Supporting Information for

Original article

Sorting nexin 3 exacerbates doxorubicin-induced cardiomyopathy *via* regulation of TFRC-dependent ferroptosis

Wenjing Yu[a,b,†], Yuehuai Hu[a,b,†], Zhiping Liu[c,†], Kaiteng Guo[a], Dinghu Ma[c], Mingxia Peng[a], Yuemei Wang[a], Jing Zhang[a], Xiaolei Zhang[a], Panxia Wang[a], Jiguo Zhang[d], Peiqing Liu[a,b,d,*], Jing Lu[a,b,*]

[a]*National and Local United Engineering Lab of Druggability and New Drugs Evaluation, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, China*

[b]*Guangdong Provincial Key Laboratory of New Drug Design and Evaluation, Guangdong Province Engineering Laboratoty for Druggability and New Drug Evaluation, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, China*

[c]*School of Pharmacy, Jinan University, Guangzhou 510632, China*

[d]*School of Pharmaceutical Sciences, Shandong First Medical University & Shangdong Academy of Medical Sciences, Taian 271016, China*

Received 17 January 2023; received in revised form 6 June 2023; accepted 13 June 2023

*Corresponding authors.

E-mail addresses: liupq@mail.sysu.edu.cn (Peiqing Liu), lujing28@mail.sysu.edu.cn (Jing Lu).

[†]These authors made equal contributions to this paper.

1) Tables S1–S5

2) Figure S1–S13

Table S1 The primer sequences were used for genotyping *Snx3*-flox and the activity of *Myh6-Cre*.

Genotype	Primer	Sequences
*Snx*3-flox	P5 (Forward)	5′-CTTTTAGAGGAGACGATGGAATA-3′
	P6 (Reverse)	5′-AGAAAGGCTGGAAGTGGCTAAA-3′
Myh6-Cre	P7 (COM)	5′- TTCCCAAGGGCATTTTATTAG-3′
	P8 (WT)	5′-CTTTGGGCTTGGCATCATCTGGT-3′
	P9 (Mut)	5′-CAGCCCCTTGTTGAATACG-3′

Abbreviations: *Myh6-Cre*, C57BL/6J-*Myh6*$^{em1(IRES\text{-}Cre)Smoc}$.

Table S2 The primer sequences of mouse *Snx3* gene for genotyping *Snx3-cTg* mice.

Primer	Sequences
Forward	5′-GCGTTGGCTACCCGTGATATT-3′
Reverse	5′-CACGACATTCAACAGACCTT-3′

Table S3 The duplex shRNAs for rats *Snx3* gene.

Primer	Sequences
shRNA-1	GGTCAAGACCAATCTTCCTATTTCAAGAGAATAGGAAGATTGGTCTTGACCTTTTTT
shRNA-2	GACTTTGAGTGGCTTCGAAGTTTCAAGAGAACTTCGAAGCCACTCAAAGTCTTTTTT
shRNA-3	GAGGAGACGATGGAATATTTGTTCAAGAGACAAATATTCCATCGTCTCCTCTTTTTT
shRNA-4	GGAACAGTTCATAAACAAGGTTTCAAGAGAACCTTGTTTATGAACTGTTCCTTTTTT
scrambled shRNA	GCCTAAGGTTAAGTCGCCCTCGTTCAAGAGACGAGGGCGACTTAACCTTAGGCTTTTTT

Table S4 The duplex siRNAs for rats *TFRC* gene.

Primer	Sequences
siRNA-1	Forward: 5′-CCUAAAUCUUCUCGCUUAUTT-3′
	Reverse: 5′-AUAAGCGAGAAGAUUUAGGTT-3′
siRNA-2	Forward: 5′-CCAUAGAUUCACUGACAUTT-3′
	Reverse: 5′-AUGUCAGUGAACUCUAUGGTT-3′
siRNA-3	Forward: 5′-GCUGGAACUUUCACAGAAUTT-3′
	Reverse: 5′-AUUCUGUGAAAGUUCCAGCTT-3′

Table S5 The primer sets used for qPCR.

Species	Gene	Gene sequence	Primer sequence
Rattus norvegicus	*Snx3*	NM_001044283.1	FP: 5′-AAGAGGCAAACCCCAAGACC-3′ RP: 5′-TTAGTACATCCAGGGCGTGC-3′
	Fth1	NM_012848.2	FP: 5′ TGCCATCAACCGCCAGATCAAC-3′ RP: 5′-AAAGTTCTTCAGGGCCACATCATCC-3′
	Slc7a11	NM_001107673.3	FP: 5′-CCATCATCATCGGCACCGTCATC-3′ RP: 5′-TACTCCACAGGCAGACCAGAACAC-3′
	Ptgs2	NM_017232.3	FP: 5′-ATCTGTGGTGGGTTTCTCATAG-3′ RP: 5′-ATCCACAGCAGAAGTCAAGTTA-3′
	Tfrc	NM_022712.1	FP: 5′-GTTCCCCGTTGTTGAGGCAGAC-3′ RP:5′-GATGACTGAGATGGCGGAAACTG-3′
	Beta-actin	NM_031144.3	FP: 5′-GCTGTGCTATGTTGCCCTAGACTTC-3′ RP: 5′-GGAACCGCTCATTGCCGATAGTG-3′
Mus musculus	*Snx3*	NM_017472.4	FP: 5′-TCGCTCCAGCCTCTCTAAGT-3′ RP: 5′-AGCAAGTCGGCAGCTTTAGT-3′
	T	NM_011638.4	FP: 5′-TCGTGGAGACTACTTCCGTGCTAC-3′ RP: 5′-TCTTGGAGATACATAGGGCGACAGG-3′
	Ptgs2	NM_011198.4	FP:5′-CTGGTGCCTGGTCTGATGATGTATG-3′ RP:5′-GGATGCTCCTGCTTGAGTATGTCG-3′
	Beta-actin	NM_007393.5	FP: 5′-TATGCTCTCCCTCACGCCATCC-3′ RP: 5′-GTCACGCACGATTTCCCTCTCAG-3′

(FP: forward primer; RP: reverse primer)

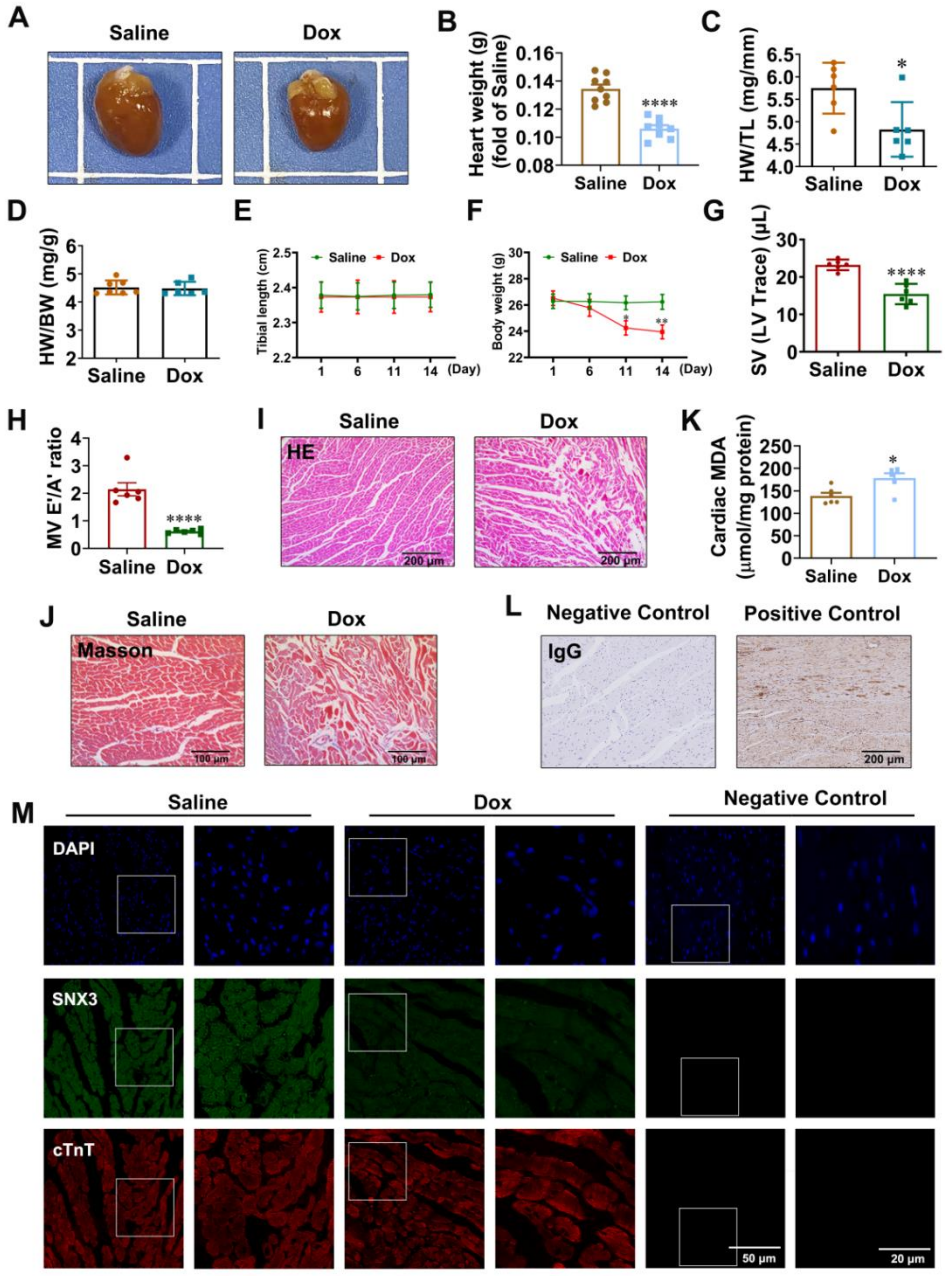

Figure S1 SNX3 expression is downregulated in Dox-induced cardiomyopathy *in vivo*, related to Figure 1.

Male C57BL/6 mice were i.p. injected with Dox or saline three times (on days 1, 6, and11, respectively). **(A)** Gross observations of heart morphology were shown. **(B)** The heart weight (HW) was measured. **(C)** The HW/TL ratio was measured. **(D)** The HW/BW ratio was measured. **(E, F)** The tibia length and body weight of mice in Dox or saline group were measured dynamically. **(G, H)** The echocardiographic parameters, including HW/TL, stroke volume and myocardial performance index (MV E'/A' ratio) were measured. **(I–J)** HE dyeing (scale bar: 200 μm) and Masson's trichrome dyeing (scale bar: 100 μm) were offered. **(K)** The intracellular MDA was assayed by the malondialdehyde assay kit. **(L)** The negative and positive control of IHC staining. **(M)** Representative immunofluorescence images of SNX3 and cTnT staining (scale bar: 20 μm) in myocardial tissues. Five independent experiments' typical pictures were offered. The data are shown as the mean ± SEM, n = 6–9. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs.* Saline group.

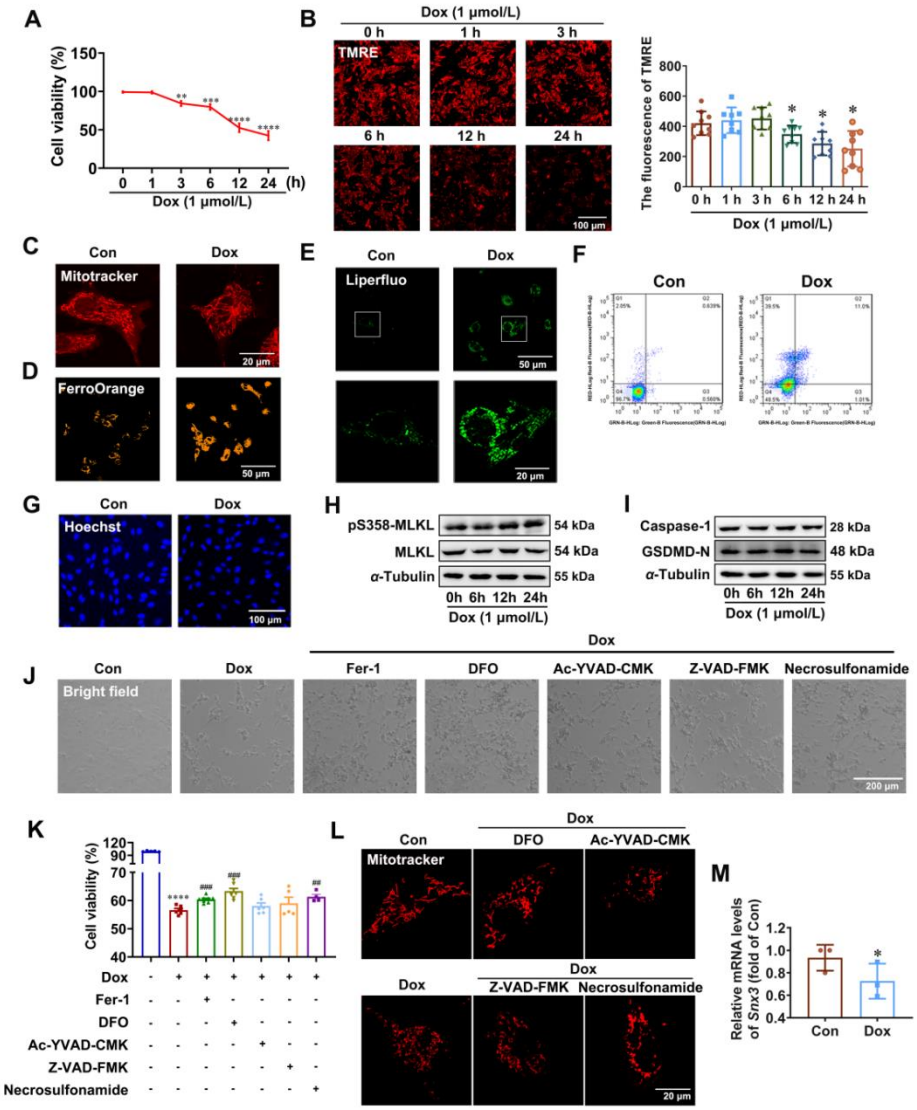

Figure S2 SNX3 expression is downregulated in Dox-induced cardiomyopathy in NRCMs, related to Figure 1.

NRCMs were handled with Dox (1 μmol/L) for the indicated time. And NRCMs were pre-infected with Vector or Ad-SNX3 for 24 h and then were treated with Z-VAD-FMK(10 μmol/L), Ferrostatin-1(3 μmol/L), DFO(100 μmol/L), Necrosulfonamide(1 μmol/L) and Ac-YVAD-CMK (1 μmol/L) for an additional 24 h or Dox(1 uM) for an extra 12 h in the presence of Vector or Ad-SNX3. **(A)** CCK-8 assay for cell viability. **(B)** TMRE dye (scale bar: 100 μm) for mitochondrial membrane potential. **(C)** Mitotracker-red staining (scale bar: 20 μm) for mitochondrial morphology in NRCMs treated with Dox (1 μmol/L) for 12 h. **(D)** FerroOrange dyeing (scale bar: 50 μm) for intracellular Fe^{2+}. **(E)** Liperfluo staining (scale bar: 50 μm) for intracellular liperROS. **(F)** The ratio of apoptosis was measured by FCM, Q1: non-viable cell, Q2: late apoptosis cell, Q3: early apoptosis cell, Q4: normal cell. **(G)** Hochest-dye was used to assess the morphology of nuclear chromatin. **(H, I)** The apoptosis-related and necroptosis-related proteins were measured by Western blotting. **(J)** Bright field observation of cell morphology to measure cell viability. **(K)** CCK-8 assay for cell viability. **(L)** Mitotracker-red staining (scale bar: 20 μm) for mitochondrial morphology. **(M)** The mRNA expressions of SNX3 were detected by qPCR. Five independent experiments' typical pictures were offered. The typical pictures from five independent experiments were offered. The data are shown as the mean ± SEM, n = 3–5. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs* Con group.

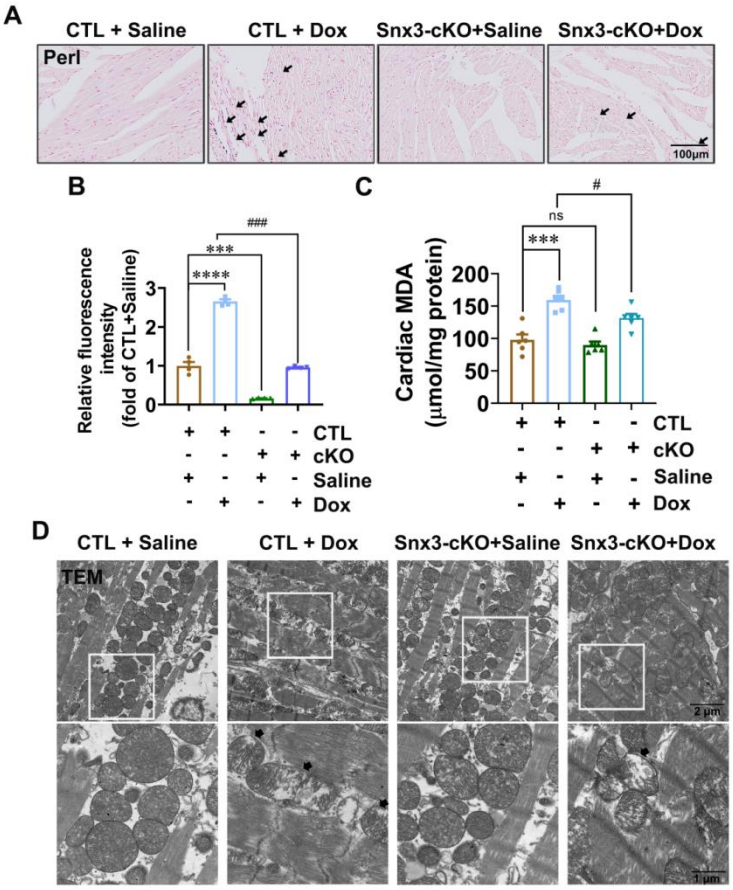

Figure S3 Deletion of cardiac Snx3 attenuates Dox-induced ferroptosis in mice, related to Figure 2.

Snx3-cKO mice and their respective *CTL* were i.p. injected with Dox or saline by three injections (on Days 1, 6, and 11). **(A)** Perl dyeing (scale bar: 100 μm) of the heart is presented. **(B)** The histograms of relative fluorescence intensity for DHE dye. **(C)** The lipid peroxidation level in hearts was assessed by measuring MDA levels. **(D)** Transmission electron microscopy of heart tissues (scale bar: 1 μm). The typical pictures from five independent experiments were offered. The data are shown as the mean ± SEM, n = 6–9. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs*. *CTL* + Saline group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001, $^{\#\#\#\#}P$ < 0.0001 *vs*. *CTL* + Dox group.

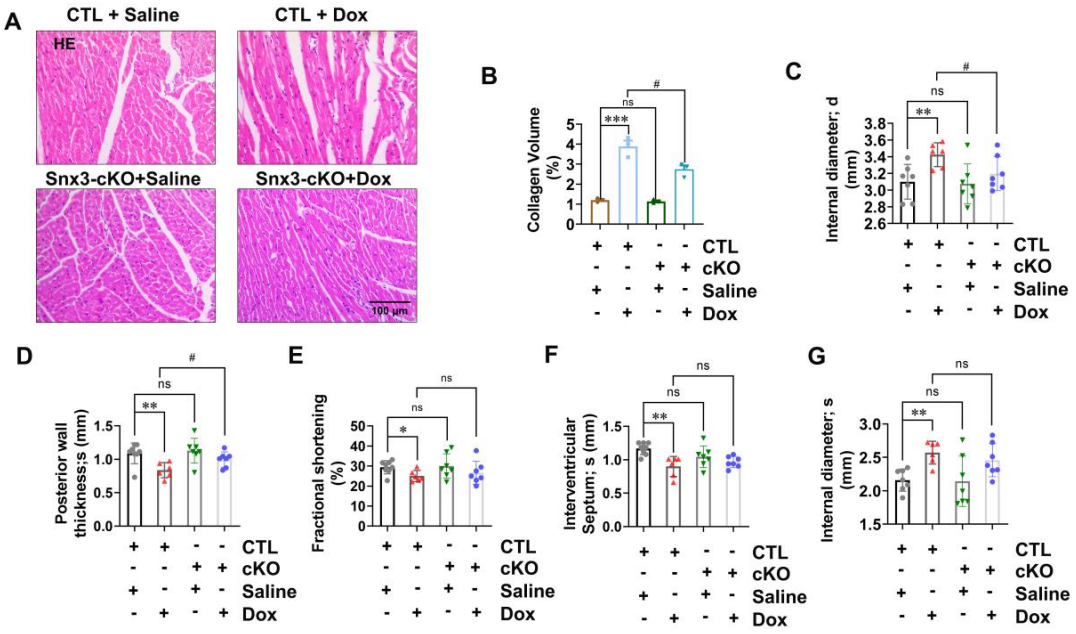

Figure S4 Deletion of cardiac Snx3 attenuates Dox-induced cardiomyopathy in mice, related to Figure 2. *Snx3-cKO* mice and their respective *CTL* were *i.p.* injected with Dox or saline by three injections (on days 1, 6, and 11). **(A)** HE dyeing (scale bar: 100 μm) were shown. **(B)** Heart sections from *CTL* and *Snx3-cKO* mice after Dox or saline treatment were stained with picrosirius red (PSR) to visualize collagen deposition. **(C-G)** The echocardiographic parameters include end-systolic interventricular septum, end-systolic/end-diastolic posterior wall thickness, end-systolic/end-diastolic internal diameter, LV trace FS %. Five independent experiments' typical pictures were offered. The data were showed as the means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 *vs*. *CTL* + Saline group, $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001, $^{\#\#\#\#}P$ < 0.0001 *vs*. *CTL* + Dox group. *n* = 6-9.

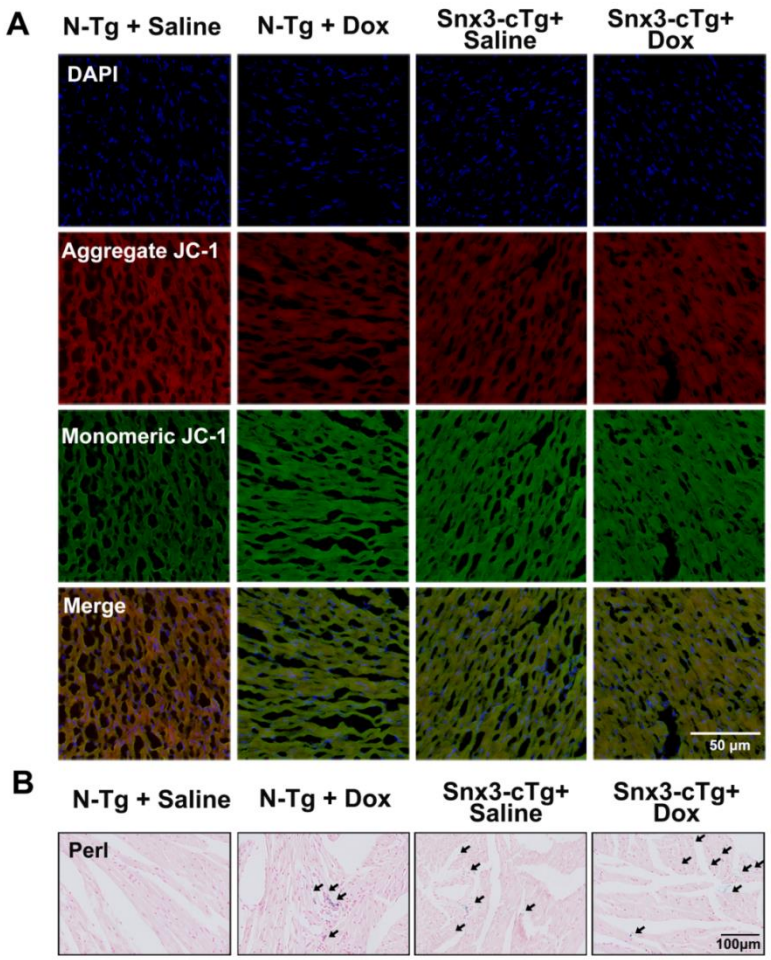

Figure S5 Cardiac-specific Snx3 transgene aggravates Dox-induced ferroptosis in mice, related to Figure 3.

Snx3-cTg mice and their respective *N-Tg* were i.p. injected with Dox or saline three times (on Days 1, 6, and 11). **(A)** Typical pictures of cardiac by JC-1 dyeing (scale bar: 50 μm) in frozen heart slides. **(B)** Perl dyeing (scale bar: 100 μm) of the heart was presented. The typical pictures from five independent experiments were offered. The data are shown as the mean ± SEM, n = 6–9. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs. N-Tg* + Saline group.

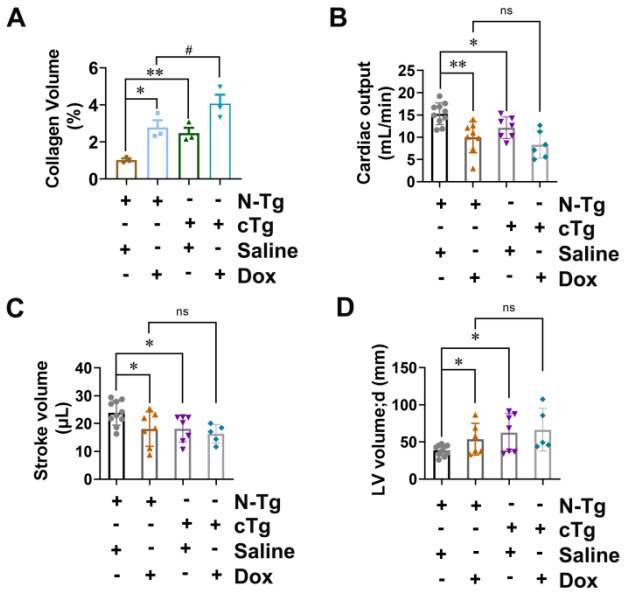

Figure S6 Cardiac-specific Snx3 transgene aggravates Dox-induced cardiomyopathy in mice, related to Figure 3. *Snx3-cTg* mice and their respective *N-Tg* were *i.p.* injected with Dox or saline three times (on Days 1, 6, and 11). **(A)** Heart sections from *N-Tg* and *Snx3-cTg* mice after Dox or saline treatment were stained with picrosirius red (PSR) to visualize collagen deposition. **(B–D)** The echocardiographic parameters were calculated, including cardiac output, stroke volume, and LV end-diastolic volume. The typical pictures from five independent experiments were offered. The data are shown as the mean ± SEM, n = 6–9. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs. N-Tg* + Saline group.

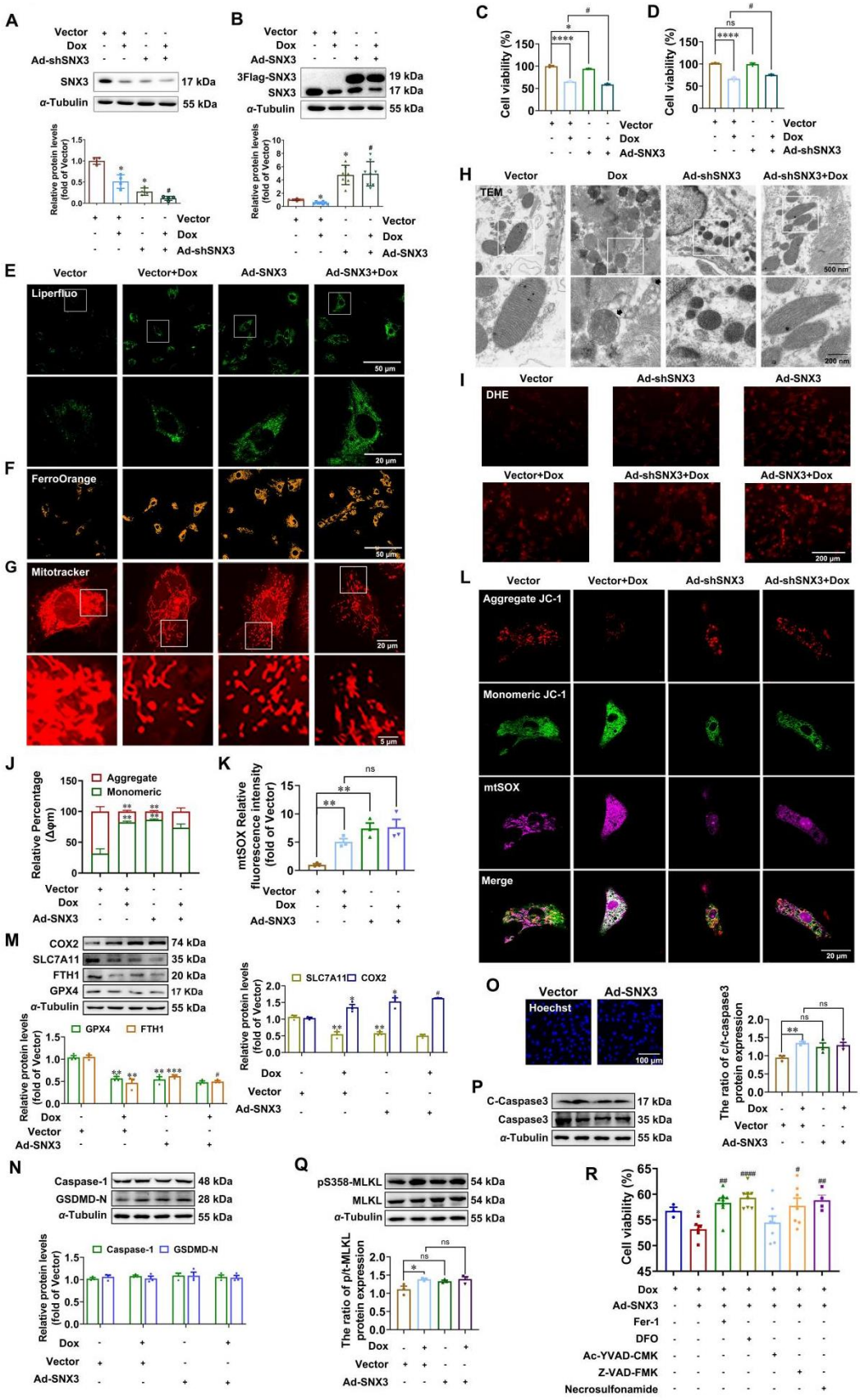

Figure S7 SNX3 exacerbated Dox-induced cardiomyopathy by inducing ferroptosis in cardiomyocytes, related to Figure 4.

NRCMs were pre-infected with Ad-shSNX3 or Ad-SNX3 for 36 h and then were treated with Dox (1 μmol/L) for an extra 12 h in the presence of Ad-shSNX3 or Ad-SNX3. **(A, B)** The SNX3 protein was measured by Western Blotting. **(C, D)** CCK-8 assay to measure cell viability. **(E**–**H)** Liperfluo dyeing (scale bar: 50 μm) for intracellular liperROS; FerroOrange dyeing (scale bar: 50 μm) for intracellular Fe^{2+}; Mitotracker-red staining (scale bar: 5 μm), TEM (scale bar: 200 nm). **(I)** The typical pictures (scale bar: 200 μm) for DHE dye. **(J, K)** The histograms of relative fluorescence intensity for JC-1 dyes and MitoSox-Red. **(L)** Representative images of NRCMs were live stained with JC-1 dyes and MitoSox-Red (scale bar: 20 μm) for mitochondrial ROS. **(M)** Western blotting evaluated the ferroptosis-related proteins expression in NRCMs. **(N)** Western blotting evaluated thepyroptosis-related proteins expression in NRCMs. **(O)** Hochest-dye was used to assess the morphology of nuclear chromatin. **(P**–**Q)** The apoptosis-related and necroptosis-related proteins were measured by Western blotting. **(R)** CCK-8 assay to measure cell viability. The typical pictures from five independent experiments were offered. The data are shown as the mean ± SEM, n = 3–5. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs.* Vector group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001, $^{\#\#\#\#}P$ < 0.0001 *vs*. Vector + Dox group.

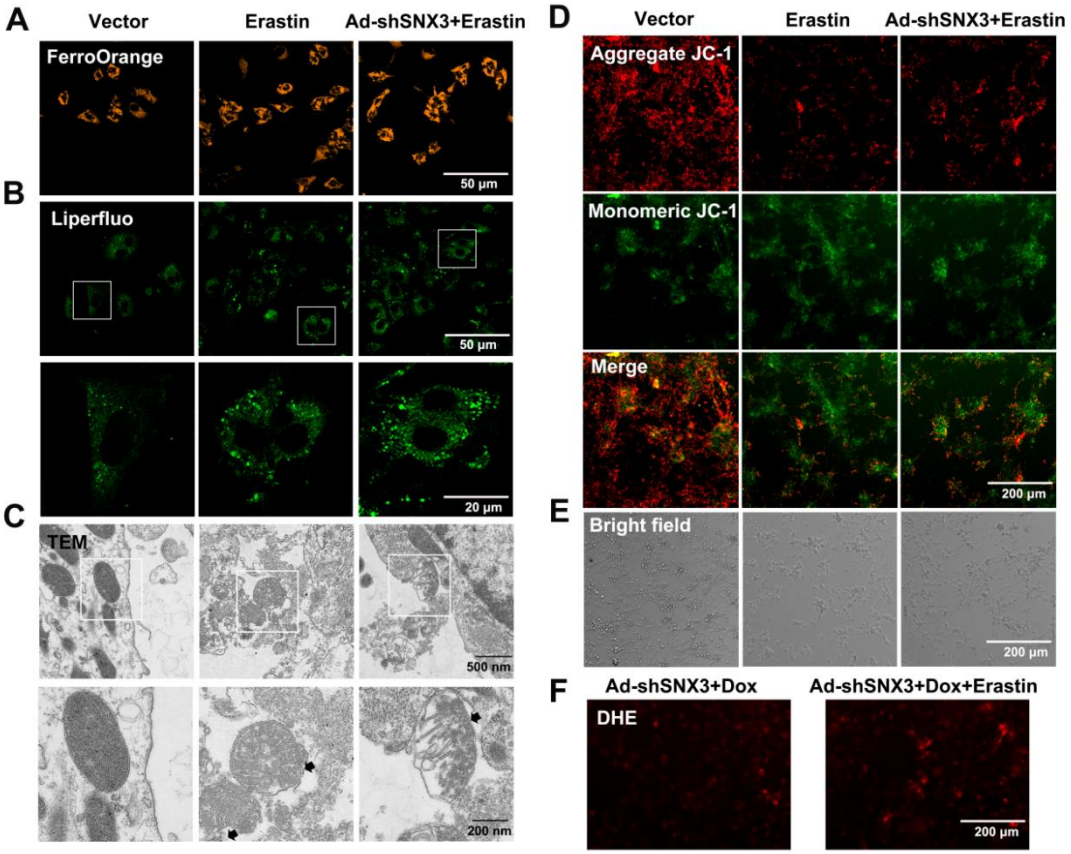

Figure S8 Ad-shSNX3 alleviates doxorubicin-induced cardiomyopathy by inhibiting ferroptosis *in vitro*, related to Figure 5.

NRCMs were pre-infected with Ad-shSNX3 for 24 h and then were treated with Erastin(10 μmol/L) for an additional 24 h or Dox(1 μmol/L) for an extra 12 h in the presence of Ad-shSNX3. **(A**–**D)** The typical pictures of FerroOrange dyeing (scale bar: 50 μm) , Liperfluo dyeing(scale bar: 20 μm), TEM (scale bar: 200 nm), JC-1 dyeing (scale bar: 50 μm). **(E)** Cell morphology in bright field. **(F)** The typical pictures and fluorescence intensity of DHE dyeing (scale bar: 200 μm). The data are shown as the mean ± SEM, n = 3–5.

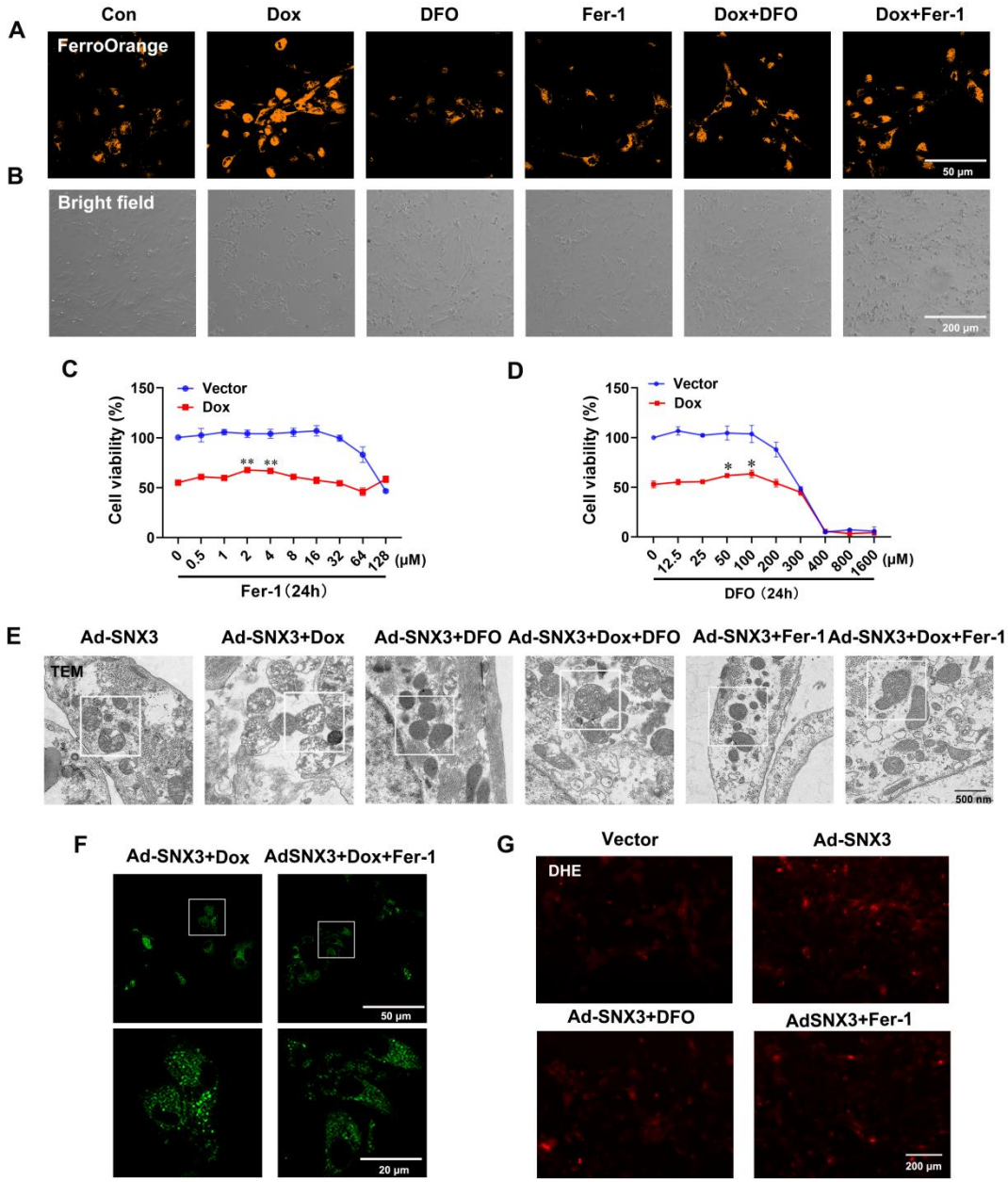

Figure S9 SNX3 exacerbates doxorubicin-induced cardiomyopathy by promoting ferroptosis *in vitro*, related to Figure 5.

NRCMs were pre-infected with Ad-SNX3 for 24 h and then were treated with DFO (100 μmol/L) or Fer-1 (3 μmol/L) for an additional 24 h or Dox (1 μmol/L) for an extra 12 h in the presence of Ad-SNX3. **(A, B)** FerroOrange staining (scale bar: 50 μm) for intracellular Fe^{2+}, cell morphology (scale bar: 200 μm) in bright field. **(C, D)** CCK-8 assay to measure cell viability. **(E–G)** TEM (scale bar: 200 nm) for mitochondrial morphology, Liperfluo staining (scale bar: 20 μm) for liperROS, DHE staining (scale bar: 200 μm) for total ROS. Five independent experiments' typical pictures were offered. The data are shown as the mean ± SEM, n = 3–5. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs*. Vector group.

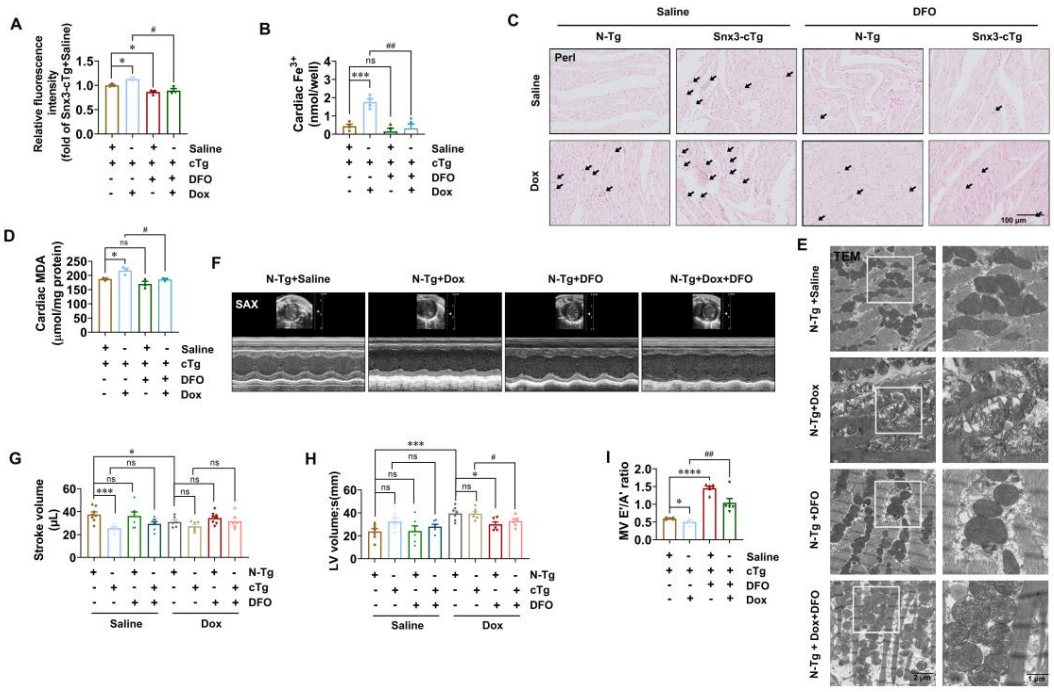

Figure S10 Forced expression of cardiac-specific Snx3 aggravates Dox-induced cardiomyopathy by induction of ferroptosis, related to Figure 6.

Snx3-cTg mice were i.p. injected DFO (100 mg/kg) or corresponding vehicle (15% castor oil) thrice a week for 4 weeks. 3 weeks after therapy of DFO, the animals were randomized assigned to treat with Dox or saline for last week. 3 days after the Dox treatment, mice were analyzed by echocardiography. **(A)** The histogram of relative fluorescence intensity for DHE staining (scale bar: 50 μm) of frozen heart slides. **(B)** The Fe^{3+} contents of the cardiac tissues. **(C)** Perl dyeing (scale bar: 100 μm) were exhibited. **(D)** The malondialdehyde assay kit was used to detect lipid peroxidation in cardiac tissues. **(E)** Transmission electron microscopy of heart tissues (scale bar: 1 μm), and representative images for cardiac tissue. The typical pictures from five independent experiments are offered. **(F)** The typical graphs of echocardiography. **(G**–**I)** The parameters, including stroke volume, LV end-systolic volume, and myocardial performance index (MV *E′*/*A′* ratio), were measured. The data are shown as the mean ± SEM, *n* =6-9. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 *vs*. *N-Tg* + Saline group or *N-Tg* + Dox group or *Snx3-cTg*+Saline group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001, $^{\#\#\#\#}P$ < 0.0001 *vs*. *Snx3-cTg* + Saline group or *Snx3-cTg* + Dox group.

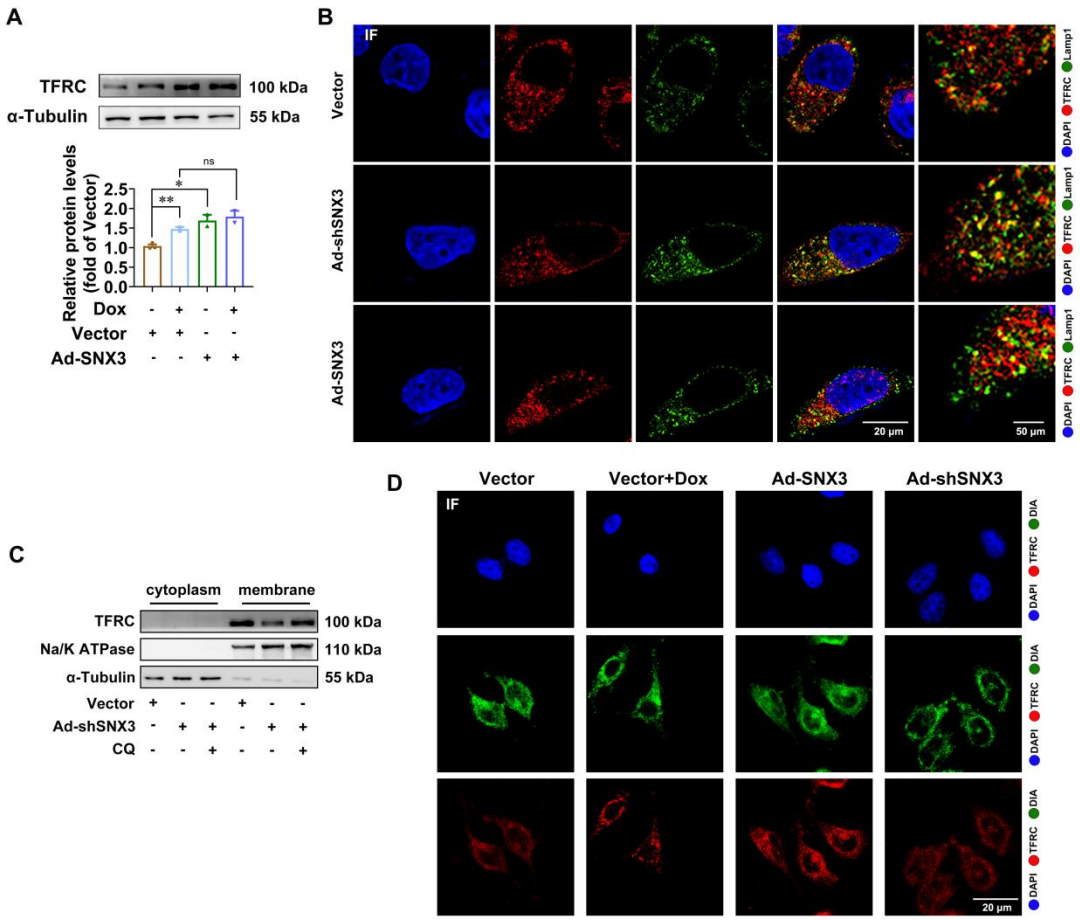

Figure S11 SNX3 facilitates the recycling of TFRC by direct interaction to trigger ferroptosis, related to Figure 7. NRCMs were pre-infected with Ad-shSNX3 or Ad-SNX3 for 36 h and then were treated with Dox (1 μmol/L) for an extra 12 h in the presence of Ad-shSNX3 or Ad-SNX3. Other groups of NRCMs were infected with Ad-SNX3 before transfecting siRNAs of siTFRC and were infected with Ad-shSNX3 before transfecting plasmids of TFRC. **(A)** Western blotting evaluated the protein expression of TFRC in NRCMs. **(B)** Intracellular colocalization of lysosome Lamp1 (green) and TFRC (red) was identified by confocal immunofluorescence microscopy (scale bar: 20 μm). **(C)** Western blotting evaluated the protein expression of TFRC in CQ-treated NRVMs infected with Ad-shSNX3. **(D)** Intracellular colocalization of membrane probe DIA (green) and TFRC (red) was identified by confocal immunofluorescence microscopy (scale bar: 20 μm). The typical pictures from five independent experiments are offered. The data are shown as the mean ± SEM, n =3–5. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs*. Vector group.

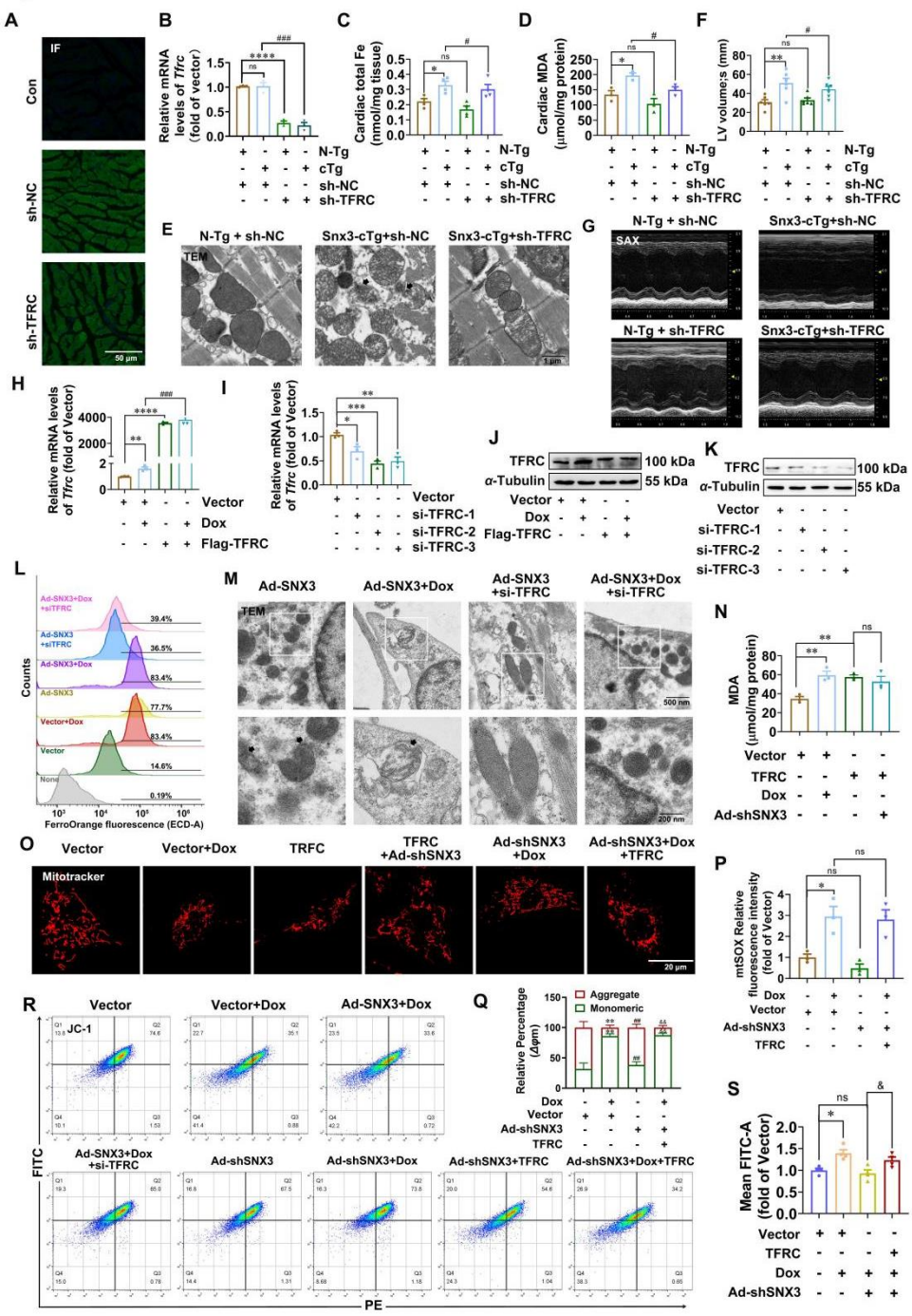

Figure S12 Schematic diagram illustrating SNX3 exacerbates doxorubicin-induced cardiomyopathy by promoting TFRC-dependent ferroptosis, related to Figure 8.

Snx3-cTg mice were intramyocardially injected at three to five sites with sh-TFRC or an empty vector sh-NC at a dose of 10^{11} viral genome particles per mouse. Mice were anesthetized using echocardiography 4 week after the Advinjection; NRCMs were pre-infected with Ad-shSNX3 or Ad-SNX3 for 36 h and then were treated with Dox (1 μmol/L) for an extra 12 h in the presence of Ad-shSNX3 or Ad-SNX3. Other groups of NRCMs were infected with Ad-SNX3 before transfecting siRNAs of siTFRC and were infected with Ad-shSNX3 before transfecting plasmids of TFRC. **(A)** Representative cardiac sections showing the successful delivery of sh-TFRC or sh-NC into mouse myocardium *in vivo* the significant presence of fluorescence elicited by GFP attached to the viral vectors. **(B)** The knockdown efficiency of TFRC

in mouse myocardial tissue was verified by qPCR. **(C, D)** The total Fe contents and lipid peroxidation level of the cardiac tissues. **(E)** Transmission electron microscopy of heart tissues (scale bar: 1 μm). **(F)** The echocardiographic parameter (LV end-systolic volume) was measured. **(G)** The typical graphs of echocardiography. **(H, I)** The *Tfrc* mRNA was measured by qPCR. **(J, K)** The TFRC protein was measured by Western blotting. **(L)** Flow cytometry analysis for intracellular Fe^{2+} by FerroOrange staining. **(M)** Representative TEM mitochondria images of NRCMs at 200 nm. **(N)** The intracellular MDA was assayed by the kit. **(O)** The histogram of relative mean FITC-A fluorescence intensity by C11-BODIPY$^{581/591}$ staining. **(O–Q)** Mitochondrial morphology by mitotracker-red dye (scale bar: 20 μm) and measurement $\Delta\Psi$m by flow cytometry using JC-1staining. **(R, S)** Flow cytometry analysis for JC-1 staining and mitochondrial membrane potential relative histogram. The data are shown as the mean ± SEM, n =3–5. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs*. Vector group; $^{\&}P$ < 0.05, $^{\&\&}P$ < 0.01, $^{\&\&\&}P$ < 0.001, $^{\&\&\&\&}P$ < 0.0001 *vs*. Ad-shSNX3 + Dox group.

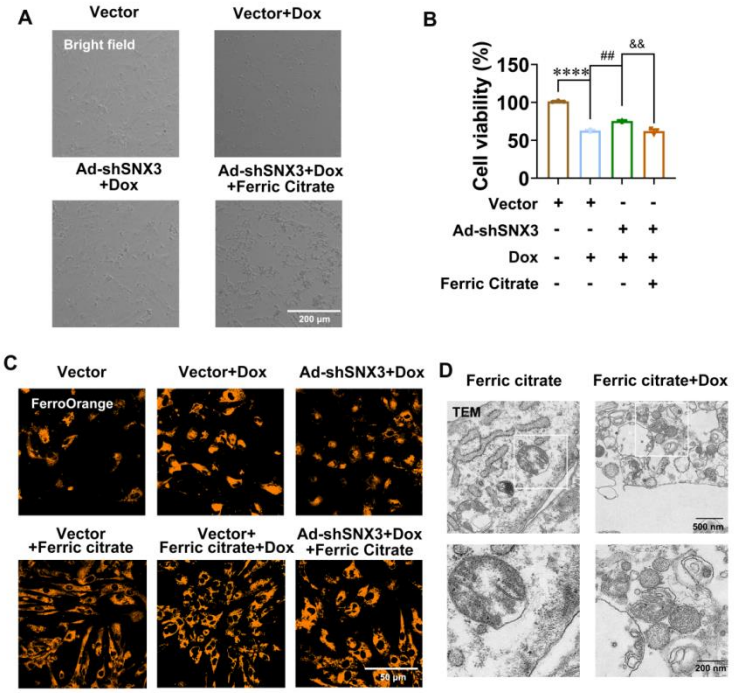

Figure S13 Schematic diagram illustrating SNX3 exacerbates doxorubicin-induced cardiomyopathy by promoting TFRC-dependent ferroptosis, related to Figure 8.

NRCMs were pre-infected with Ad-shSNX3 for 36 h and then were treated with Ferric citrate (100 μmol/L) for an extra 12 h in the presence of Ad-shSNX3. **(A, B)** CCK-8 assay to measure cell viability. **(C, D)** FerroOrange dyeing (scale bar: 20 μm) for intracellular Fe^{2+} and TEM (scale bar: 200 nm) for mitochondrial morphology. The typical pictures from five independent experiments were offered. The data are shown as the mean ± SEM, n =3–5. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs*. Vector group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001, $^{\#\#\#\#}P$ < 0.0001 *vs*. Dox group; $^{\&}P$ < 0.05,$^{\&\&}P$ < 0.01, $^{\&\&\&}P$ < 0.001, $^{\&\&\&\&}P$ < 0.0001 *vs*. Ad-shSNX3 + Dox group.