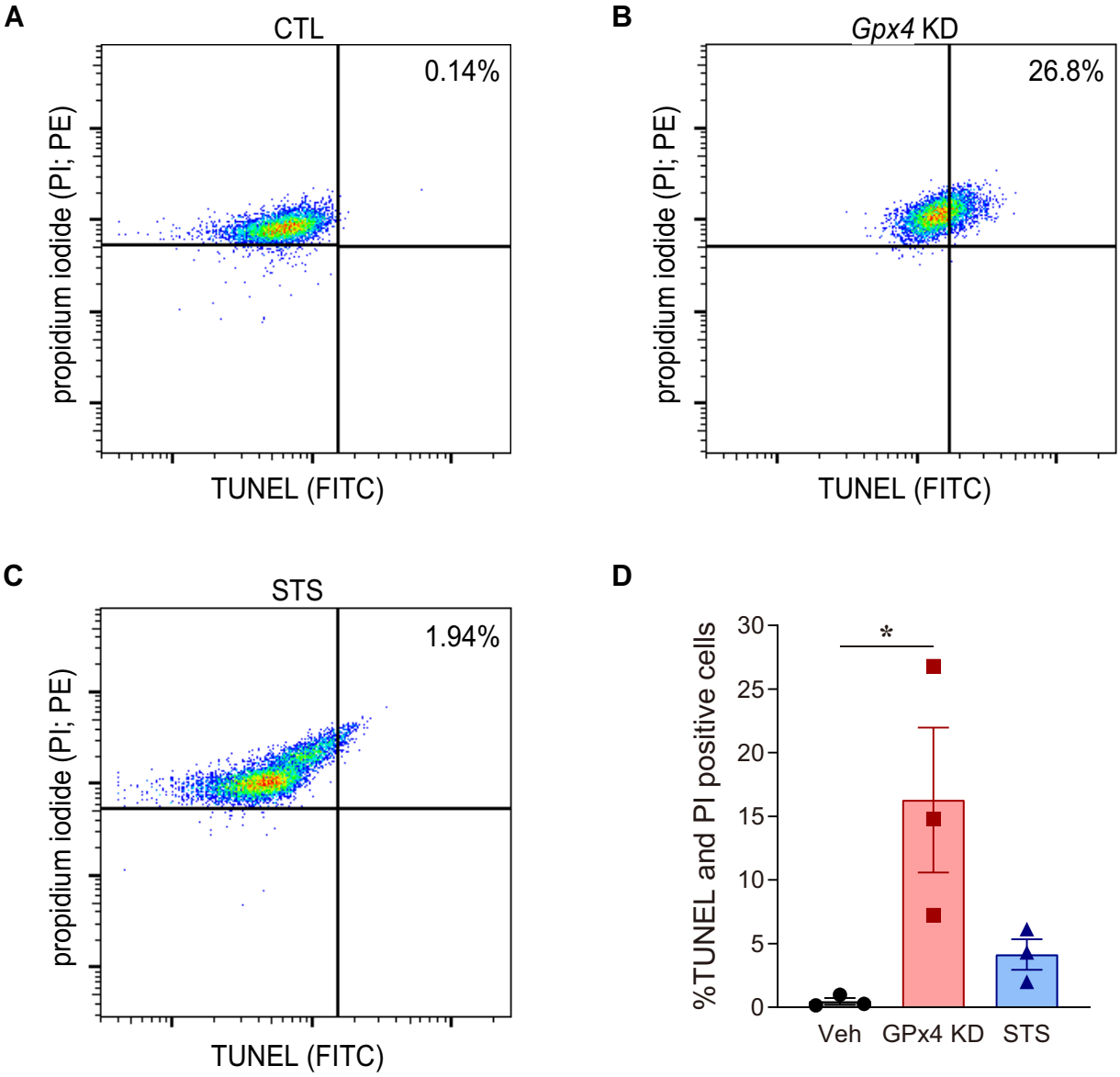
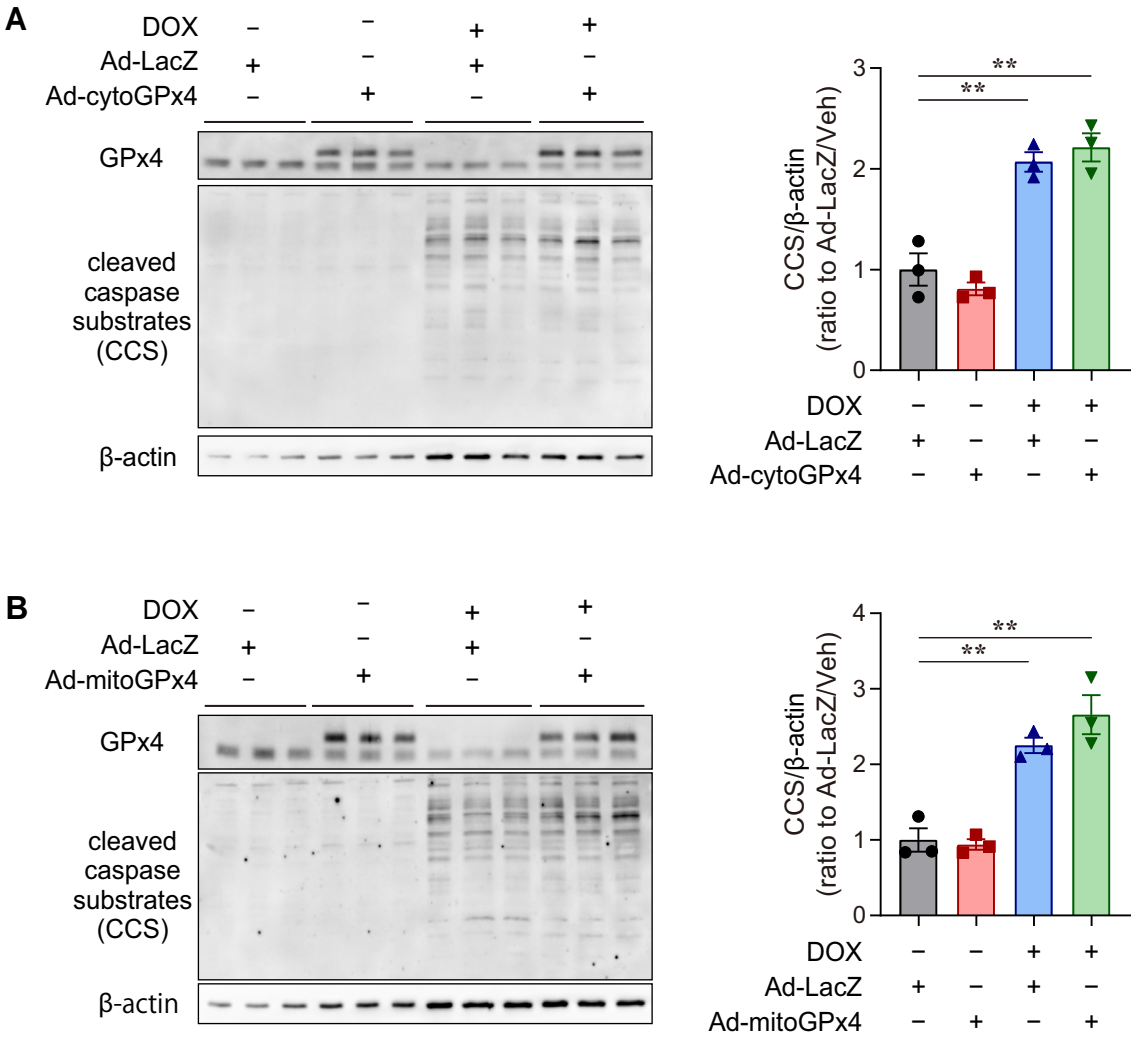
Supplemental Figure 1



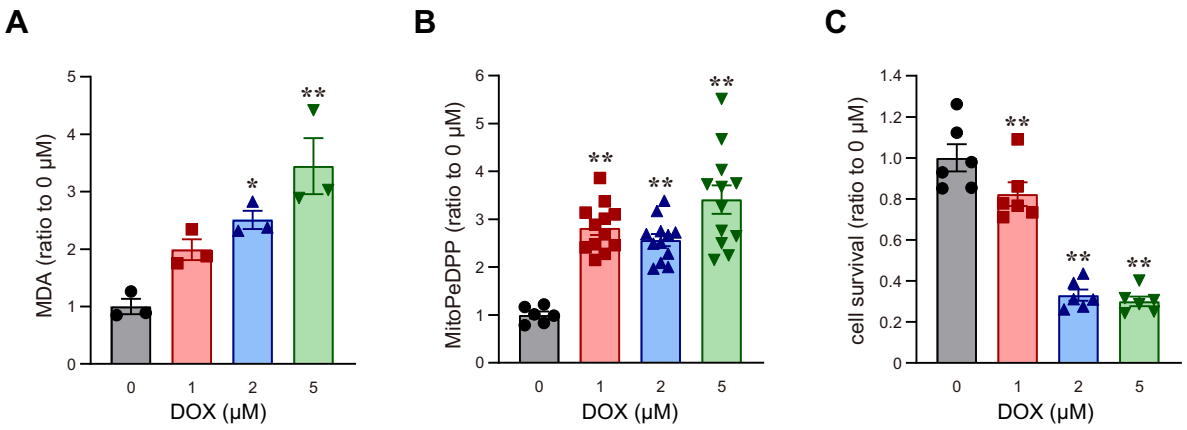
Supplemental Figure 1. TUNEL assay for H9c2 cells. Representative analysis of flow cytometry for TUNEL-positive nuclei in H9c2 cell line (rat myoblast) transfected with siRNA targeting *Gpx4* and treated with staurosporine (STS); (A) control (CTL), (B) *Gpx4* knockdown (KD), (C) STS treatment (n =3, each). (D) Quantification of the percent of TUNEL-positive nuclei per total nuclei. Data are shown as the mean ± SEM. Statistical significance was determined using one-way ANOVA with a post-hoc Tukey HSD test. *P < 0.05.

Supplemental Figure 2



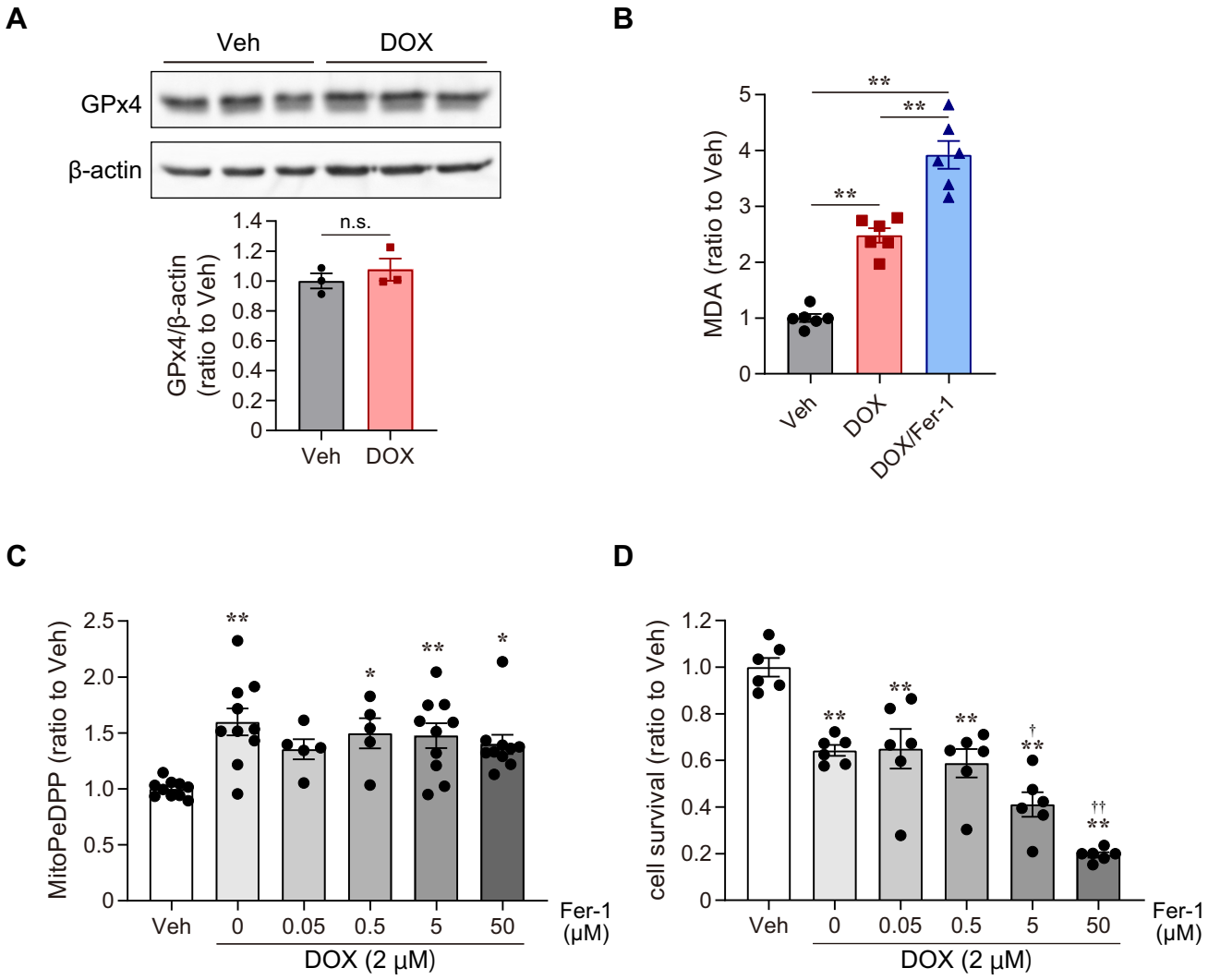
Supplemental Figure 2. Effect of GPx4 overexpression on cleavage of caspases in cultured cardiomyocytes under DOX treatment. Western blot of GPx4 and cleaved caspase substrates (CCS) in cardiomyocyte cell lysates, infected with (A) Ad-cytoGPx4 and (B) Ad-mitoGPx4 (n = 3). Data are shown as the mean ± SEM. Statistical significance was determined using one-way ANOVA with a post-hoc Tukey HSD test. **P < 0.01.

Supplemental Figure 3



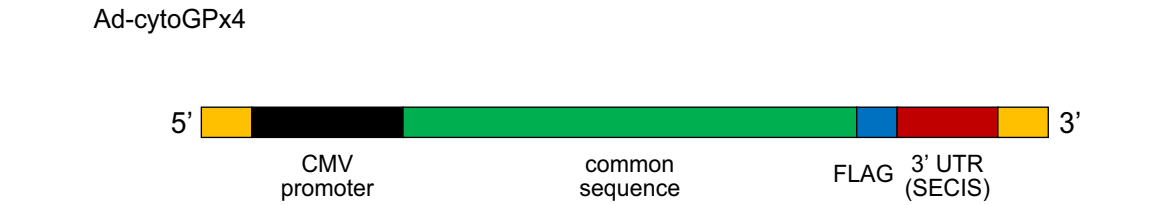
Supplemental Figure 3. DOX concentration-dependent behaviors of LPs (MDA and MitoPeDPP) and cell death in DOX-treated cardiomyocytes. (A) Malondialdehyde (MDA) levels in the cultured cardiomyocytes were measured by thiobarbituric acid reactive substances (TBARs) assay (n = 3). (B) Mitochondrial lipid peroxidation was measured using MitoPeDPP (n = 6 to 12). (C) Cell viability was assessed 30 h after treatment with DOX (n = 6). Data are shown as the mean ± SEM. Statistical significance was determined by Dunnett' s test. *P < 0.05, **P < 0.01 vs. DOX 0 μM.

Supplemental Figure 4



Supplemental Figure 4. The role of ferroptosis in non-myocyte cells. (A) Western blot of GPx4 in non-myocyte cells under DOX treatment (n=3). (B) Malondialdehyde (MDA) levels in non-myocyte cells, treated with ferrostatin-1 (Fer-1, 50 μM), were measured by thiobarbituric acid reactive substances (TBARs) assay (n = 6). (C) Mitochondrial lipid peroxidation was measured using MitoPeDPP in non-myocyte cells, treated with Fer-1 (n = 5 to 10). (D) Effect of Fer-1 on DOX-induced cell death in non-myocyte cells (n = 6). Data are shown as the mean ± SEM. Statistical significance was determined using Student' s t-test or one-way ANOVA with a post-hoc Tukey HSD test. *P < 0.05, **P < 0.01 vs. Veh, †P < 0.05, ††P < 0.01 vs. DOX. n.s.: not significant vs. Veh.

Supplemental Figure 5



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CAGGAGCCAGGAAGTAATCAAGAAATCAAGGAGTTTGCAGCCGGCTACAATGTCAGGTTT

GACATGTACAGCAAGATCTGTGTAAATGGGGACGATGCCCACCCACTGTGGAAATGGATG

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GTGATAGAGAAGGACCTGCCGTGCTATCTCGACTACAAAGACGATGACGACAAGTAGCCC

FLAG

stop codon

TACAAGTGTGTGCCCCTGCACCGAGCCCCCCTGCCCTGTGACCCCTGGAGCCTTCCACCC

3' UTR (SECIS sequence)

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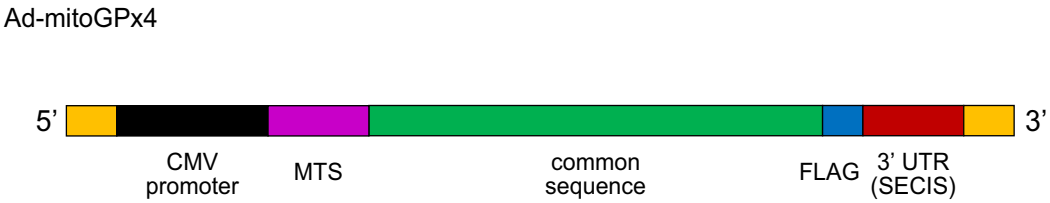
Supplemental Figure 5. Nucleotide sequence of the rat cytosolic *Gpx4* cDNA that was cloned into the adenovirus vector.

Supplemental Figure 6



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start codon

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mitochondria targeting signal

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GGTCCCATGGAGGAGCCCCAGGTGATAGAGAAGGACCTGCCGTGCTATCTCGACTACAAA

FLAG

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stop codon

3' UTR (SECIS sequence)

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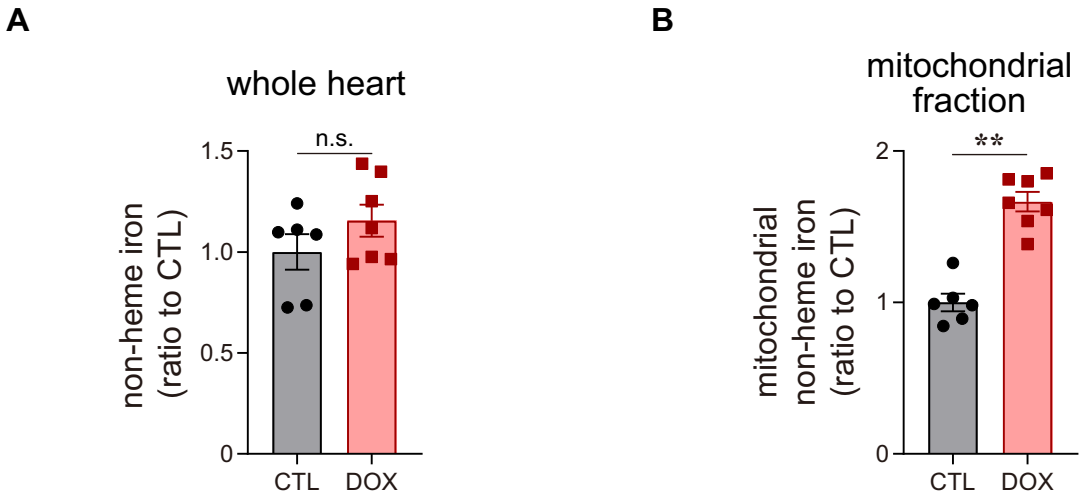
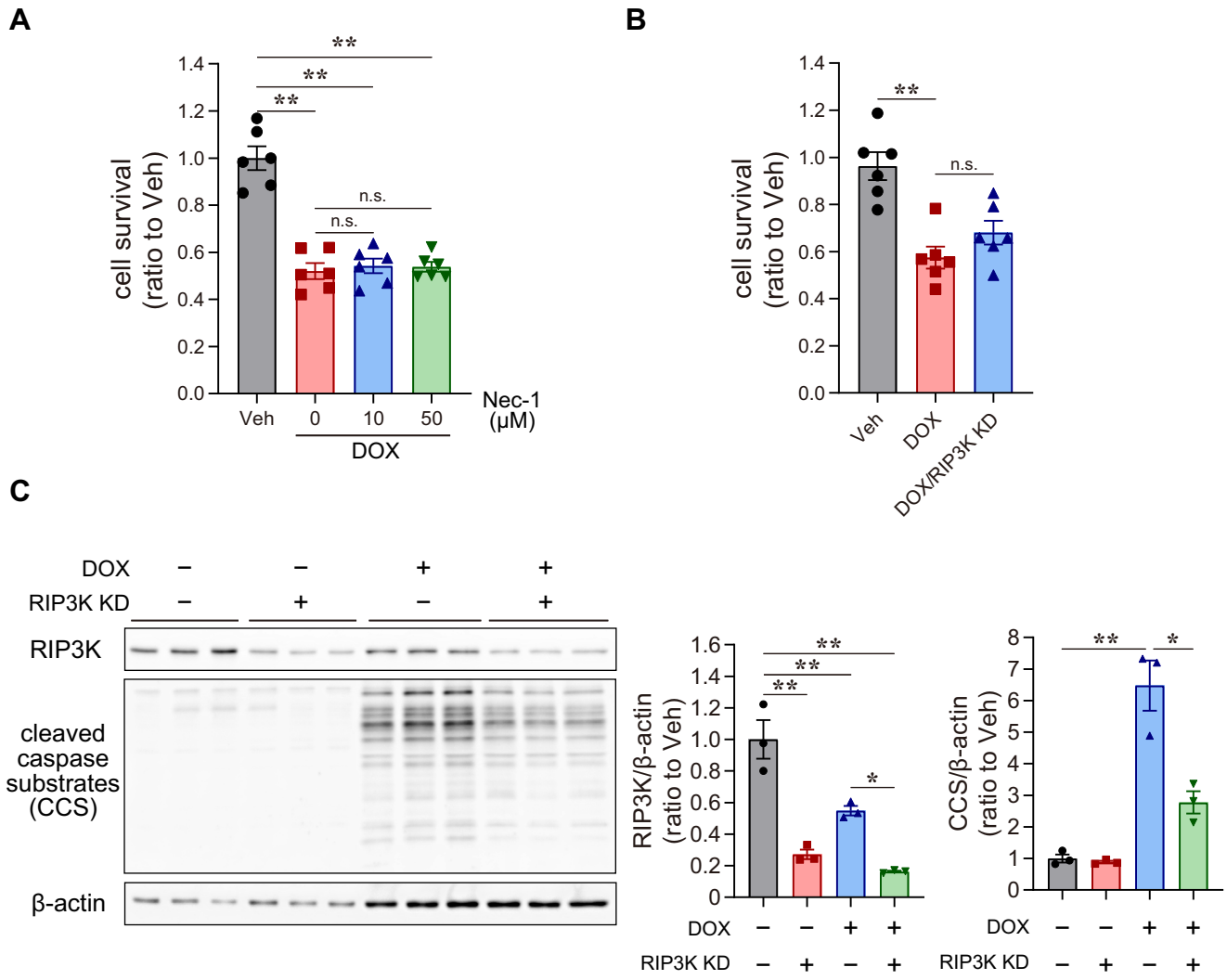
Supplemental Figure 6. Nucleotide sequence of the rat mitochondrial *Gpx4* cDNA that was cloned into the adenovirus vector.

Supplemental Figure 7



Supplemental Figure 7. Iron in DOX-induced cardiomyopathy model. (A) Non-heme iron in the whole heart at day 14 (n = 6 to 7). (B) Non-heme iron in the mitochondria of the heart at day 14 (n = 6 to 7). Data are shown as the mean ± SEM. Statistical significance was determined using Student’ s t-test. **P < 0.01. n.s.: not significant.

Supplemental Figure 8



Supplemental Figure 8. The role of necroptosis in DOX-induced cell death. (A) Cell viability was assessed 30 h after treatment with DOX and/or Nec-1 (10 and 50 μM; n = 6). (B) Cell viability was assessed 30 h after treatment with DOX in cardiomyocytes transfected with siRNA targeting *Ripk3* (n = 6). (C) Western blot of RIP3K and cleaved caspase substrates in cardiomyocyte cell lysates (n = 3). Data are shown as the mean ± SEM. Statistical significance was determined using one-way ANOVA with a post-hoc Tukey HSD test. *P < 0.05, **P < 0.01, n.s.: not significant.

Supplemental Figure 9

Calculations of detailed ratio in total cell death after DOX treatment:

We showed an example of calculations with data sets of 30 hr after DOX treatment. Ferroptosis is defined as the percentage of cell death rescued by Fer-1 in DOX-induced cell death (72-47% = 25%). Apoptosis is defined as the percentage of cell death rescued by zVAD in DOX-induced cell death (67-47% = 20%). Others are defined as the percentage of cell death that was not rescued by a concomitant inhibition with Fer-1 and zVAD (100-90% = 10%). Total % (100%) was divided into three categories (ferroptosis, apoptosis, and others) in accordance with their ratios (25:20:10).
We the detailed calculations are shown below.

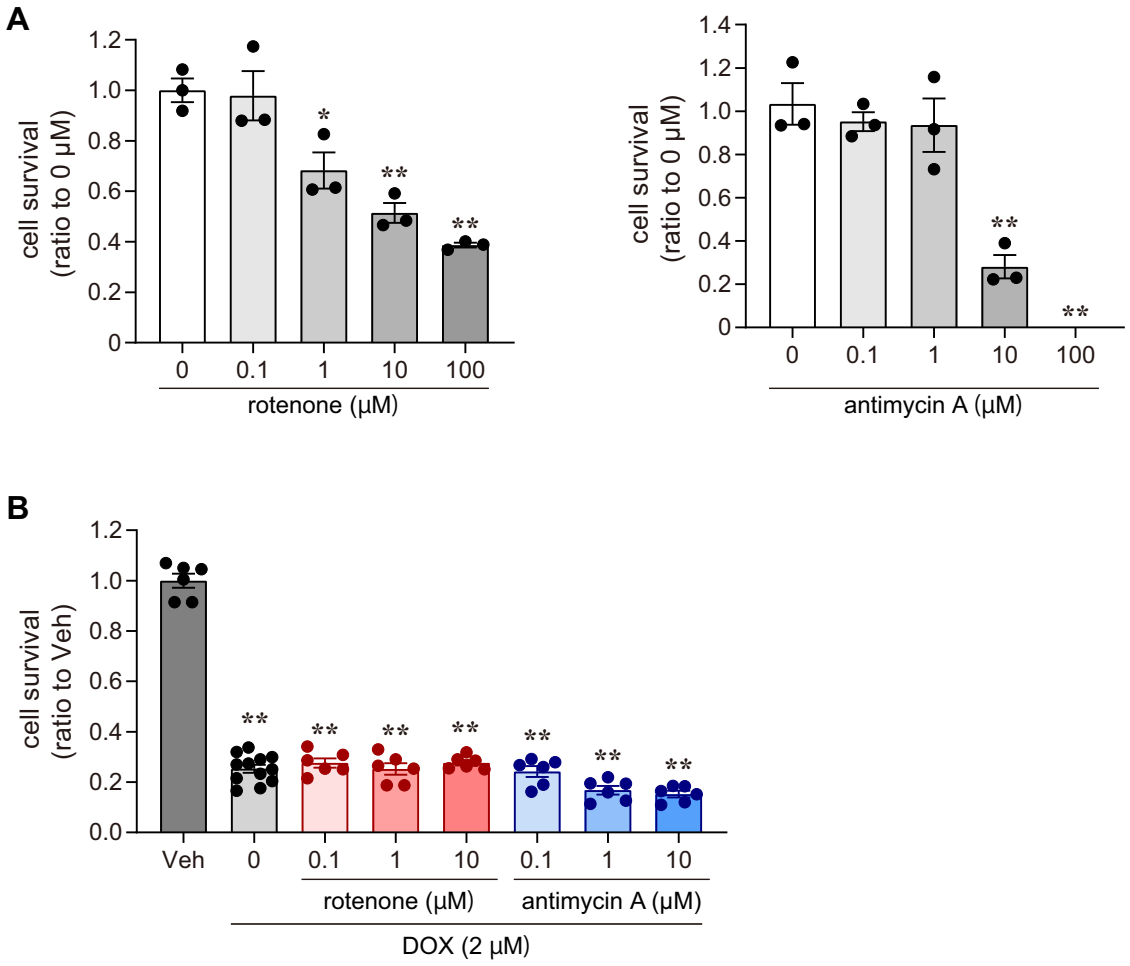
Average percentage of cell survival is as follows.
A. 100% in DOX (-), Fer (-), zVAD (-) group
B. 47% in DOX (+), Fer (-), zVAD (-) group
C. 72% in DOX (+), Fer (+), zVAD (-) group
D. 67% in DOX (+), Fer (-), zVAD (+) group
E. 90% in DOX (+), Fer (+), zVAD (+) group

We defined the percentage of ferroptosis; C-B = 25%
We defined the percentage of apoptosis; D-B = 20%
We defined the percentage of others; A-E = 10%

If we assume that DOX-induced cell death consisted of ferroptosis, apoptosis, and others, the total % (100%) was divided into three categories (ferroptosis, apoptosis, and others) in accordance with their ratios (25:20:10).
Ferroptosis = 100 * (25/25 + 20 + 10) = 46%
Apoptosis = 100 * (20/25 + 20 + 10) = 36%
Others = 100 * (10/25 + 20 + 10) =18%

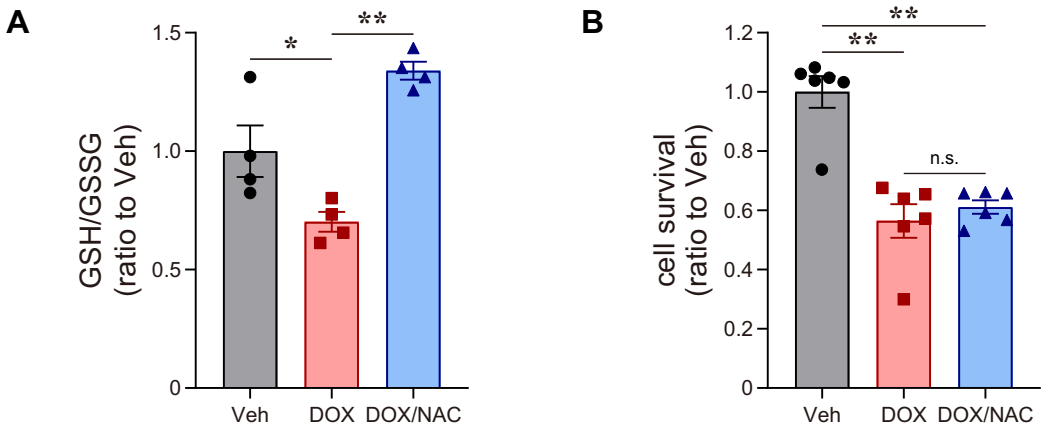
Supplemental Figure 9. Calculations of details of cell death induced by DOX.

Supplemental Figure 10



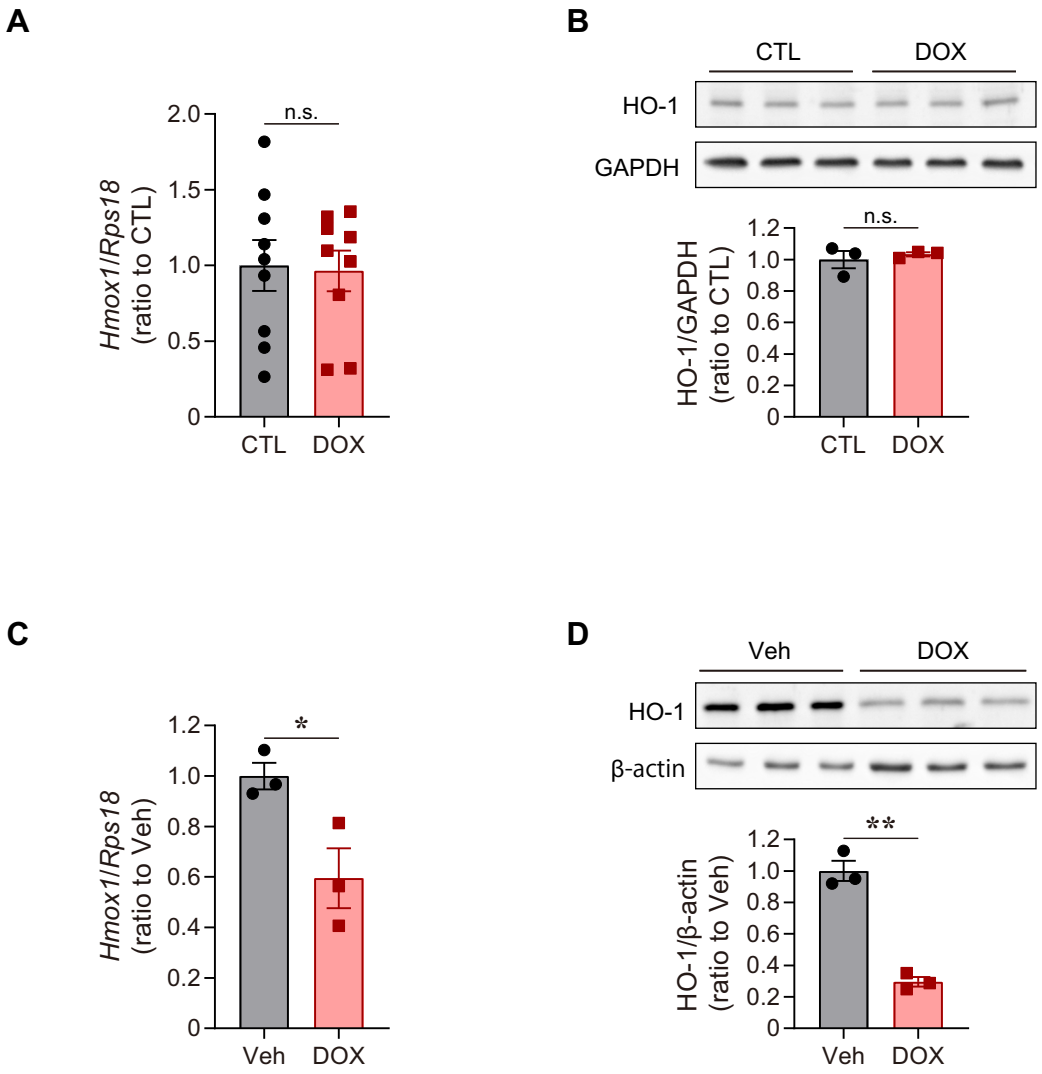
Supplemental Figure 10. Effect of inhibitors against electron transport chain (ETC). (A) Cell viability was assessed 30 h after treatment with rotenone and antimycin A (n = 3, each). Data are shown as the mean ± SEM. Statistical significance was determined by Dunnett's test. **P < 0.01 vs. 0 μM. (B) Cell viability was assessed 30 h after treatment with after DOX treatment (2 μM), with rotenone and antimycin A (n = 6 to 12). Data are shown as the mean ± SEM. Statistical significance was determined by one-way ANOVA with a post-hoc Tukey HSD test. **P < 0.01, vs. Veh.

Supplemental Figure 11



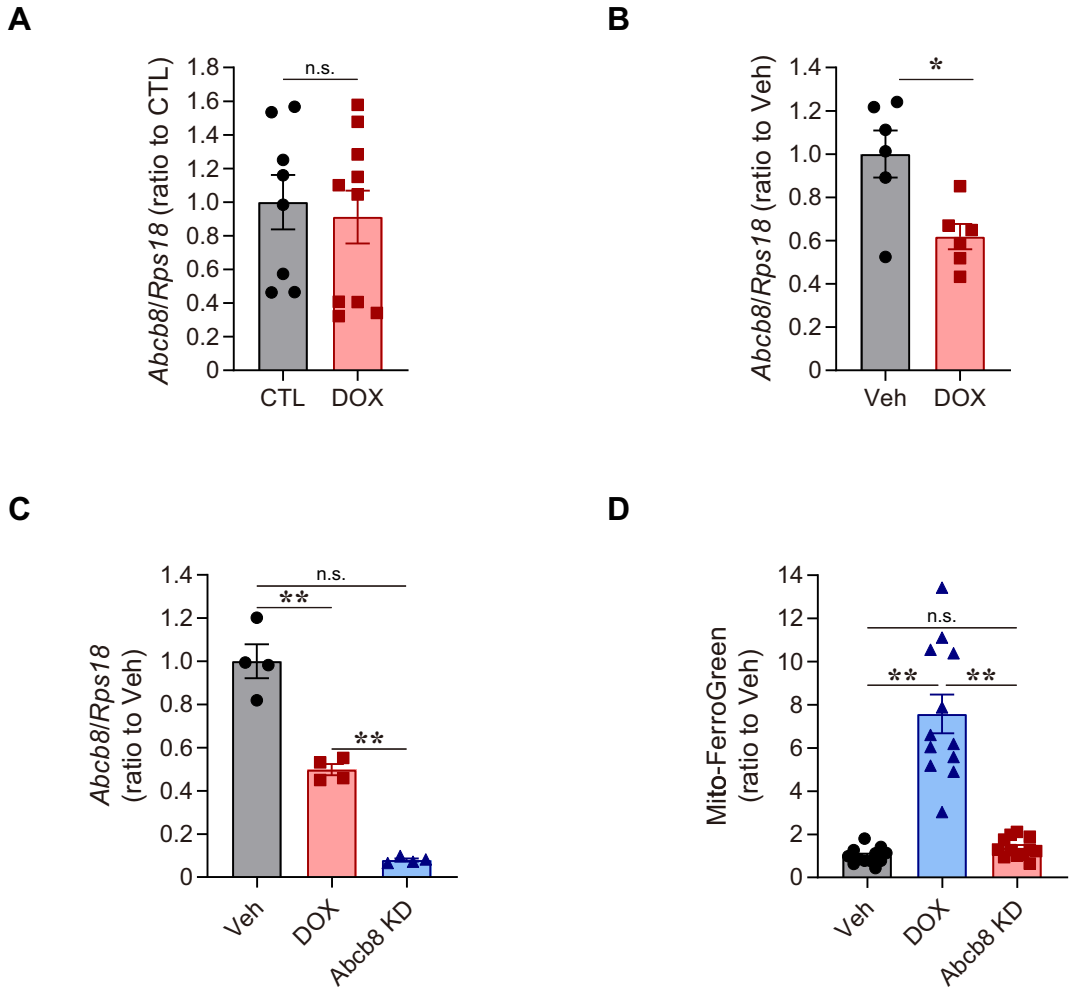
Supplemental Figure 11. The role of glutathione (GSH) in DOX-induced ferroptosis. (A) GSH levels in the cultured cardiomyocytes, treated with DOX and N-acetyl-L-cysteine (NAC; 2 mM), were measured using GSSG/GSH Quantification Kit (n = 4). (B) Cell viability was assessed 30 h after treatment with after DOX treatment (2 μM), with NAC (n = 6). Data are shown as the mean ± SEM. Statistical significance was determined by one-way ANOVA with a post-hoc Tukey HSD test. **P < 0.01, vs. Veh.

Supplemental Figure 12



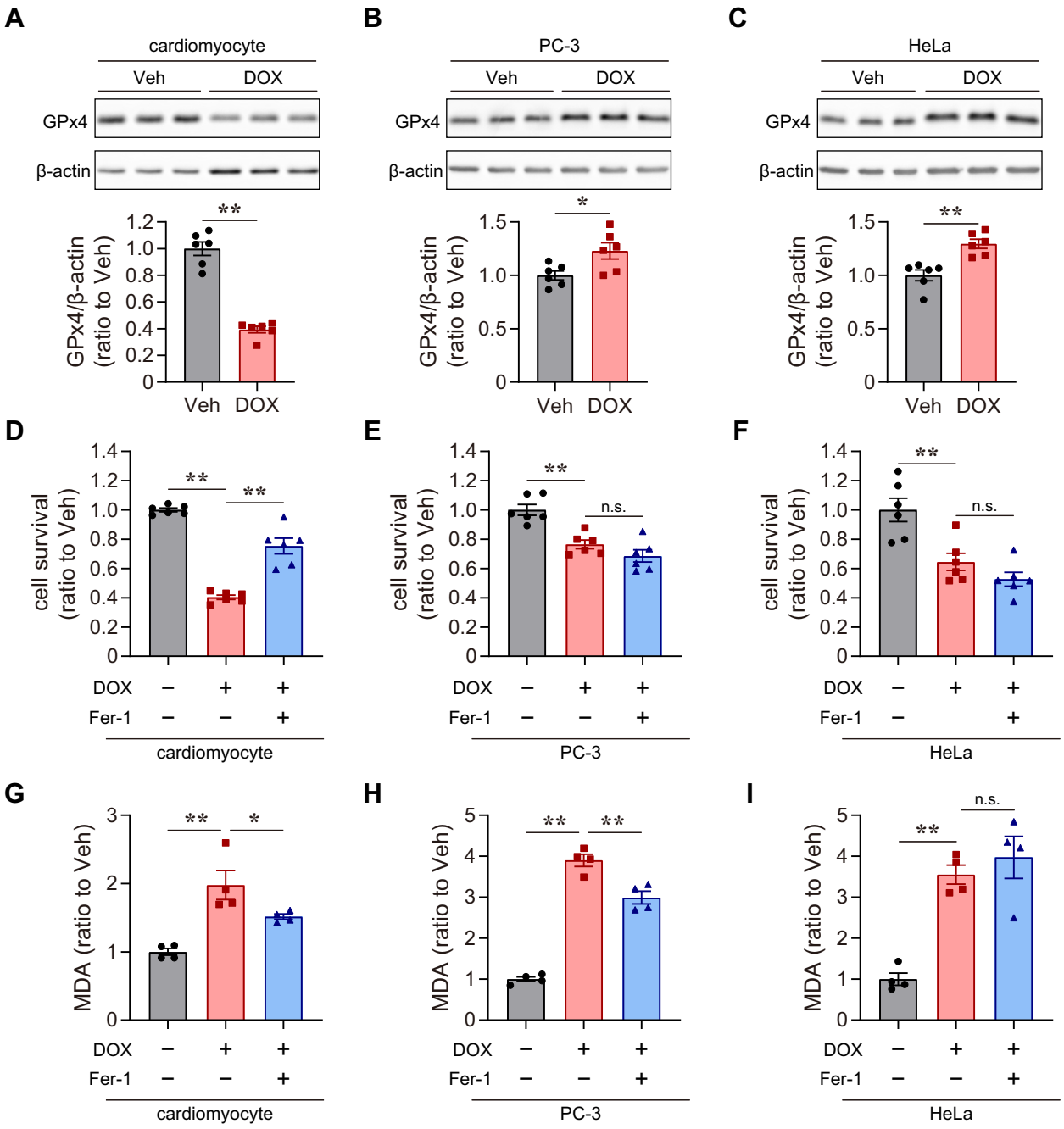
Supplemental Figure 12. Heme oxygenase-1 (HO-1) expression in response to DOX. (A) *Hmox1* expression in the myocardium at day 14 was quantified by real-time PCR (n = 9, each). (B) Western blot of HO-1 from heart tissue lysates at day 14 (n = 3, each). (C) *Hmox1* expression in cultured cardiomyocytes treated with vehicle or doxorubicin (DOX) was quantified by real-time PCR (n = 3, each). (D) Western blot of HO-1 in lysates from cultured cardiomyocytes (n = 3, each). Data are shown as the mean ± SEM. Statistical significance was determined using Student' s t-test. *P < 0.05, **P < 0.01, n.s.: not significant.

Supplemental Figure 13



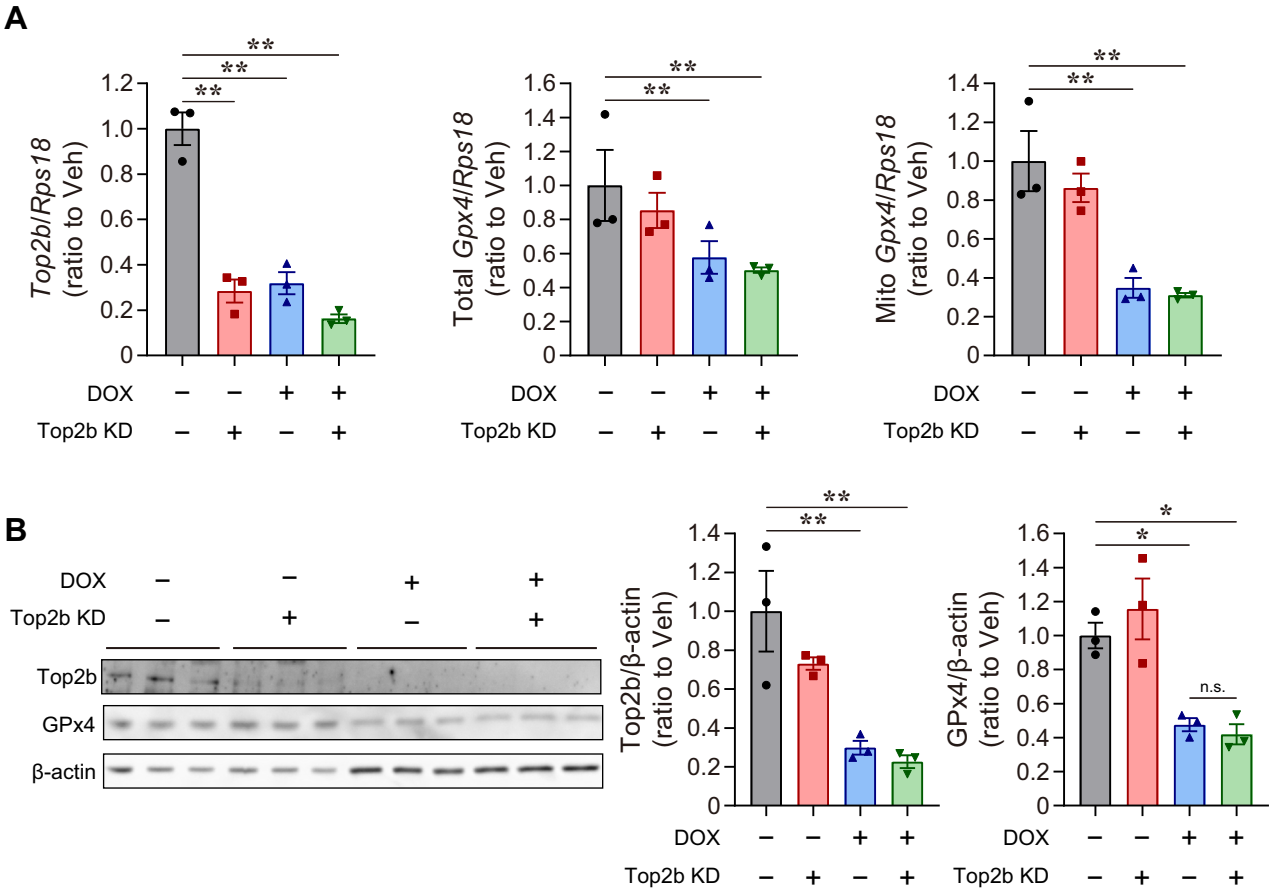
Supplemental Figure 13. Abcb8 expression in response to DOX. (A) *Abcb8* expression in the myocardium at day 14 was quantified by real-time PCR (n = 8 and 10, respectively). (B) *Abcb8* expression in cultured cardiomyocytes treated with vehicle or doxorubicin (DOX) was quantified by real-time PCR (n = 6, each). (C) Silencing of *Abcb8* in cultured cardiomyocytes with siRNA transfection (1 nM, 48 h) and DOX (2 μM, 30 h) (n = 4). (D) Mitochondrial iron level measurement using Mito-FerroGreen in cultured cardiomyocytes, transfected siRNA against *Abcb8* (1 nM, 48 h) and treated with DOX (2 μM, 30 h) (n = 12). Data are shown as the mean ± SEM. Statistical significance was determined using Student' s t-test or one-way ANOVA with a post-hoc Tukey HSD test. *P < 0.05, **P < 0.01, n.s.: not significant.

Supplemental Figure 14



Supplemental Figure 14. Differences in GPx4 behaviors depending on cell type. (A-C) Western blot of GPx4 from the lysates of cultured cardiomyocytes, PC-3 cells, and HeLa cells (n = 6). (D-F) Cell viabilities of cultured cardiomyocytes, PC-3 cells, and HeLa cells, and were assessed 30 h after treatment with DOX (n = 6). (G-I) Malondialdehyde (MDA) in cultured cardiomyocytes, PC-3 cells, and HeLa cells was measured by thiobarbituric acid reactive substances (TBARs) assay (n = 4). Data are shown as the mean ± SEM. Statistical significance was determined using Student's t-test and one-way ANOVA with a post-hoc Tukey HSD test. *P<0.05, **P < 0.01, n.s.: not significant.

Supplemental Figure 15



Supplemental Figure 15. Effect of *topoisomerase IIβ* (*Top2b*) knockdown with siRNA targeting *Top2b* on *Gpx4* downregulation induced by DOX. (A) Real-time qPCR quantification of *Top2b* and total and mitochondrial *Gpx4* expression in cultured cardiomyocytes treated with siRNA targeting *Top2b* (n = 3). (B) Western blot of Top2b and GPx4 in cultured cardiomyocytes treated with siRNA targeting *Top2b* (n = 3). Data are shown as the mean ± SEM. Statistical significance was determined using one-way ANOVA with a post-hoc Tukey HSD test. **P < 0.01, n.s.: not significant.