expression of STUB1 in CVB3-induced NMCs, and found that due to the hysteresis of STUB1 expressions, the STUB1 level was obviously down-regulated. Previous studies have shown that STUB1 expression is indeed dynamically correlated with the time after viral infection (Shi et al. 2022), indicating that the direct effect of STUB1 protein synthesis and degradation on its expression cannot be ignored. However, host-virus interactions cannot be ruled out without further validation, which is also one of the future research directions. Accordingly,

STUB1 overexpression plasmid was transfected into NMCs in the study. By assessing the LDH release, apoptosis and ER stress of the CVB3-infected NMCs, we corroborated that Sigmar1 overexpression and STUB1 overexpression could mutually counteract their effects on CVB3-induced NMCs. In particular, STUB1 overexpression stimulated the ER stress, which was reversed by Sigmar1 overexpression. Evidence has proved that CVB3-induced ER stress in NMCs and CVB-induced myocarditis could be relieved by suppressing the ER stress (Shi et al. 2021; Wang Y et al. 2021). Therefore, we could conclude that STUB1 stimulated ER stress *via* degrading Sigmar1. Of note, some limitations exist in this study. Firstly, the effect of Sigmar1 on NMCs has only been verified at the cellular level and through short-term observations, which may not fully replicate the complex *in vivo* environment, necessitating animal experiments and long-term experiments. Secondly, this study concentrated on specific pathways without exploration on other relevant pathways that could influence the outcomes, such as oxidative stress, apoptosis, or inflammatory signaling. More extensive approaches are needed to confirm this study more fully in the future. Thirdly, there is no study regarding differentiation between cardiomyocyte subtypes, which could respond differently to CVB3 infection. Finally, small sample sizes may restrict the generalizability of the results in a wider population, and disease models with larger sample sizes are needed to make future studies more credible.

In summary, our study has demonstrated that Sigmar1 overexpression alleviates the ER stress in CVB3-induced NMCs. Additionally, STUB1 could mediate Sigmar1 ubiquitination, and STUB1 and Sigmar1 overexpression could mutually counteract their effects on CVB3-induced NMCs. This is the first study evidencing that the STUB1-Sigmar1 pathway is critical in the development of targeted therapies against myocarditis, and that regulating this pathway can alleviate ER stress and thus enhance cardiomyocyte protection or reduce inflammation. This study confirms the potential of STUB1 and Sigmar1 as therapeutic biomarkers of CVB3 infection-induced myocarditis.

Conflict of interest. The authors declare no conflicts of interest.

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