

# Supporting Information

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## SI Materials and Methods

**Mice.** All protocols and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Miami Miller School of Medicine.

**Integrated Hemodynamic Analysis.** Mice (female) were anesthetized with etomidate (1.3 mg/g), urethane (0.9 mg/g), and morphine (0.5 mg/g) and were instrumented with a micromanometer-conductance catheter (SPR-719; Millar Instruments). Volume measurements were calibrated by correlation with stroke volume (measured with an ultrasonic flow probe) and by saline calibration of EDV. Myocardial systolic and diastolic performance was assessed from pressure–volume data. Transient occlusion of the inferior vena cava was used to generate the end-systolic pressure–volume relationship (ESPVR) from which ventricular elastance ( $E_{es}$ ) is determined. Cardiac preload was indexed as the left ventricular (LV)-end diastolic volume (EDV) and pressure (EDP); cardiac afterload was evaluated as effective arterial elastance ( $E_a$ ; ratio of LV-end systolic pressure to stroke volume), and myocardial contractility was indexed by the slope of the ESPVR ( $E_{es}$ ) and by the peak rate of rise in LV pressure ( $dP/dt_{max}$ ) divided by EDV ( $dP/dt$ -EDV). Isoproterenol (ISO) dissolved in 0.9% NaCl was infused into the jugular vein in escalating dosage using an infusion pump (Harvard Apparatus 22). Measurements were obtained under steady-state conditions achieved after a 5-min infusion at each concentration (10–40 ng/ml).

**Isolated Myocyte Preparation.** Myocytes were isolated from hearts perfused with  $Ca^{2+}$ -free bicarbonate buffer containing 120 mM NaCl, 5.4 mM KCl, 1.2 mM  $MgSO_4$ , 1.2 mM  $NaH_2PO_4$ , 5.6 mM glucose, 20 mM  $NaHCO_3$ , 20 mM 2,3 butanedione monoxime (Sigma), and 5 mM taurine (Sigma), gassed with 95%  $O_2$ /5%  $CO_2$ , followed by enzymatic digestion with collagenase type II (1 mg/ml) (Worthington) and protease type XIV (0.1 mg/ml; Sigma). Myocytes were obtained by mechanical disruption of digested hearts, filtering, centrifugation, and resuspension in 0.125 mM  $CaCl_2$  Tyrode buffer containing 144 mM NaCl, 1 mM  $MgCl_2$ , 10 mM Hepes, 1.2 mM  $NaH_2PO_4$ , 5.6 mM glucose, and 5 mM KCl, adjusted to pH 7.4 with NaOH. Cells were resuspended in 0.25 mM  $Ca^{2+}$  Tyrode buffer and stored in Tyrode buffer containing 0.5 mM probenecid and 1.8 mM  $CaCl_2$ . For calcium measurements, cells were incubated in 5  $\mu$ M Fura-2/acetoxymethyl ester (Molecular Probes) and then transferred to a laminin-treated slide in a Lucite chamber on the stage of an inverted microscope (Nikon Eclipse TE 200) continuously superfused with Tyrode buffer at 37 °C.

Sarcomere shortening and  $Ca^{2+}$  transients ( $[Ca^{2+}]_i$ ) were measured in myocytes stimulated with increasing concentrations of ISO at 1 Hz, or at rising pacing frequencies (1, 2, 4, 6, and 8 Hz). Sarcomere length was recorded with an IonOptix intensified CCD camera. Change in average sarcomere length was determined by fast Fourier transformation of the Z line density trace to the frequency domain. Intracellular  $Ca^{2+}$  was measured with the  $Ca^{2+}$  sensitive dye Fura-2 and a dual-excitation spectrofluorometer (IonOptix), alternatively excited with a xenon lamp at wavelengths of 365 and 380 nm. The emission fluorescence was reflected through a barrier filter ( $510 \pm 10$  nm) to a photomultiplier tube. The ratio of photon live count detected by the excitation at 365 nm compared with 380 nm represents the Fura-2 fluorescence ratio. The degree of sarcomere shortening is expressed as the ratio between the amplitude of the shortening ( $\Delta L$ ) and the baseline sarcomere length ( $L_0$ ).

**GST–S-Nitrosogluthathione Reductase (GSNOR) Binding Column.** We used full-length recombinant GSNOR with a GST tag at the N terminus (Abnova Corporation). The product (10  $\mu$ g) was first dialyzed to eliminate glutathione from the media by using Slide-A-Lyzer MINI Dialysis Units (Thermo Scientific) with a molecular mass cut off of 2,000 Da. Recombinant proteins were dialyzed against-Tris buffered saline for 2 d at 4 °C. After dialysis, the fusion protein was immobilized with 25  $\mu$ L of glutathione resin (ProFound System; Pierce) overnight. After immobilization, the fusion protein was incubated with 40  $\mu$ g of human recombinant neuronal NOS (NOS1, nNOS) or endothelial NOS (NOS3, eNOS) (Cayman Chemical) overnight at 4 °C. The resin was then washed five times by spinning at  $1,250 \times g$  for 1 min. The complexes were eluted with Laemli buffer, resolved in 7% Tris-acetate gels, transferred to PVDF membranes, and probed with either NOS1 or NOS3 monoclonal antibodies (BD Biosciences).

**S-Nitrosylation.** For the biotin-switch assay, hearts were homogenized in HEN buffer [250 mM Hepes (pH 7.7), 1 mM EDTA, and 0.1 mM neocuproine]. Free cysteine (Cys) residues were blocked with S-methyl methanethiosulfonate (MMTS) and labeled with N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio) propionamide (HPDP-biotin) with or without sodium ascorbate. Biotinylated cardiac ryanodine receptor 2 (RyR2), sarcoplasmic reticulum (SR)  $Ca^{2+}$  ATPase (SERCA), and L-type  $Ca^{2+}$  channel (LTCC) were individually immunoprecipitated with protein G-Sepharose beads, electrophoretically resolved, and immunoblotted with anti-biotin antibody. Blotted membranes were re-probed with related antibody for detection of protein load.

**GSNOR Activity.** GSNOR activity was assayed in cardiac and skeletal muscle SR-enriched fractions and crude homogenates. With equal concentrations of protein, absorbance at  $\lambda_{max} = 340$  nm was measured over time in the presence of NADH (100  $\mu$ M final) and after the addition of GSNO (100  $\mu$ M final). NADH-dependent GSNOR activity is the difference obtained in the presence and absence of GSNO.

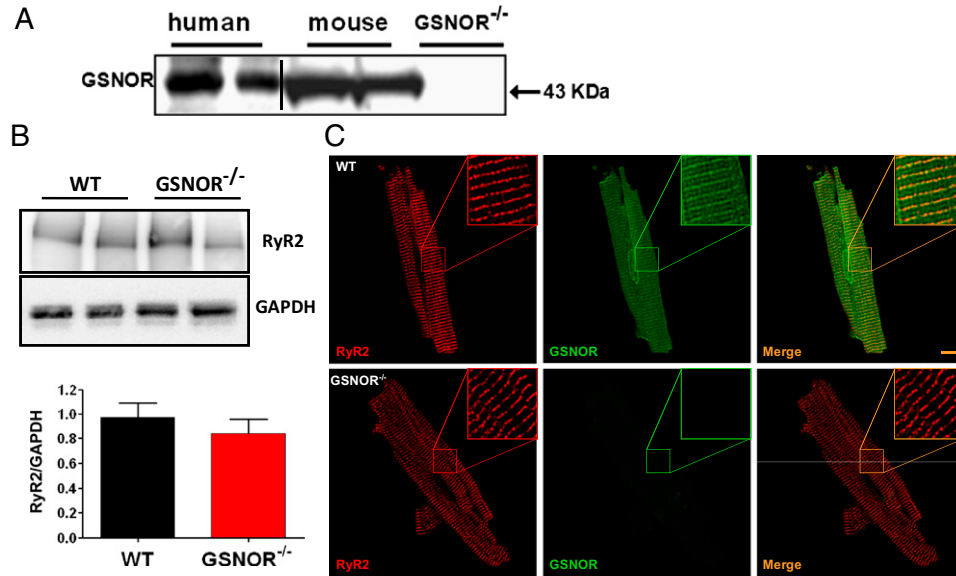
**Confocal Microscopy and Deconvolution Analysis.** Isolated myocytes were prepared as described above. Myocytes were pretreated with 1  $\mu$ M ISO for 10 min, 2 mM L-NMMA for 2 h, or 1 mM SNAP (positive control for NO donor) for 20 min (Fig. S4). In additional control experiments, the isolated myocytes were pretreated with 1 mM diethylenetriamine NONOate (NOC-18; positive control for NO donor) for 20 min or 3.5 mM p-chloromercuribenzoate (PCMB; negative control for reduction of thiols) for 30 min at room temperature (Fig. S5). Isolated myocytes were fixed in 2% paraformaldehyde and dual-stained for RyR2 and Cys-NO by incubating with mouse monoclonal primary antibody for RyR2 (Affinity BioReagents) and rabbit polyclonal antibody against Cys-NO (Sigma and A. G. Scientific) overnight at 4 °C. Fluorescence secondary antibody incubation was performed at 37 °C for 1 h with anti-mouse TRITC and anti-rabbit FITC (Jackson ImmunoResearch). Negative control images for the FITC and TRITC secondary antibodies are shown in Fig. S5.

In separate experiments, isolated myocytes were dual-stained for GSNOR and nNOS, eNOS, or RyR2 by incubating with goat polyclonal antibody against GSNOR (Santa Cruz Biotechnology) and rabbit polyclonal antibodies against eNOS or nNOS (Santa Cruz Biotechnology) or mouse monoclonal antibody against RyR2 (Affinity BioReagents). Fluorescence secondary antibody

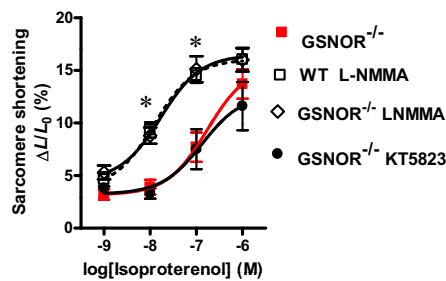
incubation was performed at 37 °C for 1 h with anti-rabbit or anti-mouse TRITC and anti-goat FITC (Jackson ImmunoResearch).

Images were obtained using a Zeiss LSM-510 confocal microscope (University of Miami). Fluorescence quantification was performed after the images were deconvolved with Image-

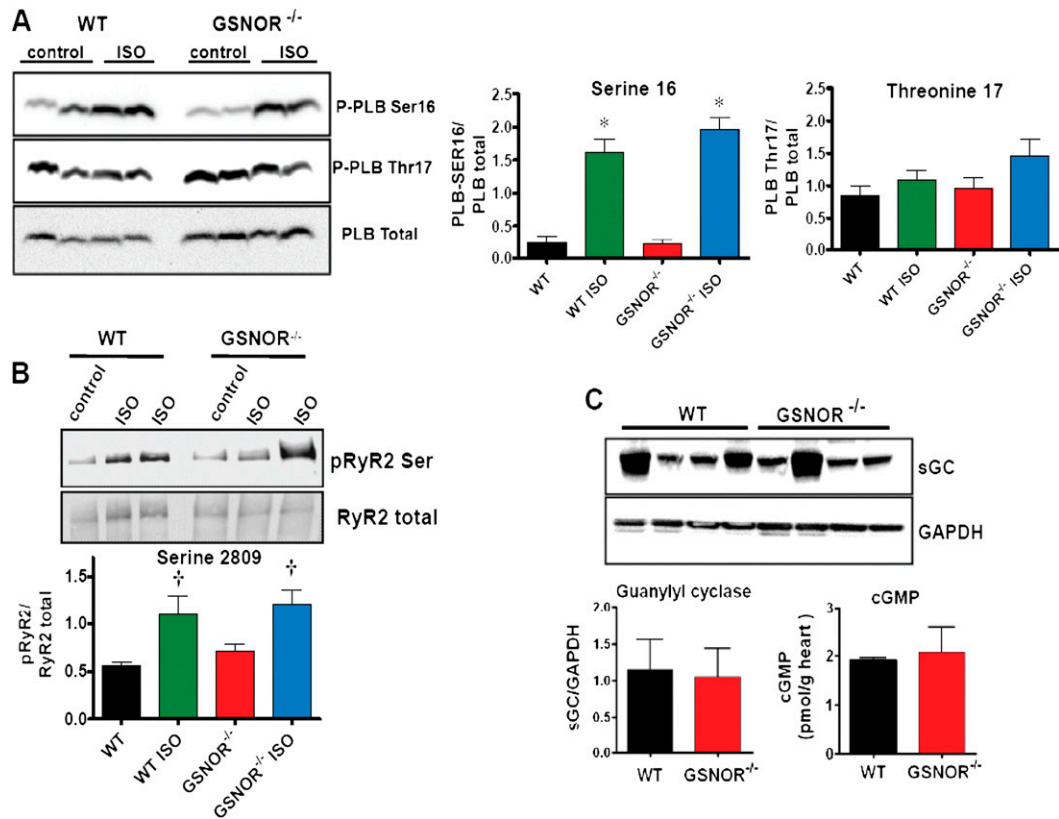
ProPlus version 6.3 (Media Cybernetics). Quantitative colocalization analysis was also performed after the images were deconvolved with Huygens Essential software, version 3.4 (Scientific Volume Imaging) for computing the Pearson correlation coefficient.



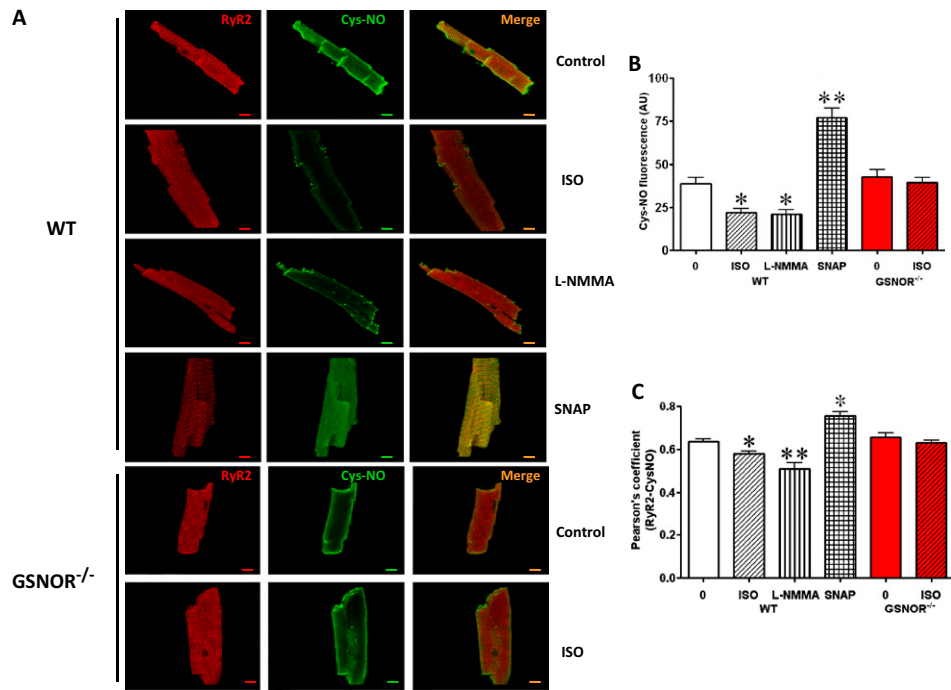
**Fig. S1.** Localization of RyR2 and GSNOR in the heart. (A) Western blot for GSNOR in heart extracts from mouse and human. Grouping of images from within the same gel is indicated by the dividing line. (B) Western blot analysis for RyR2 expression, which is similar in WT ( $n = 9$ ) and GSNOR<sup>-/-</sup>-deficient mouse hearts ( $n = 10$ ). (C) Confocal fluorescent microscopy images depict GSNOR and RyR2 colocalization along the T-tubular invaginations of the cardiac myocyte. Insets show higher magnification of the indicated sections. (Scale bar: 10  $\mu$ M.)



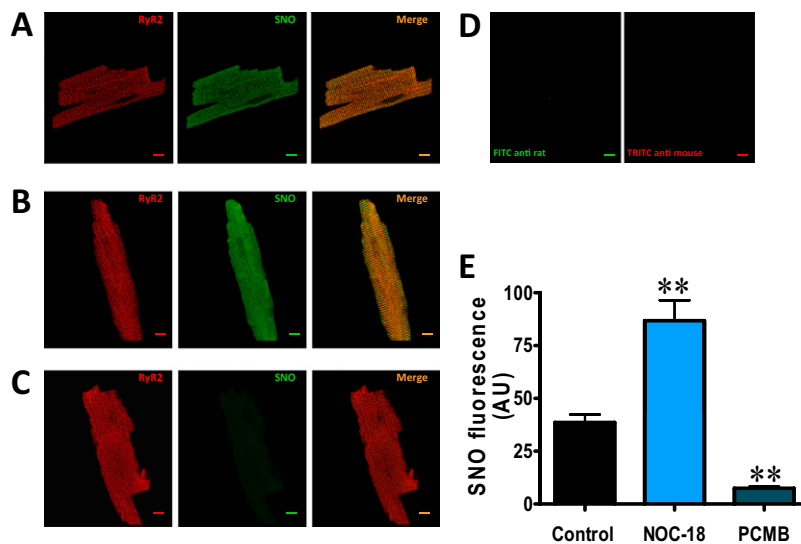
**Fig. S2.**  $\beta$ -Adrenergic inotropic response in GSNOR<sup>-/-</sup> mice myocytes. NOS inhibition [ $N^G$ -monomethyl-L-arginine monoacetate (L-NMMA); 100  $\mu$ M] but not inhibition of PKG (KT5823; 1  $\mu$ M) normalizes the  $\beta$ -adrenergic response in GSNOR<sup>-/-</sup> myocytes (\* $P < 0.05$  vs. control).



**Fig. 53.** Intact  $\beta$ -adrenergic receptor signaling and unaffected levels of guanylyl cyclase (sGC) and cGMP in  $GSNOR^{-/-}$  hearts. (A) Western blot analysis of the phosphorylation levels of phospholamban (PLB) at Ser-16 and Thr-17. (B) Phosphorylation of RyR2 at Ser-2809 in isolated perfused hearts at control conditions and after 5 min of application of ISO (2.5 nM). (C) Western blot analysis of guanylyl cyclase expression (*Upper*) and enzyme immunoassay of cGMP levels in WT and  $GSNOR^{-/-}$  hearts (*Lower*).



**Fig. 54.** S-nitrosylation and denitrosylation of RyR2. (A) Confocal images of single cardiac myocytes immunostained for RyR2 (red) and S-nitrosocysteine (green). Merged images were deconvoluted, and the colocalization analysis was performed in WT mice [control (without treatment), ISO (1  $\mu$ M for 10 min), L-NMMA (2 mM for 2 h), and S-nitroso-*N*-acetylpenicillamine (SNAP; 1 mM for 20 min)] and in GSNOR<sup>-/-</sup> mice [control (without treatment) and ISO (1  $\mu$ M for 10 min)]. (Scale bar: 10  $\mu$ M.) (B) The graph depicts the average fluorescence signal ( $\pm$ SEM,  $n = 6$ ) representative of S-nitrosothiol (SNO) in cardiac myocytes in each treatment group. \* $P < 0.01$  vs. WT and \*\* $P < 0.001$  vs. WT. (C) The graph depicts the values for the Pearson coefficient of correlation for colocalization. Number of analyzed cardiomyocytes: WT,  $n = 23$ ; WT+ISO,  $n = 10$ ; GSNOR<sup>-/-</sup>,  $n = 26$ ; and GSNOR<sup>-/-</sup>+ISO,  $n = 10$ . \* $P < 0.05$  and \*\* $P < 0.01$  compared with WT 0, ANOVA, Kruskal-Wallis posttest.



**Fig. 55.** Validation of the use of immunostaining for detection of S-nitrosylation. Confocal images of single cardiac myocyte immunostained for RyR2 (red) and S-nitrosocysteine (SNO; green) and merged. (A) WT myocyte (control) stained with anti-RyR2 from Affinity BioReagents and anti-rat S-nitrosocysteine from A. G. Scientific. (B) WT treated with NOC-18 (1 mM for 20 min). (C) WT treated with *p*-chloromercuribenzoate (PCMB; 3.5 mM for 30 min) to deplete SNO. (D) Negative control with secondary antibodies only. (E) The graph depicts the average fluorescence signal  $\pm$  SEM of six cardiac myocytes analyzed in each treatment. \*\* $P < 0.001$  vs. control. Each image is a representative of three separate animals. (Scale bars: 10  $\mu$ M.)

**Table S1. Comparison of in vivo hemodynamic variables between WT and GSNOR<sup>-/-</sup> mice**

Mice	<i>n</i>	<i>dP/dt</i> <sub>max</sub> , mmHg/s	<i>dP/dt</i> -EDV, mmHg/s per mL	<i>dP/dt</i> -IP, 1/s	PRSW, mmHg	Tau, ms
WT	6	11,658 ± 436	223 ± 14	172 ± 6	71 ± 4	4.7 ± 0.3
GSNOR <sup>-/-</sup>	4	10,828 ± 515	239 ± 35	173 ± 9	77 ± 8	4.8 ± 0.2

Conductance manometry was at 600 beats per min. PRSW, preload recruitable stroke work.

**Table S2. List of SNO proteins detected in mouse whole-heart homogenates of WT and GSNOR<sup>-/-</sup> after immunoprecipitation or MS/MS**

SNO protein	Molecular mass, kDa	Immunoprecipitation	MS/MS
RyR2	560	+	+
β-Myosin heavy chain (B-MHC)	222	+	
LTCC	210	+	
Xanthine oxidoreductase (XOR)	150	+	
NOS3	140	+	
Plasma membrane Ca <sup>2+</sup> ATPase (PMCA)	140	+	
Sodium-calcium exchanger (NCX)	120	-	
SERCA2a	110	+	+
G protein-coupled receptor kinase 2 (GRK2)	80	+	
β1-Adrenergic receptor (B1-AR)	64	+	
GAPDH	38	+	+

(+) Positive and (-) negative detection post biotin-switch assay. *n* = 2-8.

**Table S3. MS/MS peptide summary reports (from Mascot) for the 550- and 110-kDa gel-cut protein bands, obtained by biotin-switch analysis on mouse heart homogenate and identified as cardiac RyR2 and SERCA2, respectively**

Observed	<i>M<sub>r</sub></i> (expt)	<i>M<sub>r</sub></i> (calc)	Delta	Miss	Score	Expect	Rank	Peptide
Ryandine receptor 2 (RyR2), cardiac (Mus musculus)*								
586.4427	1,170.8708	1,170.4975	0.3734	0	(35)	8.5	1	R.EGDSMALYNR.T + oxidation (M)
586.4592	1,170.9038	1,170.4975	0.4063	0	50	0.24	1	R.EGDSMALYNR.T + oxidation (M)
596.0361	1,190.0576	1,189.6012	0.4564	0	37	4.4	1	K.GETGPMVAATLK.L + oxidation (M)
596.8087	1,191.6028	1,191.6321	0.0293	0	40	2.2	1	K.NVMPLSAGLFK.S + oxidation (M)
603.1577	1,204.3008	1,203.6499	0.6509	0	38	4.1	1	R.LVPYTLDDR.T
611.9487	1,221.8828	1,221.6023	0.2805	0	(38)	3.7	1	R.AIDMSNVTLR.D + oxidation (M)
612.0207	1,222.0268	1,221.6023	0.4245	0	56	0.052	1	R.AIDMSNVTLR.D + oxidation (M)
615.7842	1,229.5538	1,228.7179	0.8359	0	(47)	0.46	1	R.LAVFSQPIINK.V
615.8452	1,229.6758	1,228.7179	0.9579	0	51	0.2	1	R.LAVFSQPIINK.V
668.0392	1,334.0638	1,333.7645	0.2993	0	54	0.1	1	K.VVLPIDQYFK.N
692.4442	1,382.8738	1,382.7598	0.1141	0	46	0.53	1	R.DLYAFYPLLIR.F
722.0572	1,442.0998	1,441.6572	0.4427	0	49	0.3	1	K.EAAVPEEEGGTPEK.E
722.5046	1,442.9946	1,441.6572	1.3375	0	(38)	3.6	1	K.EAAVPEEEGGTPEK.E
807.2317	1,612.4488	1,611.8943	0.5545	0	76	0.00055	1	K.LGIAILNGNSTVQQK.M
SR ATPase, Ca <sup>2+</sup> transporting, cardiac muscle, slow twitch 2 (SERCA2) (Mus musculus) <sup>†</sup>								
704.5457	1,407.0768	1,406.7075	0.3694	0	54	0.013	1	K.SEIGIAMGSGTAVAK.T + oxidation
705.0627	1,408.1108	1,407.6881	0.4227	0	56	0.0083	1	R.IGIFGQDEDVTSK.A
781.5856	1,561.1566	1,560.8181	0.3386	0	58	0.0055	1	K.AMGVVVATGVNTEIGK.I + oxidation
788.1697	1,574.3248	1,573.8562	0.4686	0	(79)	4.2 × 10 <sup>-5</sup>	1	R.VDQSILTGESVSVIK.H
788.6841	1,575.3536	1,573.8562	1.4974	0	(89)	4.7 × 10 <sup>-6</sup>	1	R.VDQSILTGESVSVIK.H

\*GI:13569850; mass: 569,728; score: 534.

<sup>†</sup>GI:6806903; mass: 109,682; score: 502.