Sirt6 protects cardiomyocytes against doxorubicin-induced cardiotoxicity by inhibiting P53/Fas-dependent cell death and augmenting endogenous antioxidant defense mechanisms

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Abstract Sirt6, a class III NAD+−dependent deacetylase of the sirtuin family, is a highly specific H3 deacetylase and plays important roles in regulating cellular growth and death. The induction of oxidative stress and death is the critical mechanism involved in cardiomyocyte injury and cardiac dysfunction in doxorubicin-induced cardiotoxicity, but the regulatory role of Sirt6 in the fate of DOX-impaired cardiomyocytes is poorly understood. In the present study,
we exposed Sirt6 heterozygous (Sirt6+/−) mice and their littermates as well as cultured neonatal rat cardiomyocytes to DOX, then investigated the role of Sirt6 in mitigating oxidative stress and cardiac injury in the DOX-treated myocardium. Sirt6 partial knockout or silencing worsened cardiac damage, remodeling, and oxidative stress injury in mice or cultured cardiomyocytes with DOX challenge. Cardiomyocytes infected with adenoviral constructs encoding Sirt6 showed reversal of this DOX-induced damage. Intriguingly, Sirt6 reduced oxidative stress injury by upregulating endogenous antioxidant levels, interacted with oxidative stress-stirred p53, and acted as a corepressor of p53 in nuclei. Sirt6 was recruited by p53 to the promoter regions of the target genes Fas and FasL and further suppressed p53 transcription activity by reducing histone acetylation. Sirt6 inhibited Fas/FasL signaling and attenuated both Fas-FADD-caspase-8 apoptotic and Fas-RIP3 necrotic pathways. These results indicate that Sirt6 protects the heart against DOX-induced cardiotoxicity by upregulating endogenous antioxidants, as well as suppressing oxidative stress and cell death signaling pathways dependent on ROS-stirred p53 transcriptional activation, thus reducing Fas–FasL-mediated apoptosis and necrosis.

**Keywords** Doxorubicin · Cardiotoxicity · Sirt6 · p53 · Fas · FasL · Apoptosis · Necrosis

**Abbreviations**
CK Creatine kinase
CK-MB Creatine kinase-MB
CO Cardiac output
DMSO Dimethyl sulfoxide
DOX Doxorubicin
dP_max Maximal value of the first derivative of LV pressure
dP_min Minimal value of the first derivative of LV pressure
EDV End-diastolic volume
EF Ejection fraction
ESV End-systolic volume
FADD Fas associated via death domain
Fas Tumor necrosis factor receptor superfamily member 6
FasL Fas receptor-Fas ligand
FS Fractional shortening
GFP Green fluorescent protein
Gpx1 Glutathione peroxidase 1
H3K9Ac Acetylation on histone H3 lysine 9
HMGB1 High mobility group box 1
KO Knockout
LDH Lactate dehydrogenase
LV Left ventricular
LVEDD LV end diastolic diameter
LVEDV LV end-diastolic volume
LVEDP LV end-diastolic pressure
LVESP LV end-systolic pressure
LVIDs LV internal dimension in systole
LVIDd LV internal dimension in diastole
LVP LV pressure
LVPWd LV posterior wall thickness at end diastole
LVSD Left ventricle systolic diameter
LVW/TL Left heart ventricle weight/tibia length
NRCMs Neonatal rat cardiomyocytes
Prx5 Peroxiredoxin 5
ROS Reactive oxygen species
SOD2 Mn-dependent superoxide dismutase 2
SV Stroke volume
WT Wild type

**Introduction**

Doxorubicin (DOX) is a highly efficacious anticancer therapeutic drug but causes serious cardiotoxicity (Kalyanaraman, 2020; Zhang et al. 2009). Cardiomyocyte death due to apoptosis and necrosis is a critical mechanism underlying DOX-induced cardiotoxicity (Kalyanaraman 2020; Zhang et al. 2009). Free radical generation, oxidative stress, and cell death play pivotal roles in DOX-induced cardiotoxicity, and other contributors including mitochondrial function impairment, Ca²⁺ homeostasis disruption, iron regulatory protein perturbation, autophagy, inflammatory mediator release, and apoptosis-related gene and protein expression alteration participate in its pathogenesis (Prathumsap et al. 2020; Kumari et al. 2020). Most of the cardiomyocyte death can result from these cellular events (Zhang et al. 2009). Although DOX-induced cardiomyocyte death has been studied in depth for decades, the precise mechanisms are not completely elucidated (Prathumsap et al. 2020; Kumari et al. 2020; Kalyanaraman 2020; Zhang et al. 2009).

Sirtuins are an NAD-dependent deacetylase family that plays an indispensable role in delaying cell senescence and extending organismal lifespan by regulating diverse biological functions (Hall et al. 2013). There are...
seven sirtuin isoforms in mammals (Sirt1-Sirt7), with different intracellular distributions (Poulose and Raju 2015). The isoforms are involved in wide-ranging cellular processes from cell growth to longevity (Poulose and Raju 2015; Hall et al. 2013). Sirt6, specifically located in the nucleus, is a stress-responsive protein deacetylase and mono-ADP ribosyltransferase enzyme that has a prominent role in the regulation of genomic stability, cell metabolism, aging, inflammation, and stress response (Saiyang et al. 2020; Poulose and Raju 2015; Hall et al. 2013). Sirt6 has deacetylase activity toward histone H3K9Ac (acetylation on histone H3 lysine 9) and serves as a corepressor of the transcription factor hypoxia inducible factor 1α, c-Jun, and nuclear factor κB, to deacetylate histone H3K9Ac at their target promoters and downregulate the expression of a subset of their target genes (Saiyang et al. 2020; Poulose and Raju 2015). Ran et al. observed that Sirt6 was recruited to the promoter of Bcl2-associated X (BAX), where it deacetylated H3K9 and suppressed its promoter activity to reduce Bax mRNA expression, then inhibited tumor cell apoptosis (Ran et al. 2016). Peng et al. reported a deacetylase-independent protection of Sirt6 against DOX-induced cardiotoxicity, by recruiting TIP60 acetyltransferase to acetylate the transcription factor GATA4, to promote anti-apoptotic gene expression (Peng et al. 2020). These results suggest that Sirt6 can directly regulate cell death by interacting with apoptosis-related genes.

p53, a crucial tumor suppressor, is usually activated by oxidative stress inductions that are common responses to DOX exposure, and plays a crucial role in DOX-induced cardiotoxicity (Shi and Dansen 2020; Liu et al. 2020; Gambino et al. 2013). p53 is also upregulated in cardiac tissues on DOX exposure (Men et al. 2021; Shizukuda et al. 2005). Once active p53 is bound to DNA, it can stimulate the transcription of many cell death genes (Hafner et al. 2019). p53-mediated signals play a significant role in DOX-induced cardiotoxicity (Men et al. 2021; Hafner et al. 2019; Zhu et al. 2009; Shizukuda et al. 2005). p53 inactivation protects hearts against DOX-induced damage and atrophy by inhibiting oxidative stress and disinhibiting mammalian target of rapamycin signaling (Zhu et al. 2009; Shizukuda et al. 2005). McSweeney et al. used RNA sequencing (McSweeney et al. 2019) to investigate DOX-induced transcriptomic changes in human induced pluripotent stem cell-derived cardiomyocytes and suggested that p53 is a key regulator of transcriptomic changes. In addition, the increased expression of death receptors (including Fas) and enriched exogenous apoptotic pathways were significantly associated with DOX-induced cardiac injury (McSweeney et al. 2019). Fas-mediated extrinsic apoptosis proceeds through the Fas-associated death domain (FADD)/caspase-8-dependent apoptotic pathway by binding to the receptor of its ligand, Fas ligand (Holler et al. 2000; Kaufmann et al. 2012). The FasL/Fas system also leads to necrosis by activating kinase receptor-interacting protein pathways (Holler et al. 2000; Zhang et al. 2010; Amir et al. 2009).

A few existing studies have suggested that a direct interaction exists between Sirt6 and p53 (Li et al. 2018a, b; Ghosh et al. 2018). Sirt6 can serve as a co-activator of p53 (Li et al. 2018a, b) and deacetylate p53 as a substrate (Ghosh et al. 2018). Now, the role of Sirt6 in regulating cell death pathways is still unknown (McSweeney et al. 2019; Hafner et al. 2019; Hall et al. 2013; Zhu et al. 2009). In this study, we investigated the regulatory role of Sirt6 on cardiomyocyte fate in DOX-treated mice and cultured neonatal rat cardiomyocytes and identified its mechanisms of action.

Materials and methods

Reagents and antibodies

DOX, 5-bromo-2′-deoxyuridine (BrdUrd), lipid peroxidation (malondialdehyde, MDA) assay kit, catalase (CAT), and total superoxide dismutase (SOD) assay kits were from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Protein A/G plus agarose was from Santa Cruz Biotechnology (Dallas, TX, USA). MitoSOX Red was from Invitrogen (Carlsbad, CA, USA). CardioTACS in situ apoptosis detection kit was from Trevigen (Gaithersburg, MD, USA). Lipofectamine 3000 and TRizol reagent were from Invitrogen (Carlsbad, CA, USA). Annexin V-APC/7-AAD Cell Assay Kit was from BD Pharmingen (San Diego, CA, USA). Simple ChIP Enzymatic Chromatin IP Kit and antibodies anti-total p53 (1:1000, #2524), anti-phospho-p53(Ser392) (1:1000, #9281), anti-Sirt6 (1:1000, #12486), anti-caspase-3 (1:1000, #9662), anti-caspase-8 (1:1000, #9746), anti-Bcl2 (1:1000, #2876), anti-Bcl-xL (1:1000, #2764), anti-Bax (1:1000, #2772), anti-Bak (1:1000, #12105), anti-RIP1 (1:1000, #3493),
anti-RIP3 (1:1000, #15828), anti-histone H3 (1:2000, 9715), anti-H3K9Ac (1:100, #9649), anti-Myc-Tag(9B11) (1:100, #2276), anti-FLAG-tag(D6W5B) (1:1000, #14793), anti-Acetylated-Lysine (1:100, #9441), anti-β-actin (1:2000, #4970), and the negative control for mouse IgG (#5415) were from Cell Signaling Technology (Danvers, MA, USA). The antibodies anti-Fas (1:1000, #ab82419) and anti-FADD (1:1000, #ab24533) were from Abcam (MA, USA). The antibodies anti-HMGB1 (1:1000, #MAB16901-SP) and anti-FasL (1:1000, #AB16982) were from R&D Systems (Minneapolis, MN, USA) and Sigma-Aldrich Chemicals (St Louis, MO, USA), respectively. The membrane protein extraction kit was a Mem-PER™ Plus Membrane Protein Extraction Reagent Kit from Thermo Scientific (Catalog number: 89842, Rockford, IL, USA). The cytosol and nuclear protein extraction kit was a ReadyPrep™ Protein Extraction Kit (cytoplasm/nuclear) from Bio-Rad (cat no. 163-2089; Bio-Rad, Hercules, CA, USA). BCA protein assay kit was from Beyotime Institute of Biotechnology (Beijing, China). The PrimeScript RT Reagent Kit with gDNA Eraser was from Takara Biotechnology (Dalian, China). All pairs of PCR primers were synthesized by the Chengdu Qingke Biotechnology (Chengdu, China). All other chemicals were of analytical grade.

Animals

Sirt6+/− (Sirt6 heterozygous) mice on a129Sv background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Sirt6 heterozygous mice (Sirt6+/−) were backcrossed with wild-type(WT)C57BL/6J mice on a C57BL/6J background to obtain Sirt6+/− mice and littersmates (Wang et al. 2016). The mice were confirmed by PCR genotyping analysis (data not shown). The Animal Care and Use Committee of Sichuan University ensures that all procedures involving experimental animals were in compliance with the Guidelines for Animal Experiments from the Committee of Medical Ethics, National Health Department of China.

In vivo DOX-induced cardiotoxicity mouse model

Male Sirt6+/− and WT mice (8 to 12 weeks old) were treated with a 5-week protocol, which consisted of intraperitoneal injection (i.p.) of DOX at 4 mg/kg (body weight) or saline once per week for 4 weeks and maintenance for another one week. All drug doses and injection regiments were based on previous reports with some modification (Wang et al. 2014).

Cardiac echocardiography and hemodynamics

After the 5-week treatment, mice (n = 10) were anesthetized using isoflurane (2%) in oxygen and positioned in a recumbent position on a warming pad (Wang et al. 2016; Wang et al. 2014). 2D short-axis images were obtained by using a Vivid 7 ultrasound imaging system (GE Health Medical, Milwaukee, WI, USA) operating at 12 MHz. Echocardiographic image acquisition and measurements were acquired from grayscale M-mode images in the parasternal short-axis view at the mid-papillary level, and two-dimensional(2D) mode images were taken in the parasternal long- and short-axis views (Wang et al. 2016; Wang et al. 2014). After the echocardiography assessment, the mice were ventilated with a rodent ventilator (Harvard Apparatus, Holliston, MA, USA), then maintained with 2% isoflurane in oxygen for anesthesia. An incision in the neck was made and a pressure–volume(P-V) catheter with a 1.2-F transducer (serial no. 112B-B057, Scisense Inc., ON, Canada) was inserted into the left ventricle (LV) through the right carotid artery. After the P-V catheter was stabilized for several minutes, the signals were recorded by using an iWorx 308 eight-channel physiological data recorder (iWorx/CB Sciences, Inc., Dover, NH, USA). The LV performance, including the heart rate and LV peak systolic and end-diastolic pressures, was detected, while LV peak-negative developed pressure (dP/dtmin) and LV pressure at peak-positive developed pressure (dP/dtmax) were calculated (Wang et al. 2016; Wang et al. 2014). After hemodynamic measurements, mice were euthanized with an overdose of pentobarbital (i.p.), and hearts were harvested and cut into three pieces. For histology, heart slices were cut into 4-μm serial sections for immunohistochemistry after being 4% paraformaldehyde-fixed and paraffin-embedded, and the other two slices were snap-frozen using liquid nitrogen and then stored at −80°C (Wang et al. 2014).

Histology analysis

The histological sections were stained by using hematoxylin–eosin(HE) solution, wheat germ agglutinin (WGA), and Masson trichrome and underwent terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end-labeling(TUNEL) with standard
protocols (Wang et al. 2016; Li et al. 2018a, b). Cardiomyocyte size was measured from images of HE-stained sections and Alexa Fluor 488–conjugated WGA-stained sections and cardiomyocytes. About 100–200 random cardiomyocytes were measured per group (n = 10) were quantitatively assessed with Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA), and cardiomyocyte cross-sectional areas were visualized under a Zeiss microscope (AX10 imager A2 model, Germany) and presented in square micrometer (Li et al. 2018a, b). Myocardial fibrotic area was measured in five randomly selected fields per section after being Masson’s trichrome stained from each group (n = 4), and the fibrotic proportion was calculated using Image-Pro Plus as the percentage of fibrotic area to total left ventricular (LV) area (Li et al. 2018a, b; Ghosh et al. 2018; Wang et al. 2016; Zhang et al. 2010).

TUNEL staining used to assess DOX-induced cell apoptosis was analyzed by using a CardioTACS™ in situ apoptosis detection kit with the manufacturer’s instructions (Wang et al. 2014). Five randomly areas from each slice were visualized for comparison of each experimental group. The apoptotic index (%) was calculated as the percentage of TUNEL-positive cardiomyocyte nuclei to the total number of nuclei × 100 (Men et al. 2021; Wang et al. 2014).

Cardiomyocyte culture, RNA interference, adenoviral infection, and treatment

Neonatal rat cardiomyocytes (NRCMs) were isolated from 0- to 2-day-old newborn Sprague-Dawley rats, which were sacrificed by decapitation, and cardiac ventricles were obtained from all rat pups for trypsin and collagenase II digestion as described (Wang et al. 2016; Li et al. 2018a, b). After digestion, cells were suspended with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum (FBS, 10%), penicillin (100 U/mL), and streptomycin (100 U/mL); then, any non-myocyte cells were removed by pre-plate for 1 h, then plated in cell culture dishes and placed in incubator overnight maintained at 5% CO₂ in air and > 95% humidity. On the next day, cells were cultured for 24 h in DMEM supplemented with 100 μM BrdUrd to prevent non-myocyte cell proliferation.

The rat Sirt6 siRNA (siRNA no. siG150505110012), p53-siRNA(Kang et al. 2019), and the control sequence (NTC, no target control) were from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). NRCMs were transfected with Sirt6 siRNA, p53-siRNA, or NTC by using Lipofectamine 3000 following the manufacturer’s instructions (Wang et al. 2016). The Sirt6-Flag plasmids (no. 13817) were bought from Addgene (MA, USA). The replication-defective adenoviral vectors expressing Sirt6 (Ad-Sirt6) and control green fluorescent protein (Ad-EGFP) were constructed with the AdEasy1 system instructions following the manufacturer’s instruction (Wang et al. 2016). In all siRNA-treated or adenovirus-treated experiments, cardiomyocytes were transfected with Sirt6 siRNA or p53 siRNA or control NTC siRNA oligonucleotides for 48 h or with Ad-Sirt6 or control Ad-EGFP adenovirus for 48 h, then treated with DOX (2 μM) for another 18 h.

Malondialdehyde (MDA) content and SOD2 and CAT enzyme activity assays

Heart tissues or cardiomyocytes were harvested and homogenized in 0.9% saline and centrifuged at 4 °C (12,000 rpm, 15 min). The supernatant was collected, and then, the protein concentration of lysates was measured with a BCA assay kit. MDA content and both SOD2 and CAT activities in lysates of cardiac tissues and NRCMs were analyzed according to the manufacturers’ instructions (Wang et al. 2016; Wang et al. 2014).

RT-PCR assay

Total RNA was extracted by using the phenol-chloroform-based RNA extraction method with TRIzol reagent. Total RNA (1 μg) was reverse-transcribed for cDNA synthesis with the PrimeScript RT reagent kit with gDNA Eraser. All relative mRNA levels were standardized to those of β-actin. The primer sequences are in supplemental Table 1 and RT-PCR procedures involved using a CFX96™ Real-Time PCR Detection System (Bio-Rad Life Sciences).

Measurement of mitochondria reactive oxygen species production

Superoxide production was detected in living cardiomyocytes by using MitoSOX Red, a mitochondrion-specific hydroethidine-based fluorogenic dye following the manufacturer’s instructions (Wang et al. 2016). Briefly, after treatment with siRNA or adenovirus, NRCMs...
were stained with MitoSOX Red reagent (5 μM) at 37°C in the dark with or without DOX (2 μM) for 18 h and washed twice with phosphate-buffered saline (PBS) and nuclei were stained with DAPI in antifade mounting buffer and visualized by Zeiss microscopy. Fluorescence intensity was analyzed by using ImageJ (Wang et al. 2016).

Apoptosis detected by flow cytometry

Primary cardiomyocytes (5 × 10⁵/mL) were seeded in six-well plates and cultured for 24 h, then transfected with NTC-siRNA or Sirt6 siRNA for 48 h before incubation with or without DOX for 18 h or were overexpressed with Ad-EGFP or Ad-Sirt6 viruses and washed with PBS. Cells with no drugs were the control. Early/late apoptotic cells were measured by a FACSAria SORP Cell Analyzer (BD, Biosciences, San Jose, CA, USA) using the Annexin V-APC/7-AAD Cell Assay Kit.

Western blot assay and immunoprecipitation

Western blot assay was used to detect protein expression as described (Wang et al. 2016; Li et al. 2018a, b) including Sirt6, p53, caspase-8, caspase-3, RIP1, RIP3, Fas, FasL, and FADD in cardiac tissues and cardiomyocytes. Total membrane proteins and cytosolic proteins were extracted to examine the expression of membrane Fas and FasL following the protein extraction kit as described (Hou et al. 2018). Nuclear proteins were extracted to detect the protein expression levels of high mobility group box 1 (HMGB1) and H3K9Ac with a nuclear extraction kit (Wang et al. 2016). β-Actin, Na/K-ATPase α1, and Histone H3 were used for normalization in whole cell homogenates, isolated membrane proteins, and nuclei proteins, respectively.

The interacting partners of Sirt6 and p53, as well as the nuclear levels of pan-acetylated p53 in NRCMs, were assessed by co-immunoprecipitation (Wang et al. 2016; Sykes et al. 2006). Cardiomyocytes were harvested 48 h after the transfection with overexpression vectors of Flag-tagged Sirt6 (no. 13817, Addgene) and Myc-p53 (no. MG50534, Sino Biological Inc. Beijing, China). Cell extracts were prepared and then pulled down with an anti-Flag antibody together with protein A/G plus agarose. Immunoprecipitated proteins were analyzed using western blot methods as described (Wang et al. 2016, Sykes et al. 2006).

Pull-down analysis

HEK293T cells were harvested 48 h after the transfection with overexpression vectors of Flag-tagged Sirt6 (no. 13817, Addgene) and Myc-p53 (no. MG50534, Sino Biological Inc. Beijing, China). Cell extracts were prepared and then pulled down with an anti-Flag antibody together with protein A/G plus agarose. Immunoprecipitated proteins were analyzed using western blot assay with anti-Myc antibody as described previously (Amir et al. 2009)

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described before by using the Simple ChIP Enzymatic Chromatin IP kits (Wang et al. 2016). Briefly, NRCMs were formaldehyde (1%)-crosslinked and digested by micrococcal nuclease into 150–900-bp DNA fragments; then, a mouse anti-p53 antibody or rabbit anti-Sirt6 antibody or rabbit anti-H3K9Ac antibody was added to the diluted soluble chromatin solution at 4 °C for 18 h on a rotator, and a mouse IgG or rabbit IgG was further used as a negative control, 1% of the total chromatin sample before immunoprecipitation (input). After being reverse-crosslinked, eluted DNA was amplified with specific Fas and FasL promoter primers (for a 174-bp fragment of Fas promoter: forward 5′-ATGAGTTGTGGGTGTCAG-3′, reverse 5′-TAATGGAGGCAACAGAGCTAC-3′; for a 100-bp fragment of FasL promoter: forward 5′-CAGTACAGAAGCCGACTAC-3′ and reverse 5′-ACTAGAGTCCACCATCCCCA-3′). The ΔΔCt method was employed to calculate the relative abundance of the studied DNA fragments in immunoprecipitated DNA.

Statistical analysis

The data were presented as mean ± SEM. Intergroup comparisons were analyzed by using unpaired Student t test, and data of three or more repeated measures were analyzed by one-way ANOVA followed by the
Newman–Keuls post hoc comparison. A $P$ value less than 0.05 was statistically significant.

**Results**

Sirt6 is downregulated by DOX treatment

Cardiac Sirt6 level was significantly lower in Sirt6 +/- than WT mice, by 58.3% ($P < 0.01$, Fig. 1a). Five-week DOX challenge led to a significant decrease in the myocardial Sirt6 protein level, with 39.5% and 74.8% less in WT and Sirt6 +/- than control hearts (both $P < 0.01$; Fig. 1a). Sirt6 silencing induced a significant decrease in Sirt6 level of neonatal rat cardiomyocytes by 72.5%, compared with NTC-treated control NRCMs ($P < 0.01$, Fig. 1b). DOX treatment also significantly reduced Sirt6 expression, by 32.7% ($P < 0.05$) and 83.8% ($P < 0.01$) in NTC-treated and Sirt6 siRNA–transfected neonatal rat cardiomyocytes, respectively (Fig. 1b). Ad-Sirt6 infection induced significant expression of Flag-Sirt6 in cardiomyocytes, which was significantly reduced by DOX treatment (Supplementary Figure 1).

Sirt6 deficiency exacerbates body weight loss and promotes mortality in mice with DOX treatment

Mice receiving DOX showed rapid weight loss, which was deteriorated in Sirt6 +/- mice (Fig. 1c). However, the body weight for control WT and Sirt6 +/- mice gradually increased over 5 weeks, with no significant difference between the two groups. No control WT or Sirt6 +/- mice died. DOX exposure greatly decreased survival rate, with 46.5% and 76.3% WT and Sirt6 +/- mice dying 5 weeks after the first DOX injection (both $P < 0.01$ vs controls, Fig. 1d). The survival rate was lower for DOX-treated Sirt6-deficient than DOX-treated WT mice ($P < 0.05$, Fig. 1d).

Sirt6 deficiency exacerbates cardiac atrophy after DOX treatment in vivo and ex vivo

DOX exposure induced left ventricular (LV) contractility decrease and myocardial structural changes (Wang et al. 2014). Five-week DOX treatment significantly decreased the ratio of LV weight to tibial length (LVW/TL) by 12.1% (Fig. 2a, $P < 0.05$) and cross-sectional area of cardiomyocytes by 10.1% ($P < 0.05$) in WT mice (Fig. 2b, c) and increased interstitial fibrosis by 2.5-fold (Fig. 2b, d, $P < 0.01$), for a distinct atrophic phenotype. However, the LVW/TL ratios and cardiomyocyte atrophy values were significantly lower by 5.6% and 6.0% (both $P < 0.05$), respectively, and cardiac fibrosis was significantly higher by 35.7% ($P < 0.05$) for DOX-treated Sirt6 +/- mice than DOX-treated WT mice (Fig. 2b–d).

In cultured NRCMs, as compared with control NRCMs, DOX-treated NRCMs showed significantly decreased cross-sectional area by 18.3% (Fig. 2e, $P < 0.01$), which was further significantly decreased by 11.3% ($P < 0.05$) with Sirt6 silencing but increased by 11.8% ($P < 0.05$) with Sirt6 overexpression (Fig. 2f and Supplementary Figure 1).

Sirt6 ablation increases DOX-induced apoptosis and necrosis in hearts of mice and primary cultured rat cardiomyocytes

Dox treatment significantly increased the number of TUNEL-stained cardiomyocytes in the myocardium of both WT and Sirt6 +/- mice; however, Sirt6 deficiency significantly increased the number of apoptotic nuclei in the Sirt6 +/- mouse myocardium by 3.5-fold ($P < 0.01$) as compared with WT mice (Fig. 3a). Sirt6 silencing significantly exacerbated and Sirt6 overexpression significantly attenuated the apoptosis in response to DOX treatment (Fig. 3b, c): the ratios of cleaved caspase-3 to pro-caspase-3 were higher, by 41.5%, and lower, by 46.3%, respectively (both $P < 0.05$), than in DOX-treated NTC-transfected cardiomyocytes. In addition, the protein expression of the anti-apoptotic genes Bcl-2 and Bcl-xL, as well as the apoptotic genes Bax and Bak, was further detected. DOX treatment led to a significant decrease in Bcl-2 and Bcl-xL protein levels and a significant increase in Bax and Bak protein levels in both mouse hearts and cultured cardiomyocytes, which were not affected by Sirt6 knockout/silence or overexpression (Supplementary Figure 2). Flow cytometry assays exhibited that early apoptotic cardiomyocytes (APC+7-AAD−, lower right quadrant) were significantly increased in number on DOX treatment alone (Fig. 3d, e). Sirt6 silencing significantly increased by 36.7% ($P < 0.05$) and Sirt6 overexpression reduced their number by 53.8% ($P < 0.01$) as compared with DOX treatment alone (Fig. 3d, e). Both Annexin V– and 7-AAD-positive (APC+7-AAD+, late apoptosis) and only 7-AAD-positive cells (APC–7-AAD+, secondary necrosis) were significantly increased by DOX treatment.
Si rt6 silencing significantly increased the late apoptotic and secondary necrotic cell number by 177.8% \((P < 0.01)\), and Sirt6 overexpression reduced the number of both APC\(^+\)-AAD\(^+\) and APC\(^-\)-AAD\(^+\) cells by 20.8% \((P < 0.05)\), as compared with DOX-alone treated cells (Fig. 3d, e). In addition, necrotic indicators, including loss of nuclear HMGB1, and release of myocardial enzymes, such as LDH, creatine kinase (CK) and CK-MB, were investigated. DOX treatment significantly decreased nuclear HMGB1 protein level in the myocardium of both WT and Sirt6\(^{+/−}\) mice, accompanied by a significant increase in the activity of plasma LDH, CK, and CK-MB (Fig. 3f and Supplementary Figure 3A). However, after DOX treatment, the nuclear HMGB1 protein level was significantly lower, and activity of plasma myocardial enzymes was significantly higher in Sirt6\(^{+/−}\) mice than WT mice (Fig. 3f and Supplementary Figure 3A). DOX treatment significantly decreased HMGB1 protein levels in cardiomyocyte nuclei and increased the activity of myocardial enzymes in culture supernatants (Fig. 3g, h and Supplementary Figure 3B, C). Sirt6 overexpression significantly attenuated and Sirt6 silencing exacerbated the necrotic response to DOX challenge, with 43.2% higher \((P < 0.01)\), and 37.3% lower \((P < 0.05)\) HMGB1 protein level, respectively, in cardiomyocyte nuclei as compared with DOX-treated cardiomyocytes with null adenovirus infection or mock transfection (Fig. 3g, h). In addition, the activity of myocardial enzymes in culture supernatants was lower in DOX-treated NRCMs with Sirt6 overexpression and higher in DOX-treated NRCMs with Sirt6 silencing than with DOX treatment alone (Supplementary Figure 3B, C).

Sirt6 deficiency accelerates LV dysfunction after DOX treatment

DOX-induced cardiac toxicity and atrophy are characterized by a decrease in both LV mass and cardiac functions (Zhang et al. 2009; Wang et al. 2014). DOX
Fig. 2  Sirt6 deficiency exacerbates the ratio of left ventricular weight to tibial length (LVW/TL) changes, cardiac atrophy, and fibrosis following DOX injection in mice, as well as cardiac atrophy in cultured NRCMs exposed to DOX. a Changes in LVW/TL ratio in WT or Sirt6+/− mice without or with DOX (4 mg/kg weekly) injection for 4 weeks and maintenance for another one week (n = 10/group). b Representative images of hematoxylin and eosin (HE)–, wheat germ agglutinin (WGA)–, and Masson trichrome (blue represents collagen)–stained heart sections. Original magnifications, × 200, × 400, and × 100 respectively; all scale bars = 50 μm. c, d Quantitative measurement of cardiomyocyte cross-sectional area with random 100–200 cardiomyocytes (n = 10/group); the percentage of fibrosis with normalizing blue Masson’s trichrome–stained area to LV area in five randomly selected fields per section (n = 10/group). e, f Representative images of WGA-Alexa Fluor 488–stained NRCMs. NRCMs transfected with Sirt6 siRNA or NTC siRNA for knockdown for 48 h or infected with Ad-EGFP or Ad-Sirt6 adenovirus for overexpression for 48 h followed by treatment without or with DOX (2 μM) for 18 h (n = 10/group); then, cell area was measured with WGA-Alexa Fluor 488 staining. The cell membrane is green and nuclei are blue. Scale bars = 50 μm. Original magnification, × 400. Quantitative analysis of NRCM size with measurements of ≥ 50 NRCMs per group. All data were analyzed by one-way ANOVA, and values represent the mean ± SEM. In panels (a), (c), and (d), **P < 0.01 vs the WT control and ***P < 0.001 vs DOX-treated WT mice. In panel (e) *P < 0.05 and ***P < 0.01 vs NTC and #P < 0.05 vs DOX-treated NTC. In panel (f), *P < 0.05 and **P < 0.01 vs Ad-EGFP and ##P < 0.01 vs DOX-treated Ad-EGFP.
treatment significantly decreased echocardiographic LV mass in Sirt6+/− mice as compared with DOX-treated WT mice. Sirt6 deficiency also significantly impaired other parameters of cardiac remodeling, including LV end-diastolic posterior wall thickness and LV end-diastolic volume as well as the cardiac contractility indices derived from pressure volume, such as LV pressure, ±dP/dt, and LV end-systolic pressure in DOX-induced heart failure models.

Fig. 3 The effect of knockdown/overexpression of Sirt6 on DOX-induced apoptosis and necrosis in mouse hearts and NRCMs. a Representative images of TUNEL-stained sections in hearts of WT or Sirt6+/− mice without or with DOX (4 mg/kg weekly) for 4 weeks and maintenance for another one week (n = 10/group). Red arrows indicate some blue-stained TUNEL-positive nuclei. Scale bars = 50 μm. Original magnification, ×400. Quantitative analysis of TUNEL-positive cells as a percentage of positive nucleus number to total nucleus number in all groups (n = 10/group). **P < 0.01 vs the WT Control and ##P < 0.01 vs DOX-treated WT.
b, c Pro- and cleaved caspase-3 protein levels measured by western blot assay (n = 5/group) in NRCMs exposed to DOX (2 μM) for 18 h after transfection with NTC or Sirt6 siRNA for 48 h or injection of EGFP or Sirt6 adenovirus for 48 h. d, e Flow cytometry of early apoptosis and necrosis by staining with Annexin V-APC and 7-ADD. NRCMs were transfected with NTC or Sirt6 siRNA or infected with EGFP or Sirt6 adenovirus, then treated with 2 μM Dox for 18 h. Early apoptosis is Annexin V- and 7-ADD-positive (lower left quadrant). Late apoptotic and secondary necrotic cells are both APC- and 7-AAD-positive (upper right quadrant) and AAD-positive (upper left quadrant). The percentage of apoptotic and necrotic cells was quantified (n = 5). f–h HMGB1 (nuclear part) protein level in all groups by immunoblotting (n = 5). All data were analyzed by one-way ANOVA, and values represent the mean ± SEM. In panels (a) and (f), *P < 0.05 and **P < 0.01 vs the WT Control and **P < 0.01 vs NTC and #P < 0.05 vs DOX-treated WT. In panels (b), (d), and (g), *P < 0.05 and **P < 0.01 vs NTC and #P < 0.05 vs DOX-treated WT. In panels (c), (e), and (h), *P < 0.05 and **P < 0.01 vs Ad-EGFP and #P < 0.05 vs DOX-treated Ad-EGFP.
Sirt6 plays a necessary and sufficient role in inhibiting DOX-induced intracellular oxidative stress in vivo and ex vivo.

DOX induces cardiac oxidative damage and dysfunction by inducing lipid peroxidation (indicated as MDA level) (Zhang et al. 2009; Prathumsap et al. 2020). DOX treatment significantly increased MDA level in lysates of both WT mouse myocardium and NRCMs. Sirt6 deficiency significantly augmented DOX-induced MDA levels in Sirt6+/- mouse cardiac tissues and Sirt6 siRNA–transfected NRCMs over WT mice and NTC-treated cells with DOX treatment alone (Fig. 5a, b), although partial deletion of endogenous Sirt6 had no effect on baseline MDA content in Sirt6+/- mouse cardiac tissues and Sirt6-silenced NRCMs (Fig. 5a, b). In addition, Sirt6 adenoviral overexpression significantly decreased MDA content in DOX-treated cardiomyocytes as compared with DOX-treated null-adenovirus-infected cells (Fig. 5c). MitoSOX Red, a mitochondrial ROS-sensitive indicator, was used to investigate mitochondrial ROS levels. Sirt6 gene silencing induced stronger mitochondria ROS staining in DOX-treated than non-treated cardiomyocytes (Fig. 5d), whereas Sirt6 overexpression conferred less...
We further observed that DOX treatment caused cardiac oxidative damage by downregulation of endogenous antioxidant enzymes. The mRNA levels of antioxidants, including Prx 5, CAT, SOD2, and glutathione peroxidase, and activity of CAT and SOD2 were significantly decreased in DOX-treated cardiac tissues and NRCMs (Fig. 5f, g and Supplementary Figure 4A–C), parallel to increased ROS levels. Sirt6 gene silencing reduced the mRNA levels and activity of these endogenous antioxidants, and Sirt6 overexpression increased their mRNA levels and activity as compared with DOX-alone treated control counterparts (Fig. 5f, g and Supplementary Figure 4B–C).

Sirt6 mediates cardiomyocyte death partially through a p53-Fas-dependent pathway.

The most important function of p53 is as a transcription factor (Hafner et al. 2019; Sullivan et al. 2018). We observed that DOX treatment significantly increased the protein levels of total p53 and p53 phosphorylation at serine 392 (p53 Ser392-P) in cardiac tissues and NRCMs, which was not affected by Sirt6 knockout/silencing or overexpression (Supplementary Figure 4A–C). We further examined the potential interaction between Sirt6 and p53. Immunoprecipitates of endogenous p53 from NRCM lysates showed co-immunoprecipitation of endogenous Sirt6 with p53, and this binding was further weakened by Sirt6 silencing but enhanced by Sirt6 overexpression (Fig.

Table 1 Left Ventricular function in WT and Sirt6−/− Mice without or with DOX (4 mg/kg weekly) injection for 4 weeks and maintenance for another one week (Mean ± SEM)

| Echocardiographic parameters | WT (n=10) | Sirt6−/− (n=10) | WT&DOX (n=10) | Sirt6−/− DOX (n=10) |
|-----------------------------|----------|----------------|---------------|-------------------|
| LVSd (mm)                   | 0.53±0.02| 0.56±0.03      | 0.62±0.01*    | 0.64±0.02*        |
| LVDDd (mm)                  | 3.61±0.13| 3.29±0.22      | 4.04±0.19**   | 4.31±0.11***#    |
| LVIDs (mm)                  | 2.74±0.25| 2.69±0.18      | 2.99±0.23†    | 3.58±0.56**#     |
| LVWPWTd (mm)                | 0.78±0.042| 0.77±0.042     | 0.66±0.034†   | 0.60±0.046***#   |
| LVESV (μL)                  | 9.74±0.58| 9.35±0.45      | 7.99±0.23†    | 7.28±0.56**#     |
| LVEDV (μL)                  | 42.24±1.18| 41.69±2.01     | 34.14±2.24†   | 30.61±1.26‡       |
| SV (μL)                     | 39.27±1.06| 39.77±1.78     | 32.25±1.8‡    | 26.29±4.63**#    |
| EF (% Teich)                | 70.04±0.23| 75.99±3.36     | 63.85±0.34†   | 58.57±0.13***#   |
| FS%                         | 39.38±1.67| 36.93±1.83     | 29.97±1.49†   | 25.75±1.63***#   |
| LV mass (mg)                | 98.40±2.82| 96.90±3.99     | 76.30±3.03†   | 71.5±2.85**#     |

| Hemodynamics parameters    |           |               |               |                   |
|-----------------------------|-----------|---------------|---------------|-------------------|
| LVESP (mmHg)                | 84.61±4.23| 83.32±2.27    | 73.30±1.8†    | 62.95±4.63***#    |
| LVEDP (mmHg)                | 0.97±0.13 | 0.96±0.15     | 1.57±0.09‡    | 1.67±0.05‡        |
| LVP (mmHg)                  | 83.65±4.23| 83.21±2.27    | 72.9±1.8‡     | 62.93±4.63***#    |
| dP/dtmax (mmHg/s)           | 7320.10±333.74| 7365.67±284.69| 5221.90±300.81*| 4268.49±192.52***#|
| dP/dtmin (mmHg/s)           | −5914.00±347.16| −5844.19±328.58| −4505.85±183.71*| −3855.50±254.65***#|
| CO (mL/min)                 | 28.20±0.27| 29.45±0.64    | 21.47±0.51‡   | 17.31±1.90‡       |
| Heart Rate (bpm)            | 550.03±7.36| 594.79±3.45   | 517.08±25.15‡ | 484.70±24.28‡     |
| EF (%)                      | 79.01±3.42| 72.13±0.35    | 55.05±2.51†   | 49.07±1.53‡       |
| SW (mmHg/μL)                | 674.06±6.69| 708.69±3.40   | 578.88±63.40‡ | 397.59±74.51***#  |
| AE (mmHg/μL)                | 10.91±2.15| 11.18±4.85    | 8.11±2.39‡    | 7.08±3.03‡**#     |

LVSd, left ventricle (LV) end systolic diameter; LVIDd, LV internal dimension in diastole; LVIDs, LC internal dimension in systole; LVWPWTd, LV posterior wall thickness; LVESV, LV end-systolic volume; LVEDV, LV end-diastolic volume; EF (Teich)%, ejection fraction calculated by using the Teichholz method; FS%, Left ventricular fractional shortening %. LVP, LV pressure; LVESP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure; dP/dtmax, maximal value of the first derivative of LV pressure; dP/dtmin, minimal value of the first derivative of LV pressure; ESV, end-systolic volume; EDV, End-diastolic volume; SV, stroke volume; CO, cardiac output; EF, ejection fraction. All data were statistically analyzed with the one-way ANOVA, followed by Student-Neuman-Keuls t test. * P < 0.05 and ** P < 0.01 vs the WT group; # P < 0.05 and ## P < 0.01 vs the DOX-treated WT group.
Dox treatment significantly increased pan-acetylated p53 levels in cardiac tissues and NRCMs (Fig. 6d–f). Sirt6 knockout/silencing significantly boosted pan-acetylated p53 levels, which were significantly reduced by Sirt6 overexpression (Fig. 6d). We further evaluated the mRNA levels of genes regulated by p53, including the negative regulators of the p53 pathway, Mdm2, and Cdkn2a, as well as the cellular death inducers, Bax, Bak, Fas, FasL, Noxa, and Puma. DOX treatment significantly increased mRNA levels of p53, Bax, Bak, Fas, FasL, Noxa, and Puma in cardiac tissues, and the transcription of these genes, as well as Mdm2, was significantly enhanced in DOX-treated NRCMs (Supplementary Figure 5A–C). Only the mRNA levels of Fas, a death receptor, and its ligand, FasL, were regulated by Sirt6. Sirt6 knockout/silencing boosted mRNA levels of both FasL and Fas in DOX-treated cardiac tissues and NRCMs, which were blunted by Sirt6 overexpression (Supplementary Figure 5A–C). However, the changes in Sirt6 levels did not affect the expression of the other five genes (Supplementary Figure 5A–C). FasL exists in a membrane-bound form (mFasL) and a soluble form (sFasL) (Yamada et al. 2017). We confirmed that DOX treatment significantly increased both mFasL and Fas protein levels in the cellular membrane of NRCMs, which were significantly augmented by Sirt6 silencing but reduced by Sirt6 overexpression (Fig. 6f). In addition, sFasL protein change in culture supernatants of NRCMs was similar to that of mFasL (Fig. 6f). These results suggested that the p53–FasL–Fas pathway might mediate the regulation of Sirt6 on DOX-induced myocardial injury.

Sirt6 suppresses apoptosis dependent on caspase-8 and inhibits necrosis independent of RIP1 in cardiomyocytes

Fas–FasL interaction recruits FADD and further recruits and activates caspase-8 to initiate apoptosis (Akiko et al. 2017; Kaufmann et al. 2012). DOX treatment significantly increased both mFasL and Fas protein levels in the cellular membrane of NRCMs, which were significantly augmented by Sirt6 silencing but reduced by Sirt6 overexpression (Fig. 6f). In addition, sFasL protein change in culture supernatants of NRCMs was similar to that of mFasL (Fig. 6f). These results suggested that the p53–FasL–Fas pathway might mediate the regulation of Sirt6 on DOX-induced myocardial injury.

Sirt6 usually acts as a corepressor of transcription factors by modulating H3K9 acetylation and the transcription expression of FasL and Fas in cardiomyocytes

Sirt6 usually acts as a co-repressor of transcription factors by modulating H3K9 acetylation (Bosch-Preseguré and Vaquero 2014). In ChIP assays, we used an antibody for p53 and another antibody for Sirt6 to investigate the binding capacity of p53 and Sirt6 to FasL and Fas promoters. p53 bound to both FasL and Fas promoters, and this binding was enhanced by DOX treatment alone (Fig. 8a, b). Sirt6 silencing significantly augmented and Sirt6 overexpression significantly reduced p53 binding capacity to both FasL and Fas.
Fig. 6 Effect of Sirt6 deficiency/overexpression on DOX-treated cardiomyocyte death is partially mediated by a p53-Fas dependent pathway in NRCMs. **a**, NRCMs were transfected with Sirt6 siRNA or NTC or infected with Ad-Sirt6 or Ad-EGFP without or with DOX (2 μM) for 18 h. Cell lysates were extracted for Co-IP with IgG or anti-p53 antibody and probed with the indicated antibodies. Densitometry of relative p53 protein content to β-actin (n = 3). **b** HEK293T cells transfected with Myc-tagged p53 and Flag-tagged Sirt6 or Flag for 24 h. Cell lysates were incubated with IgG or anti-Flag antibody and the input and immune complexes were immunoblotted with anti-Myc antibody by western blot analysis. **c** Cardiac tissues lysates were extracted for Co-IP with IgG or anti-lysine-acylation antibody and probed with p53 antibody. Representative images are from three independent experiments. *P < 0.05 and **P < 0.01 versus the WT mice. **e**, **f** NRCMs were transfected with Sirt6 siRNA or NTC or infected with Ad-Sirt6 or Ad-EGFP without or with DOX (2 μM) for 18 h. Cell lysates were extracted for Co-IP with IgG or anti-lysine-acylation antibody and probed with p53 antibody. Representative images are from three independent experiments. *P < 0.05 and **P < 0.01 versus Ad-EGFP and #P < 0.05 vs DOX-treated Ad-EGFP.
promoters under DOX treatment, but Sirt6 silencing or overexpression had no apparent effect on control NRCMs (Fig. 8a, b). Sirt6 was also identified to bind to both FasL and Fas promoters, and these bindings were significantly reduced by Sirt6 silencing and augmented by Sirt6 overexpression under baseline and DOX-challenged conditions (Fig. 8c, d). Interestingly, Sirt6 binding capacity to both FasL and Fas promoters was significantly reduced by DOX treatment or p53 silencing (Fig. 8c, d). To reveal the role of Sirt6 in mediating the acetylation of histones, we further detected the acetylation levels of H3K9 in the nuclear protein and the promoters of Fas and FasL genes. DOX treatment significantly increased H3K9 acetylation levels in both nuclear protein and Fas and FasL promoters, which was significantly augmented by Sirt6 silencing but reduced by Sirt6 overexpression (Fig. 8e–h).

Discussion

Cardiomyocyte apoptosis and necrosis could be a fundamental part of the myocardial process that initiates or exacerbates heart failure (Prathumsap et al. 2020; Zhang et al. 2009). Although major efforts have been made to reveal the mechanism of anthracycline-induced cardiomyopathy, we still know very little about the molecular and cellular details (Kumari et al. 2020; Kalyanaraman 2020; Zhang et al. 2010). ROS scavenging of iron chelators and the topoisomerase II-β modulator dexrazoxane did not confer significant benefits, which indicates the
presence of additional mechanisms (Kalyanaraman 2020; Zhang et al. 2009). Whether Sirt6, a stress-reactive protein deacetylase and mono-ADP-ribosylation enzyme in various cardiac conditions, is involved in DOX-induced cardiotoxicity remained unclear (Saiyang et al. 2020; Poulose and Raju 2015; Hall et al. 2013; Beaubharoits et al. 2013). Sirt6 plays a crucial role in maintaining the homeostasis of the heart function. Sirt6-knockout homozygotes die around 4 weeks after birth (Kawahara et al. 2009), and cardiac-specific knockout of Sirt6 gene induces mice to develop cardiac hypertrophy spontaneously (Sundaresan et al. 2012), so we generate Sirt6-knockout heterozygous mice for the current study. Though Sirt6 ablation might aggravate DOX toxicity in other organs, including the kidney and liver, we provide the first evidence that Sirt6 deficiency in vivo and in vitro exacerbated DOX-induced cardiac atrophy, fibrosis, apoptosis, and necrosis, thus exacerbating cardiac dysfunction and impairing mouse survival, and Sirt6 overexpression in vitro mitigated DOX-induced cardiomyocyte

Fig. 8 Endogenous Sirt6 interacts with p53 and plays an important role in mediating the expression of its target gene products, FASL and FAS, transcriptionally in NRCMs. a, b Chromatin immunoprecipitation (ChIP) analysis to detect p53 binding at the promoter of Fas or FasL, with a p53-specific antibody and an immunoglobulin G (IgG) control, in NRCMs transfected with Sirt6 siRNA or NTC or infected with Sirt6 or control adenovirus for 48 h with or without DOX (2 μM) for 18 h. PCR analysis involved p53 sites in Fas or FasL promoter; IgG-ChIP was a negative control; the summarized data is relative to the IgG control antibody background (n = 5/group). e, f NRCMs were transfected with Sirt6 siRNA or NTC or infected with Sirt6 or control adenovirus with or without DOX (2 μM) for 18 h. Nuclear protein was analyzed by immunoblotting. H3K9Ac level was determined by western blot analysis. Densitometry analysis of relative protein content to H3 (n = 3/group). g, h Chromatin immunoprecipitation (ChIP) analysis to detect H3K9Ac levels in the promoters of Fas and FasL genes with a H3K9Ac-specific antibody and an immunoglobulin G (IgG) control, in NRCMs transfected with Sirt6 siRNA or NTC or infected with Sirt6 or control adenovirus for 48 h with or without DOX (2 μM) for 18 h. PCR analysis involved p53 sites in Fas or FasL promoter; IgG-ChIP was a negative control; the summarized data is relative to the IgG control antibody background (n = 5/group). All data were analyzed by one-way ANOVA. Values represent the mean ± SEM. In panels (a–d, g), and (h), *P < 0.05 and **P < 0.01 vs NTC or Ad-EGFP and #P < 0.05 vs DOX-treated NTC or Ad-EGFP.
These results suggest that Sirt6-mediated suppression of cell death is a major suppressor of DOX-induced cardiotoxicity. Our findings would be of great clinical relevance that if cancer patients have defects in Sirt6 gene and/or protein expression, or poor Sirt6 enzyme function, they will be at increased risk for cardiac diseases under DOX treatment.

Previous studies indicate that cardiotoxicity is both an energy metabolism disturbance and oxidative stress injury after DOX chemotherapy (Prathumsap et al. 2020; Kumari et al. 2020; Kalyanaraman 2020; Zhang et al. 2009). Sirt6 plays an essential role in genomic stability, glucose and lipid metabolism, and inflammation and is involved in the initiation and development of various cardiovascular diseases, including cardiovascular remodeling, heart failure, and atherosclerosis (Saiyang et al. 2020; Poulouse and Raju 2015; Hall et al. 2013; Beauharnois et al. 2013). Sirt6 can contribute to redox homeostasis by upregulating the antioxidant response (Saiyang et al. 2020; Beauharnois et al. 2013). In a previous study, we revealed that in ischemic hearts, Sirt6 interacted with FoxO3a to promote the transcription and activity of the FoxO-dependent antioxidant enzyme genes SOD2 and CAT (Wang et al. 2016). In this study, we confirmed that Sirt6 reduced DOX-induced excessive oxidative stress injury by upregulating endogenous antioxidants.

Accumulating evidence suggests that apoptosis and necrosis-induced cardiomyocyte death is an essential mechanism underlying DOX-induced cardiotoxicity and cardiac dysfunction, so the mechanisms underlying the regulation of Sirt6 on cell fate have aroused our interest (Prathumsap et al. 2020; Zhang et al. 2009). Sirt6 is considered an important protective factor in cardiac ischemia/reperfusion injury, hypertrophy, and atherosclerosis (Saiyang et al. 2020; Beauharnois et al. 2013); the downregulation of Sirt6 in DOX-treated hearts and cardiomyocytes may suggest a potential defect in the endogenous protective system. The severe injury induced by DOX treatment in cardiomyocytes, including DNA damage and oxidative stress injury, might further impair Sirt6 expression. In addition, Sirt6 consume NAD+ for exerting their enzymatic functions (Poulouse and Raju 2015; Hall et al. 2013). DOX treatment reduced the content of NAD+ in cardiomyocytes, which might contribute to the decreased activity of Sirt6 (Gorini et al. 2018). Actually, the molecular mechanism mediating Sirt6 downregulation in DOX-treated hearts remains to be elucidated (Saiyang et al. 2020; Poulouse and Raju 2015; Hall et al. 2013; Beauharnois et al. 2013).

In hearts, p53 is a regulatory factor regulating cardiomyocyte cell cycle and fate (Men et al. 2021; Hafner et al. 2019). Basal p53 activity is essential in developing embryonic hearts and maintaining the normal myo-
architecture of the heart and cardiac functions (Men et al. 2021). p53 expression and activity are upregulated in various myocardial diseases, including myocardial infarction, hypertrophic cardiomyopathy, and chemotherapy-induced cardiotoxicity (Men et al. 2021). Oxidative stress is a determining factor of DOX cardiomyopathy and a potent inducer of p53 (Shi and Dansen 2020; Prathumsap et al. 2020; Liu et al. 2020; Gambino et al. 2013). We also confirmed that DOX induced a strong oxidative stress level and a significant increase in total, phosphorylated, and acetylated p53 protein levels in cardiac tissues and cardiomyocytes, suggesting that DOX-induced excessive ROS might trigger the activation of p53 pathway. In fact, the role of p53 in anthracycline cardiotoxicity has been debated (Men et al. 2021; Li et al. 2019). Shizukuda et al. found that p53 gene disruption attenuated DOX-induced cardiotoxicity by suppressing apoptosis and collapse of endogenous antioxidant enzymes (Shizukuda et al. 2005). However, Li et al. reveal a non-canonical tumor suppressor activity of p53 protecting against low-dose DOX-induced cardiac dysfunction by retaining mitochondrial regulation (Li et al. 2019). Thus, p53 may involve a complex mechanism for controlling cell fate (Li et al. 2019; Hafner et al. 2019). We observed that as a protein deacetylase, Sirt6 did not affect p53 phosphorylation level, but could regulate p53 acetylation levels by reducing pan-acetylated p53 level with its overexpression and boosting pan-acetylated p53 level with its silencing, which is comparable to results reported by Ghosh et al. (Ghosh et al. 2018), finding that Sirt6 directly deacetylated p53 at lysine 381 but not K382 to negatively regulate the stability and activity of p53. Hu et al. also reported a similar regulatory mechanism of Sirt6, by increasing FOXO3 ubiquitination and decreasing its stability and nuclear translocation, to prevent against doxorubicin-induced liver cancer cell death (Hu et al. 2018). Our results suggest that Sirt6 may directly suppress DOX-stirred p53 activity by reducing p53 stability and activity via its deacetylation on p53.

p53 responds to various forms of cell stress as a specific transcriptional regulator for DNA sequence, which controls many genes expressions in cell outcomes, including cell death and cell cycle arrest (Antonina et al. 2019; Laptenko and Prives 2006). p53 interactions multiply with coactivators, co-inhibitors, and general transcriptional machinery’s components, which allows p53 to promote or suppress different target genes’ transcription (Antonina et al. 2019; Laptenko and Prives 2006).

We confirm the direct physiological binding and interaction between Sirt6 and p53 in cardiomyocytes, so Sirt6 may be a potential candidate co-factor for p53. This finding is comparable to those reported by Li et al. (2018a, b), showing that Sirt6 co-operated with p53 as a co-activator.

Fas and FasL are involved in the regulation of cell death (Yamada et al. 2017; Kaufmann et al. 2012). We observed that the transcription of Fas and its legend FasL were regulated by Sirt6 silencing and overexpression. These results indicate that Sirt6 might regulate DOX-induced cardiomyocyte death through Fas and FasL, which are two downstream target genes of p53. We further investigated the regulation of Sirt6 in the interaction between p53 and Fas and FasL promoters by ChiP assay. The recruitment of p53 to the Fas and FasL promoters was further enhanced by Sirt6 silencing, accompanied by increased H3K9 acetylation; these bindings were impaired by Sirt6 overexpression, which was consistent with decreased H3K9 acetylation. Sirt6 was also cognized to combine with both the Fas and FasL promoters in a p53-dependent way, and the H3K9 acetylation levels at the promoters of Fas and FasL were reduced by Sirt6 silencing and increased by Sirt6 overexpression. Acetylation of histones can increase the expression of genes via transcription activation (Yang et al. 2020). Deacetylase-induced deacetylation has the opposite effect, inducing decreased gene expression (Yang et al. 2020; Tao et al. 2014). Thus, Sirt6 may be a co-repressor of p53, recruited to promoters of Fas and FasL, and further suppresses p53 transcription activity by reducing histone acetylation.

Cardiomyocytes express both Fas and FasL (Stephanou et al. 2001). When bound by FasL, Fas initiates signaling through their cytoplasmic death domains to induce both apoptotic and necrotic cell death pathways (Kaufmann et al. 2012; Kavurma and Khachigian 2003). FADD, an essential adaptor protein in cell death, binds to the death domain of Fas via its own death domain and recruits procaspase-8 via the interaction of death effector domains (DED), then to form a death-inducing signaling complex (DISC) and induce procaspase-8 being activated by self-cleavage (Tourneur and Chiocchia 2010). Active caspase-8 leads to apoptosis by activating downstream effector caspases directly, including caspase-3 (Kaufmann et al. 2012; Tourneur and Chiocchia 2010). We confirmed that
activated caspase-8 induced caspase-3 cleavage after activation of the Fas-FADD pathway in DOX-treated cardiomyocytes, which suggests that DOX-induced apoptosis depends on Fas-FADD-caspase-8. These results were comparable to those by Ueno et al. (Ueno et al. 2006), showing increased FasL mRNA expression, caspase-8 activity, and apoptosis in the hearts of DOX-treated rats.

In addition to apoptosis, necrosis is an important type of programmed cell death (Hafner et al. 2019; Zhang et al. 2009). RIP1 and RIP3, the important serine-threonine kinases, have been identified as essential regulators of caspase-independent necrosis (DeRoo et al. 2020; Zhang et al. 2016). In general, in response to the activated signal of Fas, RIP1 binds to Fas with its death domain and acts as a multifunctional adaptor protein; then, the C-terminal RIP homotypic interaction motif domain of RIP1 allows it to combine with and phosphorylate RIP3, to form an activated RIP1/RIP3 necrosome (Zhang et al. 2016; Kaufmann et al. 2012). The necrosome then induces mixed-lineage kinase domain-like pseudokinase (MLKL) to form a large MLKL octamer that serves as the effector for cell death [20]. However, Zhang et al. revealed another mechanism in cardiomyocyte necrosis induced by DOX; that is, RIP3 mediates myocardial necroptosis via CaMKII activation rather than RIP1 and MLKL (Zhang et al. 2016). Our results were comparable to Zhang et al. (Zhang et al. 2016), confirming that DOX induced RIP3-dependent necrosis in cardiomyocytes, but RIP1 might be cleaved by activated caspase-8.

In conclusion, as summarized in Figure 9, Sirt6 plays an essential role in preserving the homeostasis of antioxidants as well as the survival and death of cardiomyocytes. It shows strong protection against oxidative stress and cell death in DOX-treated cardiac myocytes. Sirt6 reduces oxidative stress injury by upregulating endogenous antioxidants and interacts with ROS-activated p53 and serves as a co-repressor of p53. It is recruited to the gene promoters of death receptor, Fas, and its ligand, FasL, and further suppresses p53 transcription activity by reducing histone acetylation. The inhibition of Sirt6 on death receptor signaling leads to impaired Fas-FADD-caspase-8 apoptotic and Fas-RIP3 necrotic pathways. These findings have far-reaching implications for treating chemotherapy-induced cardiotoxicity and for various other diseases characterized by the activation of death receptor signaling. Now, a few inhibitors and agonists of sirt6 have been developed, but not yet been used in clinical applications. The activation/overexpression of Sirt6 promotes cell survival, suggesting that safe and effective Sirt6 agonists would protect hearts from DOX-induced cardiotoxicity.

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Author contribution SSW, JL, and LYL: investigation, experiments, data collection, and writing original draft. XXW and MMT: idea and formal analysis. LF, YJZ, JYX, XMC, and XL: animal experiment. KYX and CLZ: provision of materials and instrumentation. WJ: funding acquisition, writing, reviewing, and editing.

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Data availability The authors declare that all materials and data generated or analyzed in this study are available within this article and the supplementary materials.

Code availability Not applicable.

Declarations

Ethics approval and consent to participate N/A. There were no any human experiments in this study.

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Competing interests The authors declare no competing interests.

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