

ATRA alleviates doxorubicin-induced cardiomyocyte ferroptosis by suppressing KEAP1-mediated NRF2 degradation

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Abstract

Objective

Doxorubicin (DOX), a first-line anthracycline chemotherapeutic agent, is limited by dose-dependent cardiotoxicity. Ferroptosis is increasingly implicated in DOX-induced cardiomyocyte injury, yet effective strategies to suppress this process remain limited. This study investigated whether all-trans retinoic acid (ATRA) mitigates DOX-induced cardiomyocyte ferroptosis and defined the underlying mechanism.

Methods

We assessed the effects of ATRA on DOX-induced cardiomyocyte ferroptosis in vitro and in vivo using western blotting, quantitative real-time PCR, malondialdehyde (MDA) assays, glutathione (GSH) measurements and lipid peroxidation analysis. Loss-of-function models, cycloheximide chase assays, proteasome inhibition assays, molecular docking and co-immunoprecipitation were used to determine how the NRF2–SLC7A11 pathway mediates the protective effect of ATRA.

Results

DOX reduced cell viability, depleted GSH, increased MDA and downregulated SLC7A11 in cardiomyocytes. These effects were reversed by ATRA in vitro and in vivo. The anti-ferroptotic effect of ATRA was lost in SLC7A11-deficient and NRF2-deficient cells, indicating that the NRF2-SLC7A11 axis is required for protection. Mechanistically, ATRA stabilised NRF2 by reducing NRF2-KEAP1 interaction and proteasomal degradation. Co-immunoprecipitation showed that ATRA weakened the interaction between KEAP1 and NRF2. Molecular docking and mutagenesis suggested that KEAP1 Arg415 is functionally required for ATRA-mediated disruption of the KEAP1–NRF2 interaction. In vivo, ATRA increased myocardial NRF2 expression, suppressed DOX-induced ferroptosis and improved cardiac function, as shown by higher left ventricular ejection fraction and fractional shortening.

Conclusion

ATRA attenuates DOX-induced cardiomyocyte ferroptosis by disrupting KEAP1-mediated NRF2 degradation and activating the NRF2-SLC7A11 antioxidant pathway. These findings identify ATRA as a candidate cardioprotective strategy for anthracycline-related cardiac injury, pending further mechanistic and translational validation.

1. Introduction

Anthracyclines, including doxorubicin (DOX), remain first-line chemotherapeutic agents for many malignant tumours. Their clinical value is constrained by toxicity in normal tissues, particularly dose-

dependent cardiotoxicity¹. DOX-induced cardiotoxicity can present as acute or chronic injury, with distinct pathological features. Acute toxicity is commonly associated with arrhythmias and reversible left ventricular dysfunction, and is driven mainly by acute cardiomyocyte injury and necrosis^{2,3}. Chronic toxicity follows a progressive and often irreversible course. Primary cardiomyocyte injury can evolve into pathological hypertrophy, myocardial fibrosis and ultimately chronic heart failure⁴. Clinical evidence indicates that approximately 30% of patients receiving DOX develop chronic heart failure, and about 10% progress to symptomatic heart failure⁵. Because this toxicity compromises the long-term prognosis of patients with cancer, effective strategies to limit anthracycline-induced cardiomyocyte injury are urgently needed.

DOX-induced cardiomyocyte ferroptosis is a key driver of subsequent cardiovascular injury^{3,6,7}. DOX induces oxidative stress through several mechanisms, and sustained redox imbalance promotes lipid peroxidation and ferroptotic cell death. The quinone group of DOX generates reactive oxygen species (ROS) through continuous redox cycling⁷⁻⁹. DOX also binds cardiolipin on mitochondrial membranes and accumulates in mitochondria, impairing the electron transport chain and increasing mitochondrial ROS production¹⁰. In parallel, DOX can chelate Fe³⁺ to form complexes that are reduced to Fe²⁺, which promotes hydroxyl radical formation through the Fenton reaction¹¹. DOX further disrupts mitochondrial iron homeostasis, causing iron overload and amplifying lipid peroxidation¹². These mechanisms support the hypothesis that reducing oxidative stress may alleviate DOX-induced cardiomyocyte ferroptosis.

All-trans retinoic acid (ATRA) is an established retinoid with clinical utility in oncology¹³. Beyond its canonical role in promoting cellular differentiation, ATRA has increasingly been linked to the regulation of oxidative stress¹⁴. ATRA can bind heterodimers formed by retinoic acid receptors and retinoid X receptors, thereby modulating HO-1 and RAR β signalling and antagonising lipopolysaccharide-induced hepatic oxidative injury^{15,16}. ATRA and related analogues can also interact with acyl-CoA synthetase long-chain family member 3, promote the incorporation of medium-chain fatty acids into phospholipids and inhibit ferroptosis¹⁷. Vitamin A analogues have been reported to attenuate age-related lipid peroxidation and extend lifespan in mice, further supporting their potential antioxidant and anti-ferroptotic activity¹⁸. On this basis, we hypothesised that ATRA may protect cardiomyocytes from DOX-induced ferroptosis.

Here we show that ATRA mitigates DOX-induced cardiomyocyte ferroptosis by upregulating solute carrier family 7 member 11 (SLC7A11). Mechanistically, ATRA appears to interfere with KEAP1-dependent NRF2 degradation in an Arg415-dependent manner. Stabilised NRF2 translocates to the nucleus and enhances SLC7A11 transcription through antioxidant response elements¹⁹. These findings support ATRA as a potential cardioprotective approach in anthracycline-associated cardiac injury, while further validation is required before clinical translation.

2. Materials and methods

2.1. Reagents, antibodies and experimental treatments

Doxorubicin (DOX), all-trans retinoic acid (ATRA), ferrostatin-1 (Fer-1), erastin, RSL3, deferoxamine (DFO), cycloheximide (CHX) and MG132 were used as indicated. Unless otherwise specified, AC16 cells were treated with DOX (2 μ M) and/or ATRA (20 μ M) for 48 h. Fer-1, erastin, RSL3, CHX and MG132 were applied at final concentrations of 2 μ M, 5 μ M, 10 μ M, 5 μ M and 5 μ M, respectively; treatment durations are specified in the relevant figure legends. Detailed information on primary antibodies, including source, catalogue number and dilution, is provided in Table 3.

Table 3
Primary Antibody List for Western Blot

Target Protein	Cat. No.	Host Species	Manufacturer
SLC7A11	26864-1-AP	Rabbit	Proteintech
GPX4	67763-1-Ig	Mouse	Proteintech
β -Tubulin	61157	Mouse	Proteintech
NRF2	16396-1-AP	Rabbit	Proteintech
H3	17168-1-AP	Rabbit	Proteintech
KEAP1	10503-2-AP	Rabbit	Proteintech
Flag	66008-4-Ig	Mouse	Proteintech
P53	10442-1-AP	Rabbit	Proteintech
ATF4	10835-1-AP	Rabbit	Proteintech
ATF3	DF3110	Rabbit	Affinity

2.2. Cell culture

The AC16 human cardiomyocyte cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

2.3. Cell viability assay

Cell viability was evaluated using CCK-8 reagent. AC16 cells were seeded in 96-well plates, treated as indicated and then incubated with fresh medium containing 10% CCK-8 solution. Absorbance at 450 nm was recorded with a microplate reader, and viability was expressed relative to the corresponding control group.

2.4. Gene Expression Omnibus analysis

The GEO dataset GSE40491 was used to identify ferroptosis-related genes regulated by ATRA. GSE40491 contains gene-expression profiles of mouse aortic heart valve tissues exposed to all-trans retinoic acid (ATRA) or vehicle control dimethyl sulfoxide (DMSO). The processed expression matrix and sample annotation files were obtained from the GEO database. Samples were grouped as ATRA-treated and DMSO-treated controls according to the original GEO metadata.

Differentially expressed genes between the ATRA-treated and DMSO-treated groups were identified using the limma package in R. Probe identifiers were converted to official gene symbols according to the platform annotation file. For genes represented by multiple probes, the probe with the highest mean expression across all samples was selected for downstream analysis. Genes with $|\log_2FC| > 1.0$ and $P < 0.05$ were defined as differentially expressed genes. Adjusted P values were calculated using the Benjamini-Hochberg method to control the false discovery rate. Ferroptosis-related genes were retrieved from FerrDb and included ferroptosis drivers, suppressors and markers. The intersection between differentially expressed genes and ferroptosis-related genes was used to identify ATRA-responsive ferroptosis-associated genes. Volcano plots were generated to display the distribution of differentially expressed genes, with SLC7A11 highlighted as a candidate gene for further validation.

2.5. Lentiviral transduction and generation of stable cell lines

Stable SLC7A11-knockout cells, NRF2-knockout cells, KEAP1-overexpressing and KEAP1 point-mutant AC16 cell lines were generated using lentiviral transduction. Briefly, plasmids carrying the indicated sgRNA, overexpression or point-mutant sequences were packaged in 293T cells, and fresh lentiviral supernatants were collected for infection of AC16 cells. Successfully transduced cells were selected with puromycin. Knockdown, knockout, overexpression and point-mutation efficiencies were validated by quantitative real-time PCR and/or western blotting before functional experiments. Plasmid and targeting sequences are listed in Table 2.

Table 2
Plasmid sequences

Name	Direction	Sequence (5'-3')
SLC7A11-sh1-human	Forward	CACCGCAGGTAACCTCCTTTGGAGA
	Reverse	AAACTCTCCAAAGGAGGTTACCTGC
SLC7A11-sh2-human	Forward	CAAAGGTAACCTCCTTTGGAGATGG
	Reverse	AAACCCATCTCCAAAGGAGGTTACC
SLC7A11-sh3-human	Forward	CACCGGTGTCCACCATCTCCAAAGG
	Reverse	AAACCCTTTGGAGATGGTGGACACC
NRF2-sh1-human	Forward	CACCGATTTGATTGACATACTTTGG
	Reverse	AAACCCAAAGTATGTCAATCAAATC
NRF2-sh2-human	Forward	CAAAGTGGAGGCAAGATATAGATCT
	Reverse	AAACAGATCTATATCTTGCCTCCAC
NRF2-sh3-human	Forward	CACCGTATTTGACTTCAGTCAGCGA
	Reverse	AAACTCGCTGACTGAAGTCAAATAC
KEAP1-OE-human	Forward	CATAGAAGATTCTAGAATGCAGCCAGATCCCAGG
	Reverse	CTTTGTAGTCGGATCCACAGGTACAGTTCTGCTGGTCAA
KEAP1-R415L-human	Forward	CGTAACCTCATCGGGGTGGGGGTCATCGATGG
	Reverse	ACCCCGATGAGGTTACGGGGCACGCTCATGGG
KEAP1-R415G-human	Forward	CGTAACGGCATCGGGGTGGGGGTCATCGATGG
	Reverse	ACCCCGATGCCGTTACGGGGCACGCTCATGGG

2.6. Quantitative real-time PCR

Total RNA was extracted from cells or mouse cardiac tissues using TRIzol reagent (Takara) according to the manufacturer's protocol. Complementary DNA was synthesized using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1622). qPCR was performed using SYBR Green master mix (Vazyme) on a CFX96 system (Bio-Rad) or QuantStudio 3 real-time PCR system (Invitrogen). Each reaction was performed in triplicate. Relative mRNA expression was calculated using the comparative Ct method after normalization to GAPDH. Primer sequences are listed in Table 1.

Table 1
Primer sequences

Name	Direction	Sequence (5'-3')
GAPDH-human	Forward	ACAACTTTGGTATCGTGGAAGG
	Reverse	GCCATCACGCCACAGTTTC
SLC7A11-human	Forward	TCTCCAAAGGAGGTTACCTGC
	Reverse	AGACTCCCCTCAGTAAAGTGAC
Ptgs2-human	Forward	CTGGCGCTCAGCCATACAG
	Reverse	CGCACTTATACTGGTCAAATCCC
NRF2-human	Forward	TCAGCGACGGAAAGAGTATGA
	Reverse	CCACTGGTTTCTGACTGGATGT
GAPDH-mouse	Forward	ATCATCCCTGCATCCACT
	Reverse	ATCCACGACGGACACATT
Nppa-mouse	Forward	GCTTCCAGGCCATATTGGAG
	Reverse	GGGGGCATGACCTCATCTT
Nppb-mouse	Forward	GAGGTCACTCCTATCCTCTGG
	Reverse	GCCATTTCTCCGACTTTTCTC
Myh7-mouse	Forward	ACTGTCAACACTAAGAGGGTCA
	Reverse	TTGGATGATTTGATCTTCCAGGG
Tnnt2-mouse	Forward	CAGAGGAGGCCAACGTAGAAG
	Reverse	CTCCATCGGGGATCTTGGGT
Ldha-mouse	Forward	TGTCTCCAGCAAAGACTACTGT
	Reverse	GACTGTACTTGACAATGTTGGGA
NRF2-mouse	Forward	TCTTGGAGTAAGTCGAGAAGTGT
	Reverse	GTTGAAACTGAGCGAAAAAGGC
SLC7A11-mouse	Forward	GGCACCGTCATCGGATCAG
	Reverse	CTCCACAGGCAGACCAGAAAA
GPX4-mouse	Forward	GATGGAGCCCATTCTGAACC
	Reverse	CCCTGTACTTATCCAGGCAGA
Ptgs2-mouse	Forward	TTCAACACACTCTATCACTGGC

Name	Direction	Sequence (5'-3')
	Reverse	AGAAGCGTTTGCGGTACTCAT

2.7. Western blotting

Cells or cardiac tissues were lysed in RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of protein were resolved by SDS-PAGE, transferred to PVDF membranes and incubated with the indicated primary antibodies followed by HRP-conjugated secondary antibodies. Chemiluminescent signals were quantified using ImageJ, with β -Tubulin or the indicated loading control used for normalisation.

2.8. Nuclear and cytoplasmic protein fractionation

Nuclear and cytoplasmic proteins were isolated using a nuclear and cytoplasmic protein extraction kit (Beyotime, P0028). Briefly, treated cardiomyocytes were collected and incubated with hypotonic lysis buffer to release cytoplasmic proteins. After centrifugation, the nuclear pellet was lysed with hypertonic extraction buffer. Cytoplasmic and nuclear fractions were analysed by western blotting, with β -Tubulin and histone H3 used as cytoplasmic and nuclear markers, respectively.

2.9. Immunofluorescence staining

AC16 cells grown on coverslips were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% BSA. Cells were incubated with primary antibodies overnight at 4°C, followed by fluorescently labeled secondary antibodies (Jackson ImmunoResearch, 155400005). Nuclei were counterstained with DAPI. Fluorescence images were acquired using a fluorescence or confocal microscope. Nuclear NRF2 fluorescence intensity was quantified using ImageJ.

2.10. Lipid peroxidation assay

Lipid peroxidation was detected using C11-BODIPY 581/591. After treatment, cells were loaded with C11-BODIPY, washed and analysed by flow cytometry. Oxidised probe fluorescence was quantified with FlowJo and used as an index of lipid ROS accumulation.

2.11. Measurement of MDA and GSH

Malondialdehyde (MDA) concentrations in cell or tissue lysates were measured using an MDA assay kit (Nanjing Jiancheng Institute of Bioengineering, A003-1-2) according to the manufacturer's protocol. The MDA-thiobarbituric acid adduct was quantified by measuring absorbance at 532 nm. Reduced glutathione (GSH) levels were measured using a GSH detection kit (Nanjing Jiancheng Institute of Bioengineering, A006-2-1), with absorbance measured at 405 nm. MDA and GSH values were normalised to protein concentration determined by the BCA assay.

2.12. Co-immunoprecipitation analysis

For co-immunoprecipitation, total proteins were extracted from treated cardiomyocytes and quantified using the BCA method. Lysates containing 1 mg protein were incubated overnight at 4°C with 1 µg of the indicated primary antibody. Protein G agarose magnetic beads (Absin, abs9905) and Protein A agarose beads (Absin, abs9904) were added and incubated for 5 h. The bead-bound immune complexes were washed, denatured and analysed by western blotting.

2.13. Cycloheximide chase and proteasome inhibition assays

To assess NRF2 protein stability, AC16 cells were treated with CHX in the presence or absence of ATRA and harvested at the indicated time points. NRF2 protein abundance was examined by western blotting and normalised to β -Tubulin. For proteasome inhibition experiments, cells were treated with MG132 for 24 h before protein extraction.

2.14. Molecular docking

The molecular structure of ATRA was obtained from PubChem (CID: 444795), and the crystal structure of KEAP1 was downloaded from the RCSB Protein Data Bank (PDB ID: 4IFJ). Molecular docking was performed using AutoDockTools. Candidate binding sites and predicted binding energies between ATRA and KEAP1 were calculated, and the top-ranked poses were visualised and annotated using PyMOL.

2.15. Animal experiments

All animal procedures were approved by the Institutional Animal Care and Use Committee of Jiangsu University (approval no. UJS-IACUC-AP-2024040214). Male C57BL/6 mice aged 6–8 weeks were obtained from Cavens Laboratory Animals Company (Changzhou, China) and housed under standard specific pathogen-free conditions with free access to food and water. After one week of acclimatization, mice were randomly assigned to five groups: control, ATRA, DOX, DOX + ATRA and DOX + DFO (n = 8 per group). Mice received intraperitoneal injections of ATRA (10 mg/kg), DOX (2 mg/kg) and/or DFO (50 mg/kg) according to the experimental design. Treatments were administered every two days for 14 days. At the end of the experiment, mice were euthanized, and cardiac tissues were collected. Apical myocardial tissues were snap-frozen in liquid nitrogen or stored at -80°C for molecular analyses, whereas hearts used for histology were fixed in 4% paraformaldehyde.

2.16. Echocardiography

Cardiac function was evaluated by transthoracic echocardiography before euthanasia using a small-animal ultrasound system. Mice were anesthetized with Isoflurane, and two-dimensional and M-mode images were acquired from the parasternal short-axis view. Left ventricular ejection fraction (LVEF) and fractional shortening (LVFS) were calculated from at least three consecutive cardiac cycles. Echocardiographic acquisition and analysis were performed by an investigator blinded to treatment allocation.

2.17. Histology and immunohistochemistry

Fixed heart tissues were embedded in paraffin, sectioned and subjected to hematoxylin and eosin (H&E) or Sirius Red staining. For Sirius Red staining, sections were deparaffinized in xylene, rehydrated through graded ethanol and stained using a modified Sirius Red staining kit (Solarbio, G1472). Collagen deposition was evaluated under light microscopy.

For immunohistochemistry, paraffin sections were deparaffinized, rehydrated and subjected to antigen retrieval. Endogenous peroxidase activity was blocked, and sections were incubated with primary antibodies against the indicated proteins, including SLC7A11, followed by HRP-conjugated secondary antibodies. Signals were developed using a DAB substrate kit (Vector Laboratories, SK-4100), and nuclei were counterstained with hematoxylin. Images were captured using an optical microscope. Histological and immunohistochemical quantification was performed using randomly selected fields by investigators blinded to group allocation.

2.18. Statistical analysis

Data are presented as mean $\pm$ SD. Normality was tested using the Shapiro–Wilk method. Student's t-test or the Mann–Whitney U test was used for two-group comparisons, whereas one-way ANOVA with Tukey's post hoc test or Kruskal–Wallis analysis with Dunn's correction was applied for multiple-group comparisons, as appropriate. A two-sided p value < 0.05 was considered statistically significant.

3. Results

3.1. ATRA mitigates DOX-induced cardiomyocyte ferroptosis

To determine whether DOX induced ferroptosis in human cardiomyocytes, we treated AC16 cells with increasing concentrations of DOX. CCK-8 analysis showed that DOX induced AC16 cell death in a concentration-dependent manner, with an IC₅₀ of approximately 2 μ M (Figure S1A). Cell death induced by 2 μ M DOX was reversed by ferrostatin-1, a ferroptosis inhibitor (Figure S1B). DOX also reduced the expression of glutathione peroxidase 4 (GPX4) and SLC7A11, decreased GSH levels and increased MDA, a lipid peroxidation end product (Figure S1C–E). These findings indicated that DOX could induce ferroptosis in AC16 cells, consistent with previous reports²⁰.

We next examined whether ATRA protects cardiomyocytes from DOX-induced ferroptosis. ATRA did not significantly affect AC16 cell viability at concentrations up to 20 μ M (Figure S1F). Treatment with 20 μ M ATRA attenuated DOX-induced cardiomyocyte ferroptosis, with an effect comparable to ferrostatin-1 (Fig. 1A). ATRA also reduced ferroptosis induced by RSL3 and erastin, two classical ferroptosis inducers (Figure S1G, S1H). Consistently, ATRA reversed DOX-induced increases in MDA, lipid ROS and PTGS2, and restored GSH, SLC7A11 and GPX4 levels (Fig. 1B–E and S1I, S1J). These data show that ATRA effectively suppresses DOX-induced ferroptosis in cardiomyocytes.

3.2. SLC7A11 is required for ATRA-mediated protection against cardiomyocyte ferroptosis

To explore the mechanism by which ATRA mitigates DOX-induced cardiomyocyte ferroptosis, we analysed ferroptosis-related genes in ATRA-treated and DMSO-treated mouse aortic heart valve tissues from the GEO dataset GSE40491. Using these criteria, we identified 144 ATRA-responsive ferroptosis-related genes, including 32 upregulated and 112 downregulated genes. Among them, SLC7A11 was one of the most prominently upregulated ferroptosis suppressor genes and was selected for further validation (Figure S2A). Consistent with this screening result, SLC7A11 mRNA and protein levels were upregulated in ATRA-treated AC16 cells (Fig. 1F and S2B).

To test whether SLC7A11 is required for the protective effect of ATRA, we generated stable SLC7A11-deficient AC16 cells with knockout validation (Figure S2C and S2D). The ability of ATRA to rescue DOX-induced cell death was abolished in SLC7A11-deficient cells (Fig. 1G). Moreover, ATRA no longer restored GSH, SLC7A11 and GPX4 levels or reduced PTGS2 mRNA and MDA under DOX treatment in SLC7A11-deficient cells (Fig. 1H–K). These results indicate that SLC7A11 is a necessary mediator of ATRA-induced protection against DOX-induced cardiomyocyte ferroptosis.

3.3. ATRA increases SLC7A11 expression through NRF2 activation

NRF2, ATF3, ATF4 and p53 have been implicated in the transcriptional regulation of SLC7A11 (Figure S3A). To identify the exact regulator responsible for ATRA-induced SLC7A11 upregulation, we treated AC16 cells with ATRA and performed detection at different time points. NRF2 increased in a time-dependent manner at the protein level, whereas ATF3, ATF4 and p53 did not show comparable induction (Fig. 2A and S3B). We then generated NRF2-deficient AC16 cells to examine whether NRF2 is required for SLC7A11 induction (Fig. 2B). ATRA-induced SLC7A11 upregulation was lost in NRF2-deficient cells (Fig. 2C). Lipid ROS, GSH and MDA assays further showed that ATRA could not mitigate DOX-induced ferroptosis in the absence of NRF2 (Fig. 2D–G). Because NRF2 functions as a nuclear transcription factor, we assessed its subcellular localization. Western blotting and immunofluorescence showed enhanced nuclear accumulation of NRF2 after ATRA treatment (Fig. 2H–J). These findings indicate that ATRA promotes NRF2 nuclear translocation to activate SLC7A11 transcription.

3.4. ATRA attenuates the proteasomal degradation of NRF2 by disrupting the KEAP1-NRF2 interaction

We next investigated how ATRA enhances NRF2 nuclear accumulation. qRT-PCR showed that ATRA did not alter NRF2 mRNA expression (Fig. 3A), suggesting that ATRA regulates NRF2 mainly at the protein-stability level. Cycloheximide chase experiments revealed that NRF2 degradation was slower in ATRA-treated cells than in control cells (Fig. 3B, 3C). Treatment with the proteasome inhibitor, MG132, could maintain NRF2 protein abundance, supporting proteasome-dependent regulation (Fig. 3D). Under basal

conditions, KEAP1 is the main negative regulator of NRF2 stability and mediates most NRF2 ubiquitination and proteasomal degradation. Immunoprecipitation showed that ATRA reduced KEAP1-NRF2 binding (Fig. 3E). Together, these results suggest that ATRA weakens the interaction between NRF2 and KEAP1, thereby attenuating KEAP1-dependent proteasomal degradation of NRF2. NRF2 stabilization contributes to the suppression of ferroptosis in cardiomyocytes.

3.5. ATRA regulates NRF2 activity through KEAP1 Arg415

To determine how ATRA disrupts the KEAP1-NRF2 interaction, molecular docking was used to predict potential ATRA-binding sites on KEAP1, focusing on the ten highest-ranking sites (Fig. 4A). Overlay of predicted ATRA-KEAP1 and KEAP1-NRF2 binding interfaces identified KEAP1 Arg415 as a shared site (Fig. 4B). To test the functional importance of Arg415, we generated KEAP1-R415L and KEAP1-R415G point-mutant plasmids (Fig. 4C and S3C, S3D). Immunoprecipitation showed that ATRA reduced NRF2 binding to KEAP1 in KEAP1-overexpressing cells, whereas Arg415 mutation resulted in NRF2 expression unresponsive to ATRA (Fig. 4D). Western blotting, lipid ROS, MDA and GSH assays further showed that ATRA failed to alleviate DOX-induced cell death and oxidative stress in cells expressing Arg415-mutant KEAP1 (Fig. 4E–I). These results support KEAP1 Arg415 as a functionally important residue required for ATRA-mediated regulation of KEAP1–NRF2 binding, NRF2 stabilization and ferroptosis resistance.

3.6. ATRA reduces DOX-induced cardiomyocyte ferroptosis in vivo

To assess the *in vivo* efficacy of ATRA, 8-week-old male C57BL/6 mice were randomly assigned to five groups: control, ATRA, DOX, DOX + ATRA and DOX + DFO. Compared with control mice, DOX-treated mice showed increased gross heart size and heart weight/body weight ratios, whereas these changes were attenuated by ATRA or DFO treatment (Figs. 5A, 5B and S4A). Echocardiography further showed that DOX markedly reduced LVEF and LVFS, while ATRA and DFO partially restored these cardiac functional indices (Figs. 5C–E). Histological staining showed that ATRA and DFO alleviated DOX-induced myocardial structural abnormalities and collagen deposition (Figures S4B, S4C). Moreover, cardiac injury markers, including ANP, BNP, cTnT and MYH7, were increased after DOX treatment and reduced in the DOX + ATRA group (Figures S4D–H). These results indicate that ATRA alleviates DOX-induced cardiotoxicity and preserves cardiac function *in vivo*.

Compared with the DOX-treated group, the DOX + ATRA and DOX + DFO groups showed increased GSH levels and decreased MDA levels (Fig. 5F, 5G), reduced PTGS2 expression and enhanced SLC7A11 expression (Fig. 5H and S4I, S4J). Western blotting showed that ATRA mitigated DOX-induced downregulation of NRF2, SLC7A11 and GPX4 in cardiac tissue (Fig. 5I). Immunohistochemistry further confirmed increased SLC7A11 expression in mouse cardiac tissue after ATRA treatment (Fig. 5J). Together, these data show that ATRA activates the NRF2-SLC7A11 pathway *in vivo*, counteracts DOX-induced cardiomyocyte ferroptosis and reduces cardiac injury in mice.

4. Discussion

In this study, we show that ATRA protects cardiomyocytes against DOX-induced ferroptosis and alleviates cardiac injury. ATRA restored the SLC7A11-GPX4 antioxidant defence system, reduced lipid peroxidation, and improved cardiac function in the mouse model. Mechanistically, this protection depended on NRF2 and SLC7A11. Rather than increasing NRF2 transcription, ATRA stabilised NRF2 protein by weakening KEAP1-mediated NRF2 degradation, with KEAP1 Arg415 serving as a functional site required for this effect. These findings support an anti-ferroptotic model in which ATRA stabilises NRF2, induces SLC7A11 expression, restores GSH-dependent lipid peroxide detoxification, and consequently reduces DOX-induced cardiomyocyte injury.

Cancer therapy-related cardiac injury has become an important clinical problem as cancer survival continues to improve. DOX is a widely used anthracycline chemotherapeutic agent, but its clinical use is limited by dose-dependent cardiotoxicity, which may progress from subclinical ventricular dysfunction to symptomatic heart failure^{10, 21–23}. Cell death is a key step in this process. Fang et al. found that ferroptosis contributes to DOX-induced cardiomyopathy and that ferroptosis inhibition or iron restriction can protect the heart²⁴. Tadokoro et al. further showed that mitochondria-dependent ferroptosis plays a pivotal role in DOX-induced cardiotoxicity and represents a major form of regulated cardiomyocyte death under DOX stress²⁵. Consistent with these studies, our data show that DOX decreases GSH, SLC7A11 and GPX4, while increasing MDA, lipid ROS and PTGS2 in cardiomyocytes. Therefore, effective treatment of anthracycline-induced cardiomyocyte ferroptosis is clinically important and may help reduce chemotherapy-related cardiac injury.

Several approaches have been explored to target cardiomyocyte death or ferroptosis in DOX-induced cardiotoxicity²⁶. Fang et al. reported that ferrostatin-1, iron chelation, the mitochondrial antioxidant MitoTEMPO, Hmox1 inhibition and a low-iron diet could reduce DOX-induced ferroptotic cardiac injury²⁴. More recent studies showed that hydrogen sulfide protected cardiomyocytes through Keap1 S-sulfhydration and activation of the Nrf2/SLC7A11/GSH/GPX4 pathway, calycosin attenuated DOX-induced myocardial injury by activating Nrf2/SLC7A11/GPX4 signalling, and diacerein mitigated ferritinophagy-mediated ferroptosis partly through the same antioxidant axis^{27–29}. Compared with previous cardioprotective strategies targeting ferroptosis, the present study provides a distinct mechanistic angle. ATRA is a clinically used retinoid, and its protective effect is not limited to direct ROS scavenging or iron chelation. Instead, ATRA acts upstream by weakening KEAP1-mediated NRF2 degradation and restoring the endogenous NRF2-SLC7A11-GPX4 defence system. This mechanism may offer a broader and potentially more translatable way to reinforce the cardiomyocyte anti-ferroptotic programme.

These results also refine the understanding of retinoids in ferroptosis biology. Retinoids appear to regulate ferroptosis in a context-dependent manner. In some tumour models, ATRA can suppress NRF2-ARE activity through RAR α -NRF2 crosstalk and increase ROS-dependent cytotoxicity^{30, 31}. By contrast, vitamin A metabolites, including ATRA, have been reported to inhibit ferroptosis in neuronal and non-neuronal cells^{17, 32}, and vitamin A analogues can suppress lipid peroxidation through ACSL3-dependent

lipid remodelling¹⁸. Our findings add a cardiomyocyte-specific mechanism to this field by showing that ATRA stabilises NRF2 in a KEAP1 Arg415-dependent manner and thereby activates SLC7A11. Thus, the effect of ATRA on ferroptosis may depend on cell type, disease context, receptor state and the dominant redox regulatory machinery. This point is important for translation, because a useful cardioprotective agent should protect non-malignant myocardium without reducing the antitumour efficacy of DOX.

Several issues require further study. First, the mechanistic experiments were mainly performed in AC16 cells, and confirmation in primary cardiomyocytes, hiPSC-derived cardiomyocytes and cardiac-specific genetic models would improve physiological relevance. Second, although docking and mutagenesis implicate KEAP1 Arg415, direct biophysical evidence for ATRA–KEAP1 binding is still lacking. Third, the animal study used a short-term DOX injury protocol, whereas chronic cumulative exposure would better mimic clinical anthracycline cardiotoxicity. Finally, we did not evaluate whether ATRA alters DOX antitumour activity, which should be addressed in tumour-bearing models before translational application.

5. Conclusion

In summary, this study demonstrates that ATRA mitigates DOX-induced cardiomyocyte ferroptosis and cardiac dysfunction by blocking KEAP1-mediated NRF2 degradation. Mechanistically, ATRA weakens KEAP1-NRF2 binding through a process that depends on KEAP1 Arg415, thereby stabilising NRF2, promoting its nuclear accumulation, and restoring SLC7A11/GSH/GPX4-mediated antioxidant defence. The requirement for both NRF2 and SLC7A11 further supports the KEAP1-NRF2-SLC7A11 axis as the central pathway underlying the cardioprotective effect of ATRA. These findings provide a mechanistic basis for exploring retinoid-based cardioprotection during DOX therapy.

Abbreviations

Abbreviation	Full term
ACSL3	Acyl-CoA synthetase long-chain family member 3
ANP	Atrial natriuretic peptide
ARE	Antioxidant response element
Arg415	Arginine 415
ATF3	Activating transcription factor 3
ATF4	Activating transcription factor 4
ATRA	All-trans retinoic acid
BNP	B-type natriuretic peptide
CHX	Cycloheximide
cTnT	Cardiac troponin T
DAPI	4',6-Diamidino-2-phenylindole
DFO	Deferoxamine
DOX	Doxorubicin
FBS	Fetal bovine serum
Fer-1	Ferrostatin-1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene Expression Omnibus
GPX4	Glutathione peroxidase 4
GSH	Glutathione
HO-1	Heme oxygenase-1
KEAP1	Kelch-like ECH-associated protein 1
LVEF	Left ventricular ejection fraction
LVFS	Left ventricular fractional shortening
MDA	Malondialdehyde
MG132	Proteasome inhibitor MG132
MYH7	Myosin heavy chain 7
NIH	National Institutes of Health
NRF2	Nuclear factor erythroid 2-related factor 2

p53	Tumor protein p53
PTGS2	Prostaglandin-endoperoxide synthase 2
RAR	Retinoic acid receptor
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RSL3	RAS-selective lethal 3
RXR	Retinoid X receptor
sgRNA	Single-guide RNA
shRNA	Short hairpin RNA
SLC7A11	Solute carrier family 7 member 11

Declarations

Ethics approval and consent to participate

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Jiangsu University. The approval number was UJS-IACUC-AP-2024040214.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this manuscript and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Xu Wenjin and Xie Yuxin performed most of the experiments, analysed the data, prepared the figures, and drafted the manuscript. Guo Yaxin contributed to data collection, statistical analysis, and figure preparation. Yi Yide contributed to experimental validation and data interpretation. Hsiang-i Tsai contributed to methodology development, data analysis, and manuscript revision. Fu Shengxiang and Chen Mengxiang contributed to study design, experimental supervision, data interpretation, and manuscript revision. Zhu Haitao and Liao Xiang conceived and designed the study, supervised the project, secured funding, and critically revised the manuscript. All authors read and approved the final manuscript.

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Figures

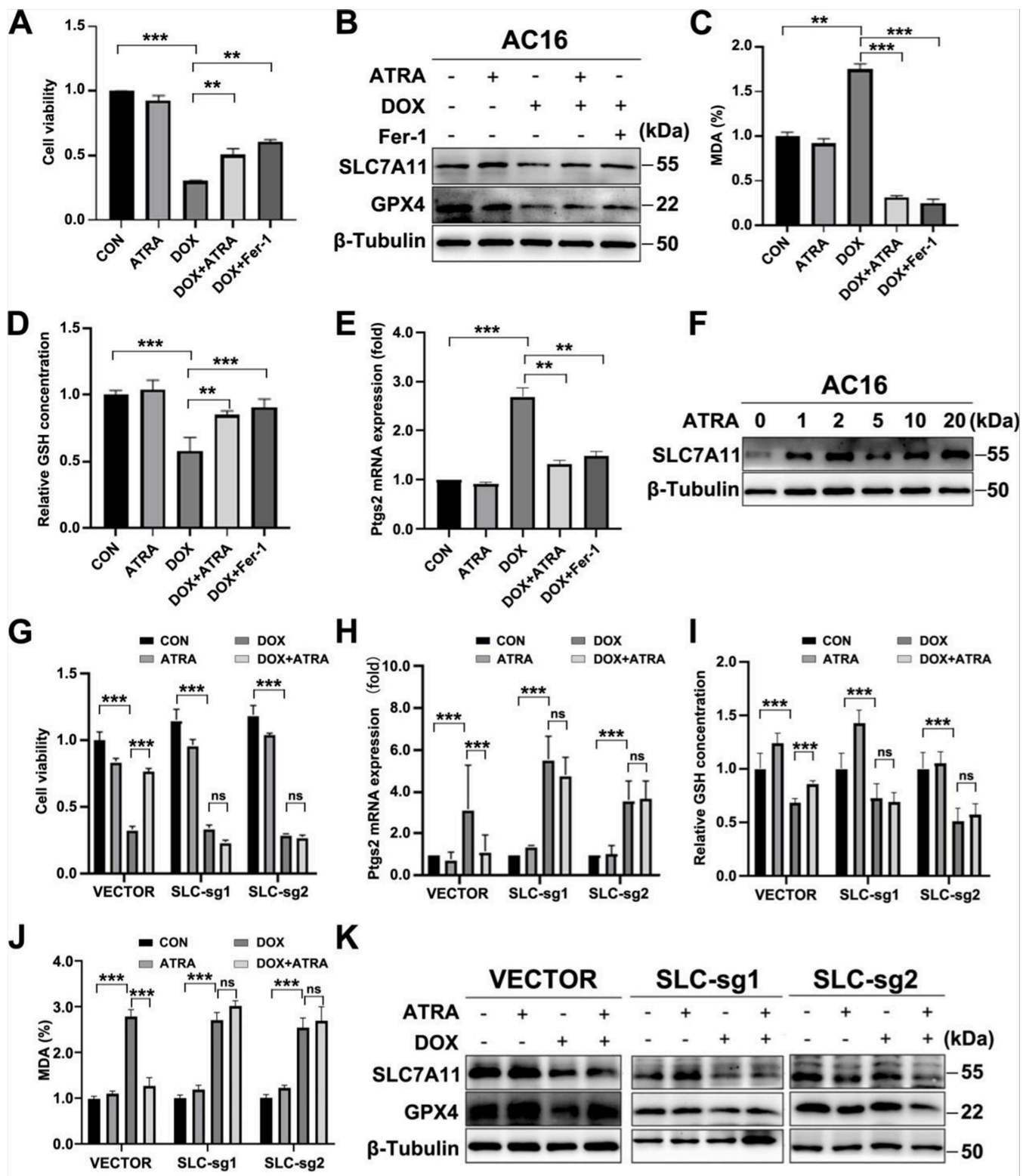


Figure 1

ATRA mitigates DOX-induced cardiomyocyte ferroptosis through SLC7A11.

(A) CCK-8 analysis of AC16 cell viability after treatment with vehicle (CON), ATRA, DOX, DOX + ATRA, or DOX + ferrostatin-1 (Fer-1). (B) Western blotting of SLC7A11 and GPX4 in AC16 cells treated as indicated; β -Tubulin was used as the loading control. (C, D) Quantification of MDA (C) and GSH (D) levels

in AC16 cells after each treatment. (E) qRT-PCR analysis of Ptgs2 mRNA expression. (F) Western blotting showing SLC7A11 expression in AC16 cells exposed to increasing concentrations of ATRA. (G) CCK-8 analysis of cell viability in vector control cells and two SLC7A11-knockout cell lines (SLC-sg1 and SLC-sg2) treated with CON, ATRA, DOX, or DOX + ATRA. (H-J) qRT-PCR analysis of Ptgs2 (H), GSH measurement (I), and MDA measurement (J) in vector and SLC7A11-knockout cells. (K) Western blotting of SLC7A11 and GPX4 in vector and SLC7A11-knockout cells after the indicated treatments. Data are mean $\pm$ SD. Statistical tests are described in Methods. ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

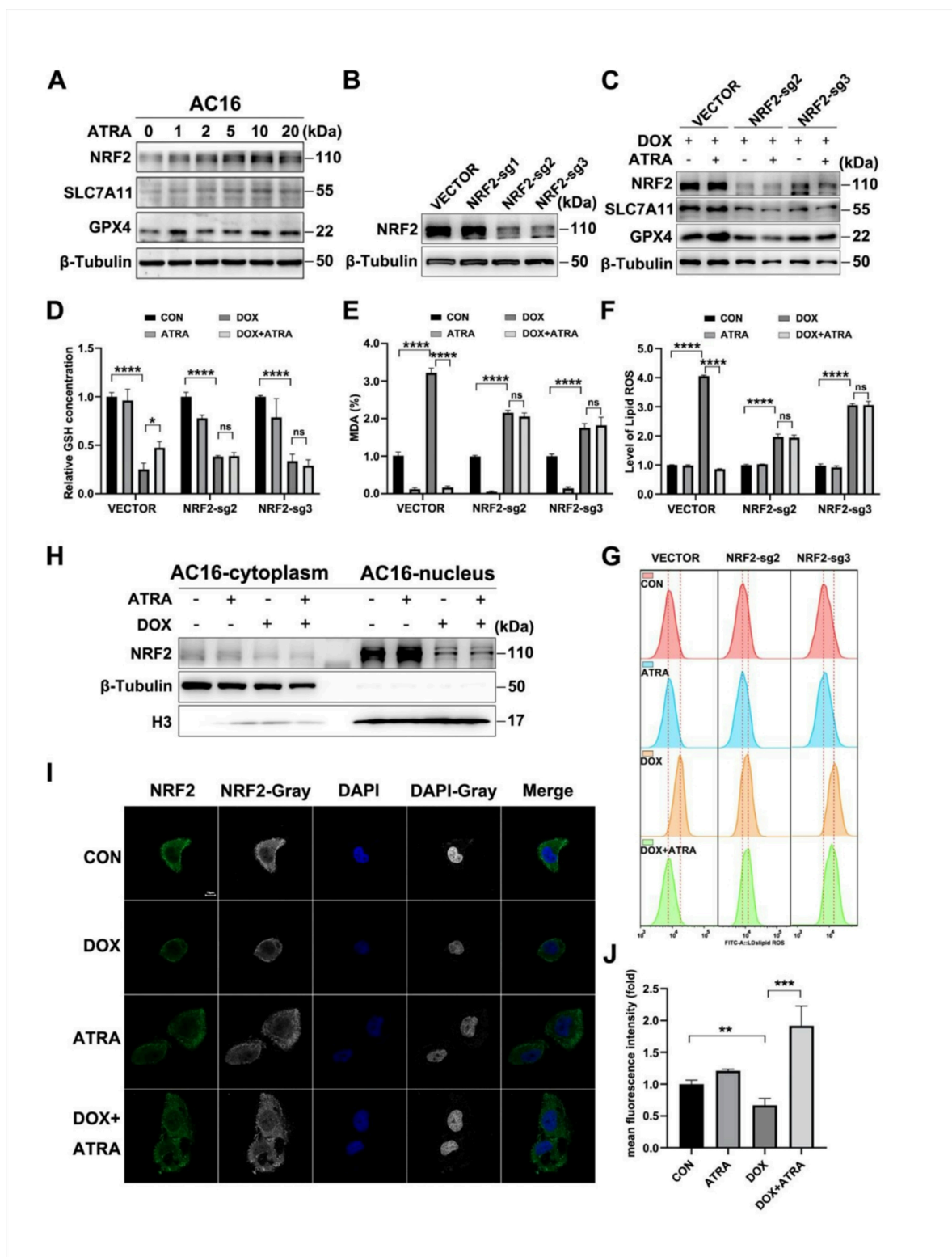


Figure 2

ATRA activates NRF2 to drive SLC7A11-dependent anti-ferroptotic signalling.

(A) Western blotting of NRF2, SLC7A11, and GPX4 in AC16 cells treated with increasing concentrations of ATRA. (B) Western blotting validating NRF2 knockout in vector and NRF2-sgRNA AC16 cells. (C) Western blotting of NRF2, SLC7A11, and GPX4 in vector and NRF2-deficient cells under DOX or DOX +

ATRA treatment. (D-F) GSH (D), MDA (E), and lipid ROS (F) levels in vector and NRF2-deficient cells treated with CON, ATRA, DOX, or DOX + ATRA. (G) Representative C11-BODIPY flow cytometry histograms showing lipid ROS accumulation. (H) Nuclear/cytoplasmic fractionation followed by western blotting to assess NRF2 subcellular distribution; β -Tubulin and H3 served as cytoplasmic and nuclear controls, respectively. (I) Confocal immunofluorescence showing NRF2 localisation in AC16 cells after the indicated treatments; green, NRF2; blue, DAPI. (J) Quantification of NRF2 fluorescence intensity. Data are mean $\pm$ SD. Statistical tests are described in Methods. ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

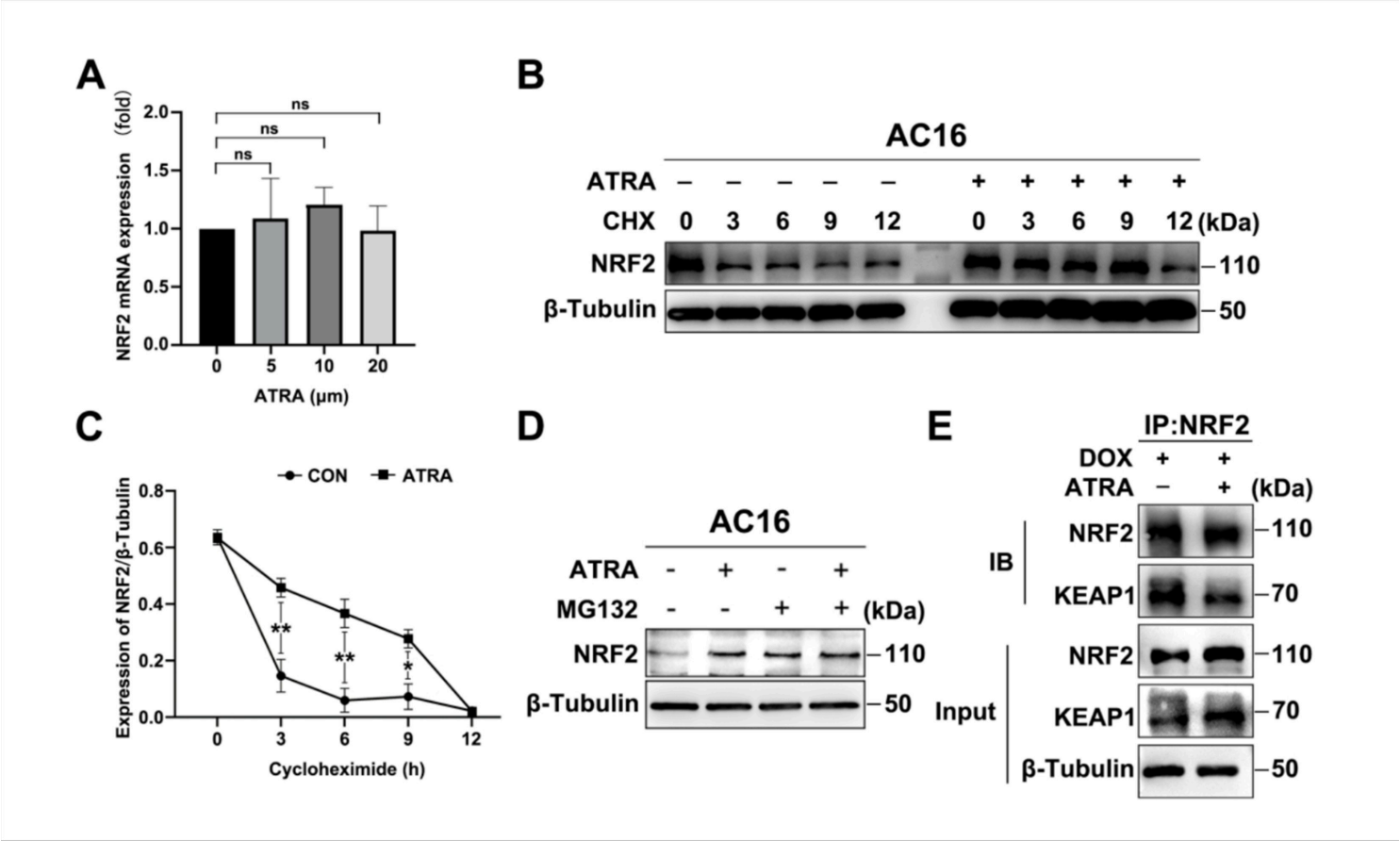


Figure 3

ATRA stabilises NRF2 by attenuating KEAP1-mediated proteasomal degradation.

(A) qRT-PCR analysis of NRF2 mRNA expression in AC16 cells treated with increasing concentrations of ATRA. (B) Cycloheximide (CHX) chase assay showing NRF2 protein degradation in AC16 cells with or without ATRA. (C) Densitometric quantification of NRF2 protein abundance normalised to β -Tubulin in (B). (D) Western blotting of NRF2 expression after ATRA and/or MG132 treatment, with β -Tubulin as the loading control. (E) Co-immunoprecipitation assay detecting the interaction between NRF2 and KEAP1 in DOX-treated AC16 cells with or without ATRA; input lysates are shown below. Data are mean $\pm$ SD. Statistical tests are described in Methods. ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

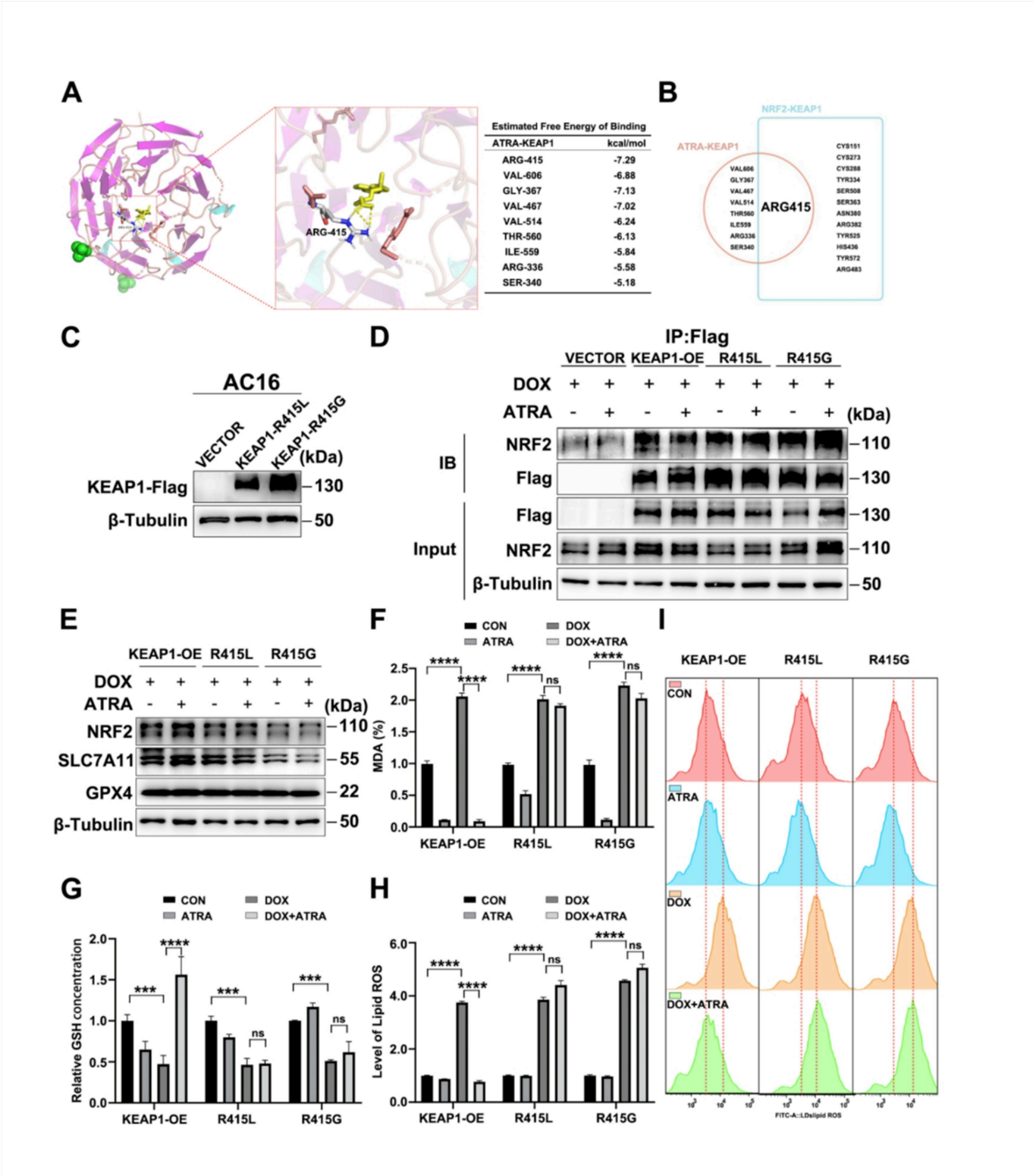


Figure 4

KEAP1 Arg415 is required for ATRA-mediated NRF2 activation and ferroptosis resistance.

(A) Molecular docking and PyMOL visualisation of predicted ATRA-binding sites on KEAP1, with estimated free energies; Arg415 is highlighted at the predicted interface. (B) Overlap between predicted ATRA-KEAP1 contact residues and reported NRF2-KEAP1 interface residues, identifying Arg415 as a

shared site. (C) Western blotting confirming Flag-tagged KEAP1 R415L and R415G expression in AC16 cells. (D) Flag co-immunoprecipitation assessing NRF2 binding to wild-type KEAP1 (KEAP1-OE) or KEAP1 Arg415 mutants after DOX or DOX + ATRA treatment; input controls are shown. (E) Western blotting of NRF2, SLC7A11, and GPX4 in KEAP1-OE, R415L, and R415G cells treated with DOX or DOX + ATRA. (F-H) MDA (F), GSH (G), and lipid ROS (H) measurements after the indicated treatments. (I) Representative C11-BODIPY flow cytometry histograms of lipid ROS. Data are mean $\pm$ SD. Statistical tests are described in Methods. ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

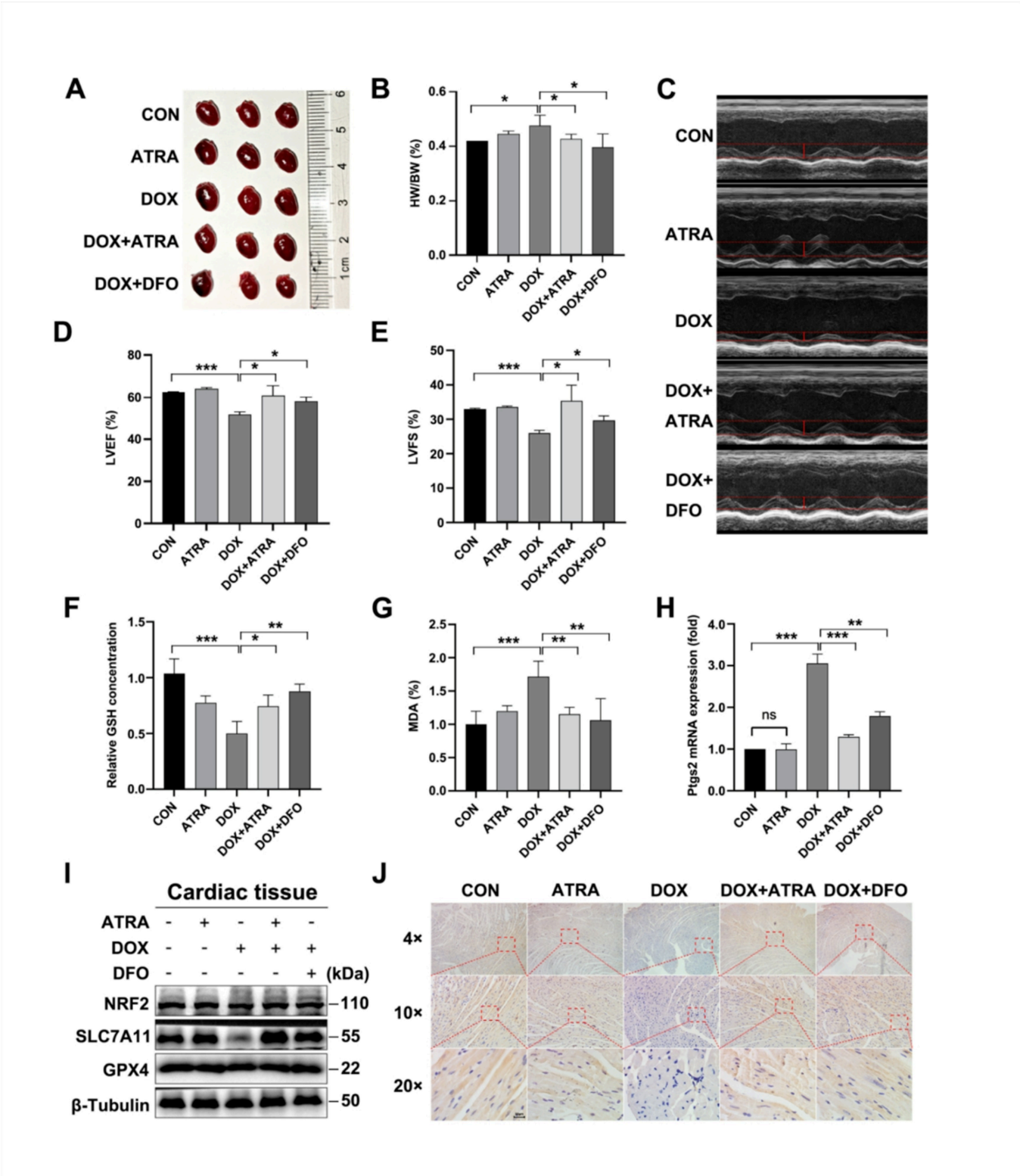


Figure 5

ATRA attenuates DOX-induced cardiac dysfunction and myocardial ferroptosis in vivo.

(A) Gross morphology of mouse hearts from the CON, ATRA, DOX, DOX + ATRA, and DOX + DFO groups. (B) Quantification of the heart weight/body weight ratio. (C) Representative M-mode echocardiographic images. (D, E) Quantification of left ventricular ejection fraction (LVEF; D) and left ventricular fractional shortening (LVFS; E). (F, G) GSH (F) and MDA (G) levels in cardiac tissues. (H) qRT-PCR analysis of Ptgs2 mRNA expression in cardiac tissues. (I) Western blotting of NRF2, SLC7A11, and GPX4 in cardiac tissues, with β -Tubulin as the loading control. (J) Immunohistochemical staining of SLC7A11 in cardiac sections at the indicated magnifications. DFO, deferoxamine. Data are mean $\pm$ SD. Statistical tests are described in Methods. ns, not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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