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Valosin-containing protein acts as a target and mediator of S-nitrosylation in the heart through distinct mechanisms

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ABSTRACT

S-nitrosylation (SNO) is an emerging paradigm of redox signaling protecting cells against oxidative stress in the heart. Our previous studies demonstrated that valosin-containing protein (VCP), an ATPase-associated protein, is a vital mediator protecting the heart against cardiac stress and ischemic injury. However, the molecular regulations conferred by VCP in the heart are not fully understood. In this study, we explored the potential role of VCP in cardiac protein SNO using multiple cardiac-specific genetically modified mouse models and various analytical techniques including biotin switch assay, liquid chromatography, mass spectrometry, and western blotting. Our results showed that cardiac-specific overexpression of VCP led to an overall increase in the levels of SNO-modified cardiac proteins in the transgenic (TG) vs. wild-type (WT) mice. Mass spectrometry analysis identified mitochondrial proteins involved in respiration, metabolism, and detoxification as primary targets of SNO modification in VCP-overexpressing mouse hearts. Particularly, we found that VCP itself underwent SNO modification at a specific cysteine residue in its N-domain. Additionally, our study demonstrated that glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis, also experienced increased SNO in response to VCP overexpression. While deletion of inducible nitric oxide synthase (iNOS) in VCP TG mice did not affect VCP SNO, it did abolish SNO modification in mitochondrial complex proteins, suggesting a dual mechanism of regulation involving both iNOS-dependent and independent pathways. Overall, our findings shed light on post-translational modification of VCP in the heart, unveiling a previously unrecognized role for VCP in regulating cardiac protein SNO and offering new insights into its function in cardiac protection.

1. Introduction

S-nitrosylation (SNO) is emerging as a pivotal post-translational modification, crucial for modulating various physiological and pathological processes [1–3]. By adding a nitrosyl group (NO-) to reactive cysteine thiols, SNO can profoundly influence protein-protein interactions and subsequent modifications, such as phosphorylation, acetylation, ubiquitination, and disulfide bond formation [2]. This regulatory mechanism plays a multifaceted role in protein stability, transcriptional regulation, DNA damage repair, cellular growth, and apoptosis [4–6]. In addition, research has demonstrated that SNO not only acts as a scavenger of nitric oxide (NO), preventing its interaction with reactive oxygen species (ROS) but also serves to protect cysteine thiols from ROS-mediated oxidation [7]; thus, it has been considered as an emerging paradigm of redox signaling protecting cells against oxidative stress in the hearts. Accumulating evidence indicates that SNO plays a vital role in preserving cardiac function in physiological and pathological conditions [3,8]. Dysregulated SNO has been increasingly associated with the development and progression of heart diseases such as myocardial ischemia and heart failure [9,10]. However, despite its significance, the precise molecular mechanisms governing SNO in the heart remain incompletely understood.

Valosin-containing protein (VCP), an ATPase-associated protein, has emerged as a key mediator of cardiac protection against heart failure induced by ischemic and pressure overload insults [11–17]. VCP, composed of distinct domains including the N-domain and two ATPase domains (D1 and D2), exhibits diverse functionality through interactions with various biological partners and cofactors [18,19]; notably, mutations in VCP have been linked to human dilated cardiomyopathy and inclusion body myopathy with early-onset Paget disease and frontotemporal dementia [20,21]. Our previous studies have shown that decreased VCP caused by cardiac stress is highly associated with

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cardiac dysfunction, and a cardiac-specific overexpression of VCP protects the heart against ischemic injury and pressure-overloaded heart failure [11–16]. In addition, our research has indicated that VCP is intricately involved in preserving mitochondrial function, preventing cardiomyocyte death, and enhancing inducible nitric oxide synthase (iNOS) expression and activity in the heart [11–13]. Given the central role of iNOS in generating NO, which can subsequently modify specific cysteine residues in target proteins via SNO, it is plausible that VCP may play a regulatory role in cardiac protein SNO modification.

We hypothesize that VCP modulates cardiac protein SNO, potentially through iNOS-dependent mechanisms, thereby influencing the biological functions of these proteins and contributing to the preservation of cardiac function. To test this hypothesis, we used a cardiac-specific VCP TG mouse model combined with liquid chromatography and mass spectrometry to determine whether overexpression of VCP affects the SNO level of target proteins in the heart. We also used a double genetically modified mouse model, in which iNOS was deleted in VCP TG mouse, to determine whether the effects of VCP on cardiac protein SNO rely on iNOS; In addition, we also used a domain mutant mouse model to identify the domain responsible for VCP SNO. This study aimed to further elucidate the biological role of VCP in the heart and provide novel insights into the underlying mechanism of VCP-mediated cardiac protection.

2. Materials and methods

2.1. Animal models

Three lines of genetically modified mouse models were employed, and the targeted gene expressions were confirmed before experiments. All the measurements were performed in male mice at an age of 3–6 months old from the following strains:

- *Cardiac-specific VCP TG mouse*: A VCP TG mouse was previously generated, and the general characteristics and cardiac protection conferred by this line of TG mice have been demonstrated [12,14,16,22]. Briefly, cardiac-specific VCP overexpression is achieved by introducing the α-myosin heavy chain (αMHC) promoter-driven VCP coding sequence into the zygotes of FVB mice. Their litter-matched WT mice were used as controls.
- *Cardiac-specific dominant-negative (DN) VCP TG mouse*: A DN VCP TG mouse was generated as described previously [23], in which a construct containing the VCP coding sequence truncated of the first 600 nucleotides was inserted downstream of αMHC promoter. Their litter-matched WT mice were used as controls.
- *VCP TG/iNOS KO bi-genic mouse:* The iNOS knockout (KO) mouse (B6/129) was purchased from Jackson Laboratory. After background cleaning via 10 times breading with FVB WT mice, this mouse was cross-mated with VCP TG to generate a VCP TG/iNOS KO bi-genic mouse model. Litter-matched pairs of VCP TG mice, iNOS KO mice, and WT mice were used as controls in this study.

All animal procedures were conducted in accordance with the guidelines established by the National Institutes of Health on the Care and Use of Laboratory Animals, as revised in 2011. The research protocols adopted in this study received approval from the Institutional Animal Care and Use Committees of Georgia State University.

2.2. Biotin Switch Technique (BST), Liquid Chromatography (LC), and Mass Spectrometry (MS)

Fifty mg of mouse heart tissue was homogenized using the biotin switch lysis buffer. The protein concentrations were detected using the BCA method and adjusted to 1 μg/μl for the biotin switch technique. Free thiols of proteins were blocked with 20 mM methyl methanethiosulfonate (MMTS) at 37 °C for 20 min. The proteins were then precipitated and washed with ice-cold acetone. S-nitrosylated cysteines were reduced with 10 mM ascorbate and labeled with N-(6-[biotinamido]hexyl)-3'-(2′-pyridyldithio) propionamide (biotin-HPDP) reagents.

Following the biotin switch assay, Western blotting was performed to detect the total SNO level of cardiac proteins with an antibody against biotin. 10 μg of proteins from each group were separated using nonreducing SDS-PAGE and transferred onto nitrocellulose membranes. The biotinylated protein was probed with an anti-biotin antibody (1:3000) (Vector Laboratories, Burlingame, CA) and visualized with an enhanced chemiluminescent substrate (PerkinElmerLife Science).

To identify SNO-peptides, LC/MS/MS analysis was performed using a Thermo Fisher Scientific LTQ-Orbitrap Velos Pro mass spectrometer paired with Ultimate™ 3000 Chromatography System, according to the manufacturer's instructions [24]. Briefly, 200 μg of Biotin-HPDP-linked proteins were enriched using streptavidin beads and then digested in 10 μg trypsin in 500 μl 50 mM ammonium hydrogen carbonate (NH_4HCO_3) at 37 °C overnight. The trypsinized peptides were collected and slowly injected into the avidin cartridge. After the removal of the non-biotinylated and nonspecifically bound peptides, the biotinylated peptides were eluted and then desalted with Pierce C18 spin columns. After complete drying, the desalted peptides were resuspended in 5ul of mobile phase A (2 % acetonitrile (ACN) and 0.1 % formic acid in H_2O). The desalted peptides were first trapped on a Dionex™ Pepmap C18 pre-column (5 mm × 300 μm) at a 2 % mobile phase B (85 % ACN and 0.1 % formic acid). The flow rate was set at 30 μl/min. Then, the peptides underwent separation on a Dionex™ PepMap100C18 column (100 Å, 3 μm, 75 μm × 150 mm) using an 85-min gradient at a flow rate of 250 nl/min. A column wash program was performed after each sample separation to minimize carry-over effects. The eluted peptides were subsequently introduced into the mass spectrometer through a Proxeon Nanospray Flex™ Ion Source. The spray voltage was set at 2.15 kV and the capillary temperature at 275 °C. Full MS scans from 300 to 2000 *m/z* were conducted in the Orbitrap analyzer at a resolution of 60,000 at 400 *m/z* using the lock mass option. The ten most intense peptide ions with charge states of 2–4 were isolated and subjected to fragmentation using collision-induced dissociation (CID) with normalized collision energy (NCE) set at 30 %. The ion selection threshold for MS/MS analysis was set at 3000. To identify the biotin-modified peptides and the located sites, the MS/MS spectra were searched against a Swiss-Prot mouse database using a local Mascot search engine (V.2.3) with biotin HPDP as a variable modification. Peptides identified with a 95 % confidence interval are displayed in Scaffold software. Protein and peptide False Discovery Rates (FDR) were less than 1 %.

2.3. Protein extraction and Western blot

Total protein was extracted from left ventricular tissues and subjected to Western blot as previously described [25]. Primary antibodies used for immunoblotting: anti-VCP-N terminal (Abcam, Cambridge, UK, ab109240), anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA, 2118), anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA, 97166), and OxPhos Rodent WB Antibody (Thermo Fisher Scientific, Waltham, MA, USA, 45–8099). Bands were visualized using Odyssey DLx Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

2.4. Streptavidin purification to detect specific SNO-proteins

Protein lysates processed by the modified biotin switch assay were treated with Dynabeads™ M − 270 Streptavidin (65305, Invitrogen) to purify and isolate biotin-labeled SNO-proteins. BST-treated protein lysates were incubated with magnetic beads at room temperature for 30 min with gentle rotation. The coated beads were washed and resuspended with elution buffer containing 0.1 % SDS, heated at 95 °C for 5 min, and then centrifuged for 30 s at 4000 rpm at 4 °C [26,27]. After magnetic separation, the supernatant containing eluted proteins was

collected and subjected to Western blot.

2.5. Statistical analysis

Data were evaluated as fold changes compared to the control group and are presented as average ± standard error (SE). Statistical analysis was done using a standard one-way ANOVA, followed by Holm-Sidak's multiple comparisons. Statistical significance was established at a p-value of less than 0.05 ($p < 0.05$).

3. Results

3.1. VCP preferentially increases SNO of mitochondrial proteins in the heart

We assessed the effect of VCP on the SNO of target proteins in the heart using the cardiac-specific VCP TG mice (Fig. 1A) [12,22] compared to their litter-matched WT mice. The total proteins extracted from the heart tissues of VCP TG and WT control were treated with the biotin switch assay and then separated using liquid chromatography and analyzed with mass spectrometry to identify and quantify protein SNO levels. In total, 208 SNO proteins were successfully detected in these mouse heart tissues. Among these, the SNO levels of 19 cardiac proteins exhibit a difference of at least 2 folds between the two groups of mice, including 14 upregulated and 5 downregulated SNO proteins in VCP TG mice vs. WT mice. In addition, there were 7 cardiac proteins whose SNO modifications were detected only in the hearts of the VCP TG mice but not in WT mice, and 2 proteins' SNO were detected only in WT but not in VCP TG mice. These most significantly different SNO proteins are presented in Table 1.

Intriguingly, among the cardiac proteins with the most pronounced SNO levels, a substantial majority are mitochondrial proteins. These SNO proteins are engaged in various cellular processes, including mitochondrial respiration (NADH dehydrogenase flavoprotein 2, NADH dehydrogenase iron-sulfur protein 6, NADH dehydrogenase 1 alpha subunit 10, NADH dehydrogenase 1 alpha subunit 9, succinate-semialdehyde dehydrogenase, cytochrome *c*, cytochrome *c* oxidase subunit 6C, and NADP-dependent malic enzyme), metabolism and detoxification (e.g., peroxisomal acyl-coenzyme A oxidase 1, 3-hydroxyacyl-CoA dehydrogenase type-2, NADP-dependent malic enzyme, citrate lyase subunit beta-like protein, microsomal glutathione S-transferase 3 and epoxide hydrolase 2), and protein degradation, calcium regulation and chaperonins, as summarized in the associated biological function in Table 2. Remarkably, the transitional endoplasmic reticulum ATPase, also known as VCP, exhibited a more than 10-fold increase in the SNO levels in the hearts of VCP TG mice compared to WT mice.

On the other hand, the top downregulated SNO proteins in VCP TG mice are related to the NO donor/carrier or scavenger/transfer of NO groups, including superoxide dismutase [Cu–Zn], ferritin heavy chain, aldehyde dehydrogenase, kynurenine oxoglutarate transaminase 3 and serum albumin; The other two are related to protein-protein interaction,

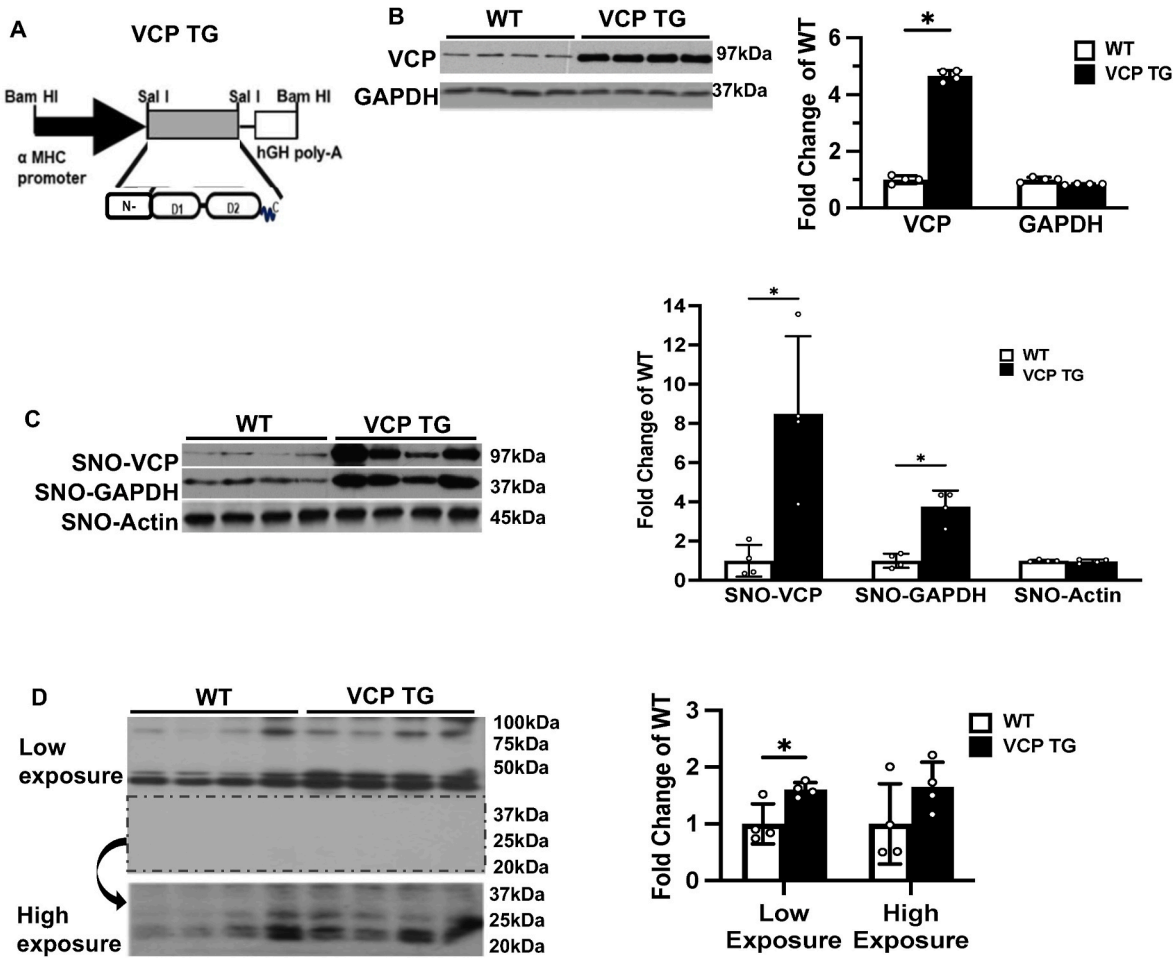

Fig. 1. Overexpressing VCP in the heart activates VCP S-nitrolysation (SNO) and increases overall SNO level of cardiac proteins. A. The construct of cardiac-specific VCP TG mice model. **B.** The total protein expression of VCP and GAPDH in heart tissues from the TG and WT mice. **C.** SNO proteins of VCP, GAPDH, and Actin from the same heart tissues of the two groups after biotin-switch assay. **D.** The overall SNO proteins detected by the antibody against biotin under low and high exposures. N = 4/group, *, $p < 0.05$ vs. WT.

Table 1
Top changed SNO proteins identified in VCP TG versus WT mouse hearts.

Protein descriptions	Protein name (Swissprot)**	MW	VCP Values	WT Values	VCP/WT Folds
Up-regulations					
NADP-dependent malic enzyme	MAON_MOUSE	67 kDa	4.7	0.0	*
Microsomal glutathione S-transferase 3	MGST3_MOUSE	17 kDa	4.7	0.0	*
Sarcalumenin	SRCA_MOUSE	99 kDa	2.8	0.0	*
Peroxisomal acyl-coenzyme A oxidase 1	ACOX1_MOUSE	75 kDa	2.8	0.0	*
Cytochrome *c*1, heme protein	CY1_MOUSE	35 kDa	1.9	0.0	*
Peptidyl-prolyl *cis*-trans isomerase A	PPIA_AOTTR	18 kDa	1.9	0.0	*
NADH dehydrogenase [ubiquinone] flavoprotein 2	NDUV2_MOUSE	27 kDa	1.9	0.0	*
Transitional endoplasmic reticulum ATPase (VCP)	TERA_HUMAN	89 kDa	22.5	2.2	10.4
Succinate-semialdehyde dehydrogenase	SSDH_MOUSE	56 kDa	6.6	1.1	6.1
60 kDa heat shock protein	CH60_CRIGR	61 kDa	3.7	1.1	3.5
Complement C3	CO3_MOUSE	186 kDa	3.7	1.1	3.5
26S proteasome non-ATPase regulatory subunit 2	PSMD2_MOUSE	100 kDa	3.7	1.1	3.5
NADH dehydrogenase [ubiquinone] iron-sulfur protein 6	NDUS6_MOUSE	13 kDa	2.8	1.1	2.6
14-3-3 protein epsilon	1433E_BOVIN	29 kDa	2.8	1.1	2.6
Estradiol 17-beta-dehydrogenase 8	DHB8_MOUSE	27 kDa	2.8	1.1	2.6
NADH dehydrogenase [ubiquinone] 1 alpha ssubunit 10	NDUAA_MOUSE	41 kDa	5.6	2.2	2.6
Citrate lyase subunit beta-like protein	CLYBL_MOUSE	38 kDa	5.6	2.2	2.6
Tubulin alpha-4A chain	TBA4A_BOVIN	50 kDa	5.6	2.2	2.6
Epoxide hydrolase 2	HYES_MOUSE	63 kDa	10.3	4.3	2.4
NADH dehydrogenase [ubiquinone] 1 alpha subunit 9	NDUA9_MOUSE	43 kDa	16.8	7.5	2.2
Cytochrome *c* oxidase subunit 6C	COX6C_MOUSE	8 kDa	4.7	2.2	2.2
3-hydroxyacyl-CoA dehydrogenase type-2	HCD2_MOUSE	27 kDa	13.1	6.4	2.0
Down-regulations					
Superoxide dismutase [Cu–Zn]	SODC_MOUSE	16 kDa	0.0	1.1	#
Ferritin heavy chain	FRIH_BOVIN	21 kDa	0.0	1.1	#
Aldehyde dehydrogenase	ALDH2_MESAU	54 kDa	0.9	4.3	−4.6
Kynurenine–oxoglutarate transaminase 3	KAT3_MOUSE	51 kDa	0.9	3.2	−3.4
Reticulon-4-interacting protein 1	RT4I1_MOUSE	43 kDa	1.9	4.3	−2.3
Serum albumin	ALBU_MOUSE	69 kDa	1.9	4.3	−2.3
Profilin-1	PROF1_MOUSE	15 kDa	2.8	6.4	−2.3
Transmembrane protein 65	TMM65_HUMAN	25 kDa	0.9	2.2	−2.3

Table 2
The relevance of the upregulated SNO proteins in VCP TG mouse heart to the potential cardiac functions.

Cardiac function relevance	Upregulated SNO-Proteins	Biological role
Mitochondrial respiration	NADH dehydrogenase [ubiquinone] flavoprotein 2	Subunit of complex I of ETC in mitochondria
	NADH dehydrogenase iron-sulfur protein 6	Subunit of complex I of ETC in mitochondria
	NADH dehydrogenase [ubiquinone] 1 alpha subunit 9	Subunit of complex I of ETC in mitochondria
	NADH dehydrogenase [ubiquinone] 1 alpha subunit 10	Subunit of complex I of ETC in mitochondria
	Cytochrome *c*1, heme protein	Subunit of complex III of ETC in mitochondria
	Cytochrome *c* oxidase subunit 6C	Subunit of complex III of ETC in mitochondria
	Succinate-semialdehyde dehydrogenase	Subunit of complex II of ETC in mitochondria
Metabolisms/ detoxification	NADP-dependent malic enzyme	Pyruvate metabolism and decarboxylation
	Peroxisomal acyl-coenzyme A oxidase 1	Enzyme catalyzing the fatty acid beta-oxidation
	Microsomal glutathione S-transferase 3	Regulating lipid hydroperoxides
	Citrate lyase subunit beta-like protein	Catalyzing citrate to acetate and oxaloacetate.
	Epoxide hydrolase 2	Converts epoxide to the corresponding dihydrodiols
	3-hydroxyacyl-CoA dehydrogenase type-2	Regulating fatty acid metabolic processes.
	Citrate lyase subunit beta-like protein	Regulating fatty acid synthesis
Calcium regulation/ER stress	Sarcalumenin	Regulating Ca^{2+} reuptake into the SR via SERCA2a.
	Transitional endoplasmic reticulum ATPase (VCP)	Regulating ER stress and mitochondrial function
Chaperonins/ adaptors	60 kDa heat shock protein	A molecular chaperone in mitochondria
	14-3-3 protein epsilon	A negative regulator of cell cycle
	Peptidyl-prolyl *cis*-trans isomerase A	Function as a protein folding chaperones
Proteasomes	26S proteasome non-ATPase regulatory subunit 2	Regulating the ubiquitinated protein degradation

including reticulon 4 interacting protein and profilin 1, a ubiquitous actin monomer-binding protein.

3.2. Overexpression of VCP triggers its SNO modification and enhances GAPDH SNO levels in the heart

To validate the VCP SNO in TG mouse hearts identified by LC-MS/MS, the biotin HPDP-linked proteins from both VCP TG and litter-matched WT mouse hearts were further detected by Western blotting with a specific antibody against VCP. The construct of VCP TG mouse was illustrated in Fig. 1A. The total protein extracted from the same animals but without biotin switch treatment was also detected by Western blot to confirm the VCP expression. As shown in Fig. 1B, VCP expression increased about **4.5-fold** in the heart tissues of VCP TG vs. WT mice. While SNO-VCP was nearly undetectable in the heart tissues of

WT mice, it was remarkably increased in VCP TG mice by more than **8-fold** vs. WT mice (Fig. 1C). This result further confirms the findings from the LC-MS/MS assay.

Notably, the SNO level of GAPDH was significantly increased in VCP TG mice vs. WT (Fig. 1C) despite the similar level in the expression of GAPDH between two groups (Fig. 1B). To further confirm these results, we detected another housekeeping protein's SNO level (Actin). There is no difference in the SNO level of actin in both groups (Fig. 1C).

In addition, we detected the total SNO level of the cardiac proteins in VCP TG and WT mice. As shown in Fig. 1D, overexpressing VCP in the heart increased the overall SNO levels of the cardiac proteins in VCP TG mice vs. WT mice.

These data together with our results from LC-MS/MS indicate that VCP not only has the potential to facilitate the SNO modification of various cardiac proteins but also serves as a target protein for SNO itself.

3.3. VCP SNO targets the specific cysteine residue located at its N-terminal domain

Next, we determined the SNO residue in VCP using the LC-MS analysis to identify the peptide sequences of each SNO protein in the heart. Our results showed that in response to VCP overexpression, only one single S-nitrosylated cysteine residue of VCP was detected in the heart tissues of the VCP TG mice. Further sequence analysis revealed the identified cysteine residue of VCP located at its N-terminal domain, as shown in Fig. 2A.

To validate this finding, we utilized a cardiac-specific DN-VCP TG mouse model [23], in which the initial 600 nucleotides in the N-terminal region were deleted (Fig. 2B). As shown in Fig. 2C, exogenous DN-VCP expression was observed as a distinct band at 75 kDa beneath the endogenous VCP bands, confirming the DN-VCP overexpression in the TG mouse model. No significant difference in endogenous VCP expression in the heart between the DN-VCP TG and WT groups was detected (Fig. 2C).

We also analyzed the SNO of the exogenous DN-VCP and endogenous VCP in the heart tissues from the same mice in the two groups using western blotting following streptavidin purification and the biotin switch assay. As shown in Fig. 2D, upon the overexpression of DN-VCP, the SNO of this exogenous DN-VCP was not detectable in the DN-VCP TG mouse heart tissues. In addition, unlike the VCP TG mice, overexpression of DN-VCP in the heart did not increase the SNO level of the endogenous VCP, but remarkably deceased SNO-VCP in the heart tissues of DN-VCP TG mice compared to their litter-matched WT group (Fig. 2D). No significant change in the SNO-GAPDH (Fig. 2D) or overall SNO levels of cardiac proteins was observed upon the overexpression of DN-VCP (Fig. 2E). These data together suggest that the N-terminal domain of VCP is required for VCP SNO.

3.4. Deletion of iNOS does not affect the VCP SNO modification in the heart

Previous studies have identified VCP as a stimulator of iNOS expression both in vitro and in vivo [11,12]. To investigate whether increased SNO of VCP in the heart depends on the iNOS induced by VCP

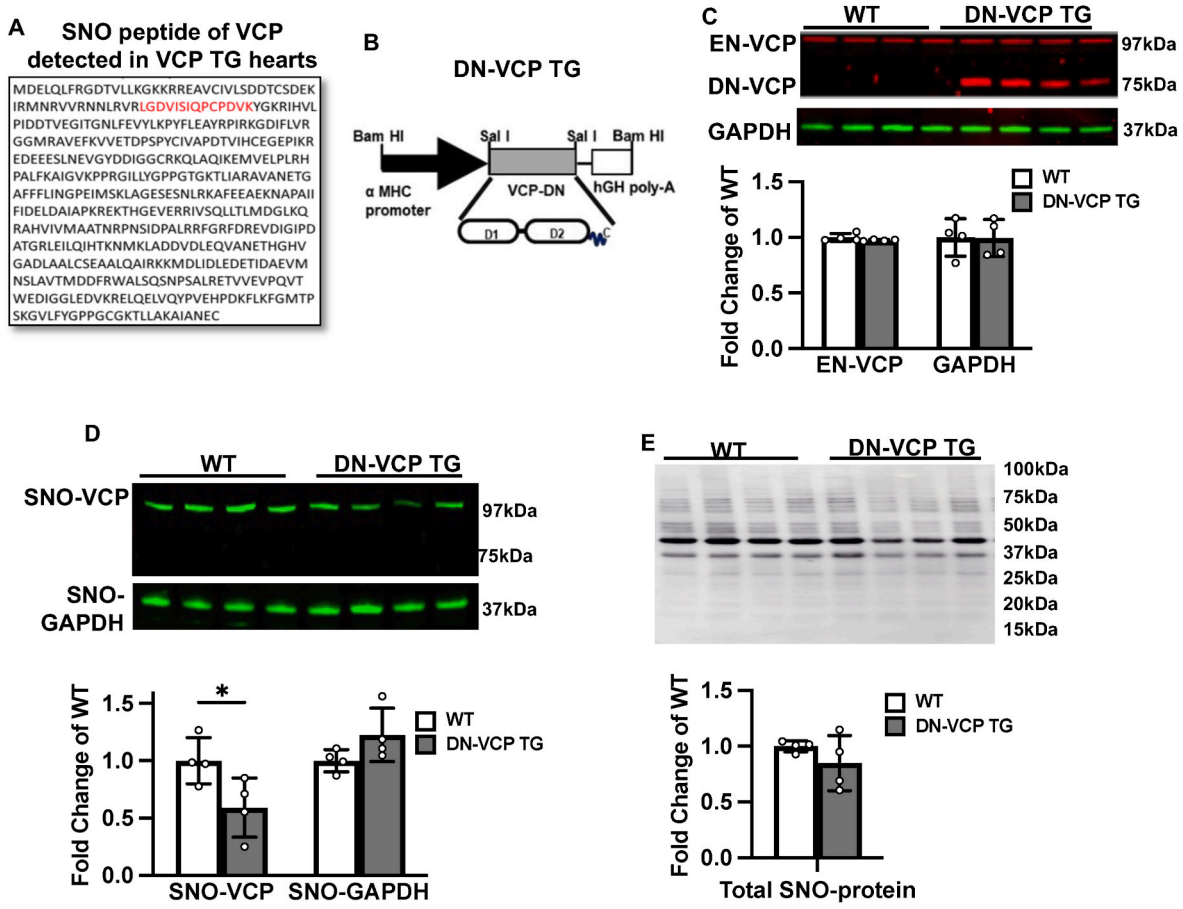

Fig. 2. Deletion of N-terminal domain of VCP eliminates VCP S-nitrosylation (SNO) in the mouse heart. A. The sequence of the SNO-peptide in VCP protein detected in VCP TG mouse heart by LC-MS (highlighted in red) located in the N-terminal domain. **B.** The construct of DN-VCP TG mouse model, in which the N-terminal domain was deleted. **C.** The total protein expression of exogenous DN-VCP, endogenous VCP, and GAPDH in the heart tissues of DN-VCP TG mice and WT mice. **D.** The SNO-proteins from the same heart tissues of mice from C. *, $p < 0.05$ vs. WT. n = 4/group. **E.** The total SNO level of cardiac proteins in the two groups detected by the antibody against biotin.

overexpression, we employed a VCP TG/iNOS KO bi-genic mouse model, in which iNOS gene was deleted in the VCP TG mice (Fig. 3A). Total proteins were obtained from the mice of four groups, i.e., VCP TG/iNOS KO mice and their litter-matched WT, VCP TG mice, and iNOS KO mice, and then VCP and the SNO VCP levels were detected by Western blotting (Fig. 3B-C). VCP SNO was significantly increased in VCP TG mice vs. WT mice, however, no significant difference was observed between VCP TG and VCP TG/iNOS KO mice (Fig. 3B-C). These data indicate that VCP SNO modification is independent of iNOS expression in the heart.

3.5. SNO modification of mitochondrial proteins relies on iNOS expression in the heart

Prior studies have demonstrated the regulatory role of VCP in preserving the functionality of mitochondrial respiration in the heart in an iNOS-dependent manner [11,12]. Our LC-MS data also revealed that VCP overexpression resulted in a significant elevation of cardiac mitochondrial protein SNO, particularly those integral to the respiratory complexes (Tables 1–2). Therefore, we sought to test the effect of VCP and iNOS on the SNO of proteins involved in the mitochondrial respiratory complexes. We used a commercially available cocktail of rodent OXPHOS antibodies for Western blot to assess the SNO levels of the five mitochondrial respiration complexes in the hearts of VCP TG/iNOS KO mice and their litter-matched WT and VCP TG mice. In the cocktail, NADH: ubiquinone oxidoreductase subunit B8 (NDUFB8**)** was probed representing complex I (CI-NDUFB8), succinate dehydrogenase complex iron-sulfur subunit B (SDHB) for complex II (CII-SDHB), ubiquinol-cytochrome c reductase core protein 2 (UQCRC2) for complex III (CIII-UQCRC2), mitochondrially encoded cytochrome *c* oxidase I (MTCO1) for complex IV (CIV-MTCO1), and ATP synthase subunit alpha (ATP5A) for complex V (CV-ATP5A).

As shown in Fig. 4A-B, there are no significant changes in the abundance of these representative proteins in the hearts among the three groups of mice (Fig. 4A and B). While the SNO levels of CI-NDUF88, CII-SDHB, and CIV-MTCO are slightly increased in the hearts of VCP TG mice compared to WT mice (Fig. 4C and D); they are significantly diminished in VCP TG/iNOS KO mice due to the deletion of iNOS (Fig. 4C and D), where they are almost undetectable. These results indicate that iNOS is essential for the SNO of these proteins.

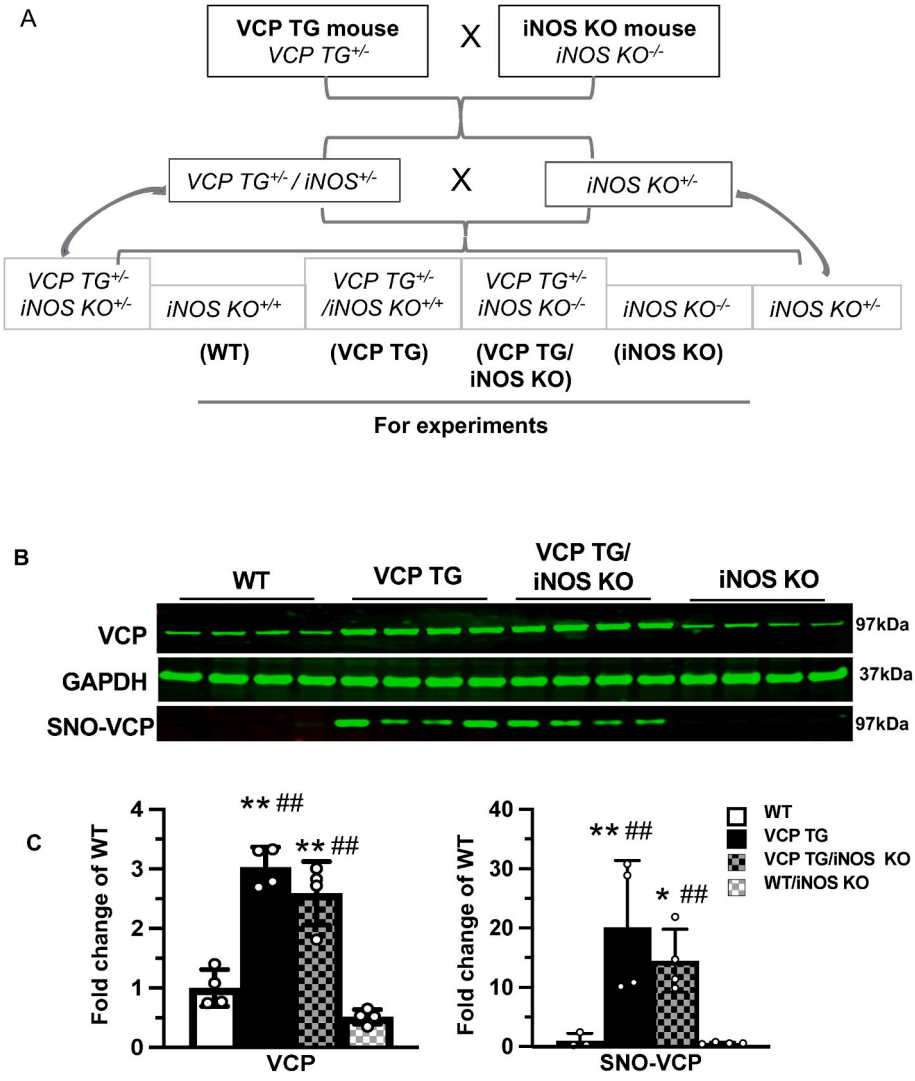

Fig. 3. Deletion of inducible nitric oxide synthase (iNOS) does not affect VCP S-nitrosylation (SNO) in the TG mouse heart. A. The scheme of the breeding strategy and the generation of VCP TG /iNOS KO mouse model and its litter-matched control mice. **B.** Representative images of Western blot showing the total protein expression of VCP and its SNO level in the heart tissues from the four groups of mice. **C.** Quantification of Western blot showing fold changes of VCP expression and SNO VCP level normalized to wild-type. *, $p < 0.05$,**, $p < 0.01$ vs. WT, ##, $p < 0.01$ vs. iNOS KO mice. n = 4/group.

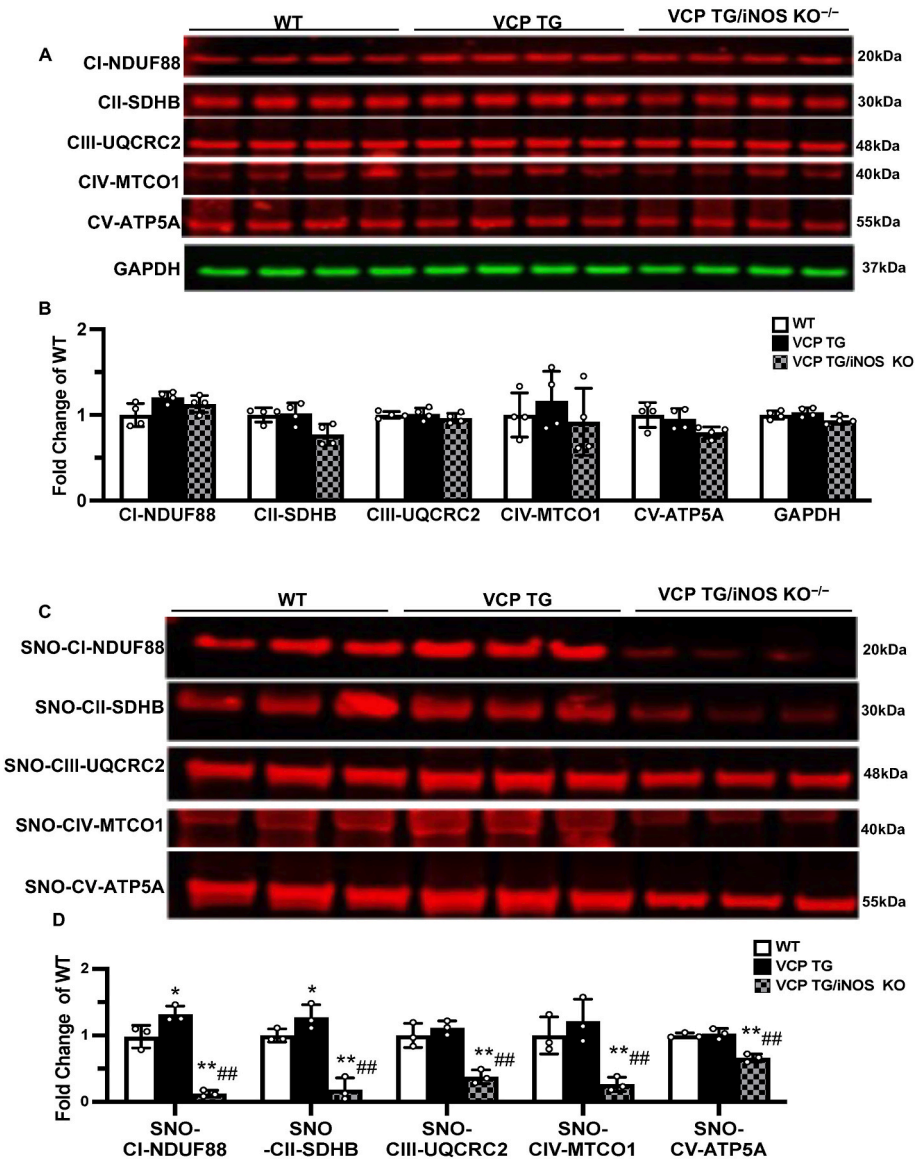

Fig. 4. Genetic knockout (KO) of inducible nitric oxide synthase (iNOS) results in a substantial reduction in the S-nitrosylation (SNO) of mitochondrial proteins. A-B. The images and the quantitated values of Western blots showing the total abundance of the representative mitochondrial proteins of each electron transport chain complex, in the heart tissues of wild-type (WT), VCP transgenic (TG), and VCP TG/iNOS KO mice. GAPDH used as a loading control in total protein extracts. **C-D**. The representative images and quantitated values of Western blots showing the SNO level of these proteins by testing the purified biotinylated proteins from the same heart tissues. *,$p < 0.05$, **, $p < 0.01$ vs. WT, ##: $p < 0.01$ vs. VCP TG mice.

4. Discussion

This present study uncovers the role of VCP in regulating cardiac protein SNO, which may be mechanistically linked to the cardiac protection conferred by VCP. In particular, this study reveals a previously unidentified mechanism of VCP as a target for SNO modification in the heart, expanding our understanding of its regulatory role in cellular signaling pathways. Additionally, the study unveils a previously unrecognized role of VCP in regulating SNO modification of essential cardiac proteins, particularly those involved in mitochondrial function. This study also reveals a dual mechanism involved in VCP-mediated regulation of cardiac protein SNO, providing insight into the multifaceted nature of VCP's role in cellular redox signaling. Overall, this study offers novel insights into the post-translational modification of VCP and its implications for cardiac health, highlighting its potential for mitigating oxidative stress-related cardiac damage.

Emerging evidence indicates that, like phosphorylation, SNO plays a multifaceted role in regulating cardiac function and protecting the heart against various pathological conditions, highlighting its significance in cardiovascular physiology and pathology [3,28–33]. It has been shown that SNO of proteins involved in cardiac contractility, such as troponins and phospholamban, modulate the sensitivity of cardiac myofilaments to calcium ions, affecting the force of cardiac muscle contraction [34,35]. SNO can also modulate the activity of ion channels in cardiac myocytes, such as potassium channels and L-type calcium channels, to maintain normal cardiac rhythm and function [36,37]. In addition, SNO can modulate mitochondrial function in cardiac cells by affecting the activity of proteins involved in mitochondrial respiration, ATP production, and apoptosis [38,39]. Importantly, SNO has been shown to confer protection against ischemia-reperfusion injury by modulating various signaling pathways involved in cell survival and apoptosis [9,40–42]. Several studies have demonstrated that SNO plays a crucial role in mediating the cardioprotective effects of ischemic pre-conditioning (IPC), a golden standard of protection against myocardial ischemia [30,43]. However, despite the power of IPC in cardiac protection, its clinical translation is limited due to its unpractical nature. Thus,

inducting a pre-emptive phenomenon of the heart to activate the survival pathways as IPC became an appealing novel therapeutic strategy for preventing myocardial ischemia.

Our previous studies have demonstrated that cardiac-specific overexpression of VCP protects the heart against ischemic injury, with an efficiency comparable to IPC [12]. These data indicate that increase of VCP plays a pre-emptive conditioning role in the heart. However, the underlying molecular mechanisms are not fully understood. The findings from the present study reveal critical aspects of the mechanism underlying the cardioprotection conferred by VCP overexpression that has not been characterized previously: *First*, overexpressing VCP in the heart induces a substantial increase in the SNO content of cardiac proteins, as observed in the IPC hearts [43]. *Secondly,* our results reveal that increased VCP in the heart can trigger or activate the SNO modification of proteins involved in mitochondrial respiration complexes. Although the impact of SNO modification on the activity of individual mitochondrial proteins is still under investigation, emerging studies have proved the protective role of this post-translational modification in stressed hearts through modulating mitochondrial complex functions to repress reactive oxygen species (ROS) production [44,45]. For example, it has been showed that SNO of a subunit of NADH dehydrogenase (ubiquinone) within complex I could change the structural foundation, disrupting its interaction with ubiquinone, consequently reducing ROS production in ischemic myocardium during reperfusion [46]. Recent clinical studies also showed that treatment with mitochondria-selective SNO agent (MitoSNO) provides cardioprotection by SNO modification of mitochondrial complex I, limiting the damage of ROS in the heart during the reperfusion of ischemia-reperfusion injury [47,48]. These results imply a potential mechanism of cardiac protection conferred by VCP in the ischemic heart through SNO of mitochondrial complexes to inhibit stress-induced ROS production. In addition, other studies indicate the protective role of SNO of mitochondrial proteins by inhibiting mitochondrial permeability transition pore (mPTP) opening [49,50]. Our previous studies also showed that overexpression of VCP in cardiomyocytes inhibits the stress-induced mPTP opening through modulating mitochondrial calcium homeostasis [13]. The increased SNO of calcium regulation proteins (sarcalumenin and VCP) in the TG mouse heart detected in this study may provide a plausible mechanism of mPTP inhibition conferred by VCP. These investigations delve into the specific functions and regulatory mechanisms governed by SNO, offering deeper insights into the complex interplay between this modification and mitochondrial function in cardiac physiology and pathology. *Thirdly,* we found that VCP overexpression significantly increases SNO-GAPDH. Notably, it has been found that SNO-GAPDH is highly involved in IPC-induced cardiac adaption [51,52]. Studies demonstrated that IPC results in an increase of mitochondrial translocation of SNO-GAPDH, which shifts its binding to partners, subsequently increases SNO of mitochondrial proteins and facilitates a protective mechanism in IPC heart [51,52]. Besides, GAPDH is a key enzyme in the glycolytic pathway, catalyzing the conversion of glyceraldehyde 3-phosphate to 1, 3-bisphosphoglycerate. SNO of GAPDH could potentially modulate its enzymatic activity directly or by altering GAPDH interaction with other cofactors and serve as a regulatory mechanism in the heart. SNO-GAPDH has also been identified as a nuclear transnitrosylase that modulates various nuclear proteins, such as sirtuin-1 and histone deacetylase-2, thereby regulating nuclear gene transcription and cellular metabolism [53]. *Fourthly*, previous studies have shown that a significant enrichment in mitochondrial SNO proteins in metabolic pathways within the heart participate in modulating cellular energy homeostasis [54]. Our results also showed increased SNO level in serveral enzymes catalyzing cardiac metabolism and oxidation processes, which may also promote cellular energy production to compensate for the energy insufficiency caused by mitochondrial dysfunction in the ischemic condition [17,55,56]. These data together indicate that, like IPC, increased VCP in the heart induces a pre-emptive mechanism via enhancing the SNO of essential cardiac functional proteins, preventing the heart from following ischemia-reperfusion injury. These findings imply a previously unrevealed mechanism of cardiac protection conferred by VCP.

The number of proteins identified to undergo SNO has been expanding rapidly due to advancements in techniques and methodologies. In this study, we discovered that VCP is a target of SNO in the heart, which was not previously identified. Our results showed that, while SNO VCP was nearly undetectable in WT mice, increased VCP triggered the SNO modification of VCP, resulting in a pronounced increase in SNO VCP in the VCP TG mouse hearts. Notably, although increasing the expression of a protein can potentially lead to an increase in its SNO content, our results revealed an inequivalent enhancement between VCP protein expression and SNO VCP level in VCP TG vs. WT mice. While still unclear, this substantial increase in SNO-VCP in VCP TG mouse hearts may be facilitated by the following various factors: First, our previous studies demonstrated that overexpression of VCP in the heart increases its mitochondrial translocation [12]. The translation would enhance its interaction with other SNO proteins within the mitochondria or promote the recruitment of VCP to specific protein complexes where SNO reactions occur, facilitating SNO-VCP formation. In addition, SNO may increase the protein stability of SNO-VCP, slowing down its degradation, which would also contribute to the higher level of SNO-VCP [57]. Furthermore, SNO-VCP may change VCP activity or functions, selectively modulating the transcription or degradation of regulatory proteins, which could potentially affect the balance between nitrosylating and denitrosylating agents [58], indirectly influencing the SNO of VCP in the heart. Lastly, it is also possible that the increase in SNO VCP levels could involve feedback regulation mechanisms that further enhance its own SNO.

Despite such an abundance of SNO VCP, our results showed that SNO occurs only at a select cysteine residue at the N-domain of the VCP proteins. Supportively, we found that overexpression of DN-VCP, a mutant protein lacking N-domain, did not induce the SNO in exogenous DN-VCP, nor increase the SNO of endogenous VCP or overall SNO content of cardiac proteins. These data support the essential role of the N-domain in VCP SNO modification. Our data also revealed no significant change in SNO-GAPDH level between the DN-VCP TG and WT mice upon the overexpression of DN-VCP, further indicating the dependency of GAPDH SNO on the VCP SNO. Remarkably, rather than the increase of SNO-VCP, our results showed a slight decrease in SNO of endogenous VCP in DN-VCP TG mice vs. WT mice. A plausible explanation is that overexpression of DN-VCP inhibits the subcellular translocation of endogenous VCP [23], potentially reducing the interaction of VCP with other SNO proteins in the specific cellular compartments, and subsequently repressing SNO of the endogenous VCP. In addition, our previous study showed that overexpression of DN-VCP in the heart markedly decreases its ATPase activity [23], which may indirectly affect the SNO of VCP in the DN-VCP heart. These findings further highlight the importance of the N-domain in VCP SNO and provide additional evidence for biological function and regulatory mechanisms mediated by VCP.

Therefore, our results from this study not only reveal the impact of VCP overexpression on mitochondrial proteins but also identify VCP as an SNO target and locate its SNO site in the heart, providing new insight into the biological function of this protein and the potential mechanism underlying the cardioprotection conferred by VCP. Although the exact mechanisms involved in mitochondrial protein SNO remain unclear, our studies imply several possibilities: First, SNO-GAPDH can serve as a mitochondrial *trans*-S-nitrosylase, facilitating the transfer of SNO groups from the cytosol to the mitochondria, thus potentially offering a mitochondrial source of NO for mitochondrial proteins. Additionally, SNO of mitochondrial proteins may be transnitrosylated through SNO donor/scavenger/carrier proteins. Our results showed that the SNO levels of several cardiac proteins are downregulated upon VCP overexpression, such as superoxide dismutase [Cu–Zn], ferritin heavy chain, aldehyde dehydrogenase, and serum albumin, which were previously identified as metal transfer, NO donor or SNO scavenger. By reducing the SNO of

these proteins, VCP may enhance the availability of NO or other reactive nitrogen species for the prioritized key proteins, such as those involved in mitochondrial functions. Such orchestrated redistribution of SNO levels among different proteins may aim at increasing the cellular capacity to combat oxidative stress and promote cardiac protection. Furthermore, our previous studies demonstrated that overexpression of VCP significantly increases iNOS expression in cardiomyocytes *in vitro* and *in vivo* [11,12] and also enhances the translocation of iNOS into the mitochondria [12]. Our results in this study further showed that VCP overexpression leads to an increase in the SNO of specific mitochondrial proteins in the heart which are substantially diminished in the absence of iNOS. This finding suggests that iNOS plays an essential role in mediating the SNO of these mitochondrial proteins, highlighting the interplay between VCP, iNOS, and mitochondrial function in the heart. However, surprisingly, we found that deletion of iNOS in VCP TG mice did not abolish VCP SNO modification induced by overexpressing VCP, suggesting a mechanism independent of iNOS. One possibility is that VCP overexpression might lead to increased production of NO through other nitric oxide synthases, such as endothelial NOS and neuronal NOS, facilitating SNO reactions. In addition, SNO of VCP may be transnitrosylated by S-nitrosylated proteins through activating nitrosating agents, such as nitrous acid and S-nitrosothiols, or transition metal ions. It may also be mediated by the intracellular thiol-disulfide exchange systems, including thioredoxin and glutathione. These systems can modulate protein function through SNO in an NOS-independent manner.

Despite the novel discoveries, there are several aspects that require further investigation, such as the mechanism by which VCP regulates cardiac protein SNO, the impact of SNO on individual mitochondrial protein activity, and the essential role of SNO-GAPDH. To gain a better understanding of these mechanisms, developing a mouse model with SNO site-specific mutants of VCP and GAPDH may be needed in the future. Additionally, although global deletion of iNOS does not affect VCP expression and SNO level in the hearts of iNOS KO mice at baseline condition, a cardiac-specific iNOS KO mouse model would be more powerful to completely exclude the potential impact from other cells, particularly in the cardiac stress conditions where iNOS may be induced and play more relevant roles. Furthermore, our presented data in this study focus on the male mice since our pilot studies showed no significant difference between the sexes of these young mice at baseline, either in the VCP TG or iNOS KO mice. However, potential sex differences will still need to be considered in future studies with aged mice or under stress conditions. It is notable that although VCP is specifically overexpressed in cardiomyocytes, it could result in alterations not only in cardiomyocytes,but also in non-cardiomyocytes through direct cell-to-cell interaction or indirectly by its secreted molecules. For these reasons, in this study, we detected the SNO level of the cardiac proteins in whole heart tissue, which allowed us to explore the comprehensive impact of the overexpressed VCP in all cell types, thus, the results will be more relevant to the cardiac physiological function. However, the isolated cardiomyocytes could be studied to gain further insights into cell-specific effects.

In summary, this study revealed a previously uncharacterized role of VCP as a target and mediator of SNO in the heart and the underlying dual mechanisms of regulation. We provide new insight into the multifaceted nature of VCP's role in cellular redox signaling, enhancing our understanding of the molecular mechanisms underlying cardiac protection.

Data availability

All data needed to evaluate the conclusions are present in the paper and all raw data and statistical p-values will be provided as requested.

CRediT authorship contribution statement

Xiaomeng Shi: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Molly O'Connor:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Hongyu Qiu:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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