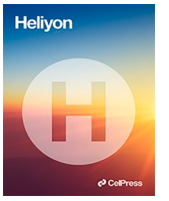
Heliyon 10 (2024) e24573

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

NAT10 promotes renal ischemia-reperfusion injury via activating NCOA4-mediated ferroptosis

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ARTICLE INFO

Keywords:
Ischemia-reperfusion injury
Acute kidney injury
NAT10
ac4C RNA modification
Renal injury

ABSTRACT

Ischemia-reperfusion injury (IRI) is a significant contributor to acute kidney injury (AKI) and is associated with substantial morbidity and mortality rates. In this study, we aimed to investigate the role of NAT10 and its ac4C RNA modification in IRI-induced renal injury. Our findings revealed that both the expression level of NAT10 and the RNA ac4C level in the kidneys were elevated in the IRI group compared to the sham group. Functionally, we observed that inhibition of NAT10 activity with Remodelin or the specific knockout of NAT10 in the kidney led to a significant attenuation of IRI-induced renal injury. Furthermore, in vitro experiments demonstrated that NAT10 inhibition and specific knockout of NAT10 in the kidney markedly suppressed global ac4C RNA modification, providing protection against hypoxia/reoxygenation-induced tubular epithelial cell injury and ferroptosis. Mechanistically, our study uncovered that NAT10 promoted ac4C RNA modification of NCOA4 mRNA, thereby enhancing its stability and contributing to IRI-induced ferroptosis in tubular epithelial cells (TECs). These findings underscore the potential of NAT10 and ac4C RNA modification as promising therapeutic targets for the treatment of AKI. Overall, our study sheds light on the critical involvement of NAT10 and ac4C RNA modification in the pathogenesis of IRI-induced renal injury, offering valuable insights for the development of novel AKI treatment strategies.

1. Introduction

Renal ischemia-reperfusion injury (IRI) is a complex pathophysiological process characterized by temporary interruption and subsequent restoration of blood flow to the kidney [1,2]. It commonly occurs during clinical scenarios such as renal transplantation, cardiac surgery, and renal artery occlusion [3]. While the restoration of blood flow is necessary, it can paradoxically result in additional tissue damage, leading to compromised renal function [4]. Extensive research has been conducted to unravel the underlying mechanisms of renal IRI and identify potential therapeutic targets to mitigate its adverse effects. This research has provided valuable insights into the cellular and molecular events implicated in renal IRI, including oxidative stress, inflammation, mitochondrial dysfunction, and activation of cell death pathways [5,6]. RNA modification refers to the process of chemically altering RNA molecules after their synthesis [7,8]. These modifications play a critical role in regulating various aspects of RNA function, including stability, localization, translation, and degradation. Understanding these mechanisms is vital for the development of effective interventions aimed at preventing or minimizing renal IRI and improving patient outcomes.

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https://doi.org/10.1016/j.heliyon.2024.e24573
Received 13 September 2023; Received in revised form 18 December 2023; Accepted 10 January 2024
Available online 18 January 2024

Recently, there has been a growing interest in understanding the impact of RNA modification on various biological processes and diseases [9]. Recent studies have revealed the significance of RNA modifications in the pathophysiology of renal IRI [10]. For example, N6-methyladenosine (m6A), one of the most abundant RNA modifications, has been found to be dynamically regulated during renal IRI [11–13]. Altered m6A modification patterns on specific mRNAs have been observed to influence their stability, translation efficiency, and subsequent protein expression, ultimately impacting the outcome of renal IRI [14]. Additionally, other RNA modifications such as 5-methylcytosine (m5C), pseudouridine (Ψ), and N1-methyladenosine (m1A) have also demonstrated potential involvement in the development and progression of renal IRI [15]. These modifications can affect RNA structure, interactions with RNA binding proteins, and gene expression profiles, thereby influencing cellular responses to ischemia-reperfusion injury [16]. The identification of specific RNA modification patterns associated with renal IRI has opened new avenues for therapeutic interventions. Modulating these modifications through targeted approaches, such as small molecule inhibitors or RNA-modifying enzymes, holds promise for ameliorating renal damage and promoting tissue repair following ischemia-reperfusion injury.

NAT10 (*N*-acetyltransferase 10), a recently discovered RNA modification enzyme, has emerged as a key player in the field of epitranscriptomics [17]. This enzyme facilitates the addition of acetyl groups to the 4-position of cytidine (C) residues in RNA molecules, leading to the formation of N4-acetylcytidine (ac4C) [18]. The ac4C modification has demonstrated diverse roles in various biological processes, including mRNA stability, translation efficiency, and cellular stress responses [17,19]. Consequently, research endeavors have concentrated on elucidating the functional significance of NAT10-mediated RNA ac4C modification and its influence on gene expression regulation. Recent studies have provided insights into the mechanisms governing the dynamic regulation of ac4C levels, the identification of RNA targets of NAT10, and the potential implications of aberrant ac4C modification in human diseases. One particularly intriguing area of investigation is the exploration of NAT10-mediated RNA ac4C modification in the context of ischemia-reperfusion injury (IRI) [20]. During IRI, the restoration of blood flow to ischemic tissues can exacerbate damage, and understanding the role of ac4C modification in this process may unveil potential therapeutic targets for mitigating IRI-induced injury. While research in this specific area is still in its nascent stages, recent studies have commenced unraveling the impact of NAT10-mediated RNA ac4C modification in IRI pathogenesis.

In this study, we observed an upregulation of NAT10 expression and an increase in RNA ac4C levels in the kidneys of mice with ischemia-reperfusion injury (IRI). To further confirm the crucial role of NAT10, we employed a specific inhibitor called Remodelin to inhibit NAT10 activity. Additionally, we generated a strain of NAT10 conditional knockout (cKO) mice with the genotype NAT10 fl/fl, PAX2-Cre. Inhibition of NAT10 using Remodelin and the NAT10 cKO approach resulted in a reduction of IRI-induced renal injury, indicating the critical involvement of NAT10 in this process. Furthermore, NAT10 inhibition and NAT10 cKO suppressed global ac4C RNA modification and provided protection against tubular epithelial cell (TEC) injury and ferroptosis induced by hypoxia/reoxygenation (H/R). Mechanistically, NAT10 facilitated ac4C RNA modification of NCOA4 mRNA, enhancing its stability and contributing to IRI-induced ferroptosis in TECs. These findings emphasize the potential of targeting NAT10 and ac4C RNA modification as therapeutic strategies for the treatment of acute kidney injury (AKI).

2. Material and methods

Ethical statement

All procedures involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Experimentation Ethics Committee from the First Affiliated Hospital of Changzhou (Jiangsu, China).

2.1. Reagents and materials

This study used antibodies against NAT10 (Abcam, ab194297) and ac4C (Abcam, ab252215). The PAS, Cr assay kit, and BUN assay kit were provided by Nanjing Jiancheng Bioengineering Institute.

2.2. Animal studies

Male C57BL/6 J mice, aged between six to eight weeks and weighing around 20–22 g, were obtained from the SLAC Animal Center located in Shanghai, China. To induce acute kidney injury (AKI) caused by ischemia/reperfusion (I/R), the mice underwent a previously described method (15). Briefly, the mice were anesthetized and placed on a thermostat plate to maintain their body temperature at 36.5 °C. Microaneurysm clamps were used to clip the bilateral renal pedicles for 40 min, inducing ischemia. After the ischemic period, the clamps were removed to allow for a 24-h period of reperfusion. All animals were sacrificed under anesthesia. The sham control group underwent the same procedure without clamping the renal pedicle. Kidney tissues and blood samples were collected for further analysis. Blood samples were used to measure BUN and Cr levels following the manufacturer's instructions. Kidney tissues were embedded in paraffin for histological analysis using HE staining.

2.3. Generation of kidney-specific NAT10 knockout mice

A mouse line with a targeted deletion of NAT10 from kidney tubular epithelial cells (TECs) was created on a C57BL/6 background. Shanghai Animal Center constructed mouse lines with the genotype NAT10 Flox/Flox (fl/fl). NAT10 Flox/Flox mice were bred with PAX2-Cre mice, which have a kidney-specific promoter called PAX2-driven Cre, to generate NAT10 knockout mice. PCR was used to

genotype all mice before and after experiments.

2.4. Renal histology

Paraffin-embedded sections of mouse kidney were prepared using routine steps, including fixation, dehydration, waxing, and embedding. H&E staining was performed, and histological evaluations were conducted using light microscopy. Renal tubular damage was assessed as previously described.

2.5. Measurement of serum levels of urea and creatinine

Serum samples were obtained, and the levels of BUN and creatinine were measured using an automated biochemical analyzer (BE-2000, Mindray) according to the manufacturer's guidelines.

2.6. Extraction of RNA and quantitative real-time PCR

otal RNA was extracted from cells or tissues using TRIzol (Invitrogen, 15,596,018) following the manufacturer's instructions. Real-time PCR analysis was performed using the Bio-Rad iQ SYBR Green Supermix with Opticon 2 (Bio-Rad) on a CFX96 real-time RT-PCR detection system (Bio-Rad) following a previously described protocol.

2.7. Western blotting

Protein lysates were obtained from kidney tissues and cultured cells using established procedures, and Western blot analysis was performed according to previously described methods. The *anti*-NAT10 antibody (Abcam, ab194297) was used, and the secondary antibody conjugated with HRP was obtained from Sangon Biotech Co. Ltd, located in Shanghai, China. The Tanon 5800 image system (Tanon, Shanghai, China) was used to detect signals, and ImageJ software (National Institutes of Health) was used for quantitative analysis.

2.8. Dot blot assay

The m6A content in the poly-A tailing of total RNA was measured using an RNA ac4C dot blot assay. Total RNA was extracted using TRIzol (Invitrogen, 15,596,018) according to the manufacturer's guidelines. After double dilution, the RNAs (300 ng) were applied onto a nylon membrane (Sigma-Aldrich, GERPN1210B). The membranes underwent ultraviolet crosslinking and were then blocked using 5 % nonfat milk. Afterward, they were incubated overnight with the anti-ac4C antibody (Abcam, ab252215). The membranes were then incubated with the secondary antibody for 1 h at room temperature. The Tanon 5800 image system (Tanon, Shanghai, China) was used to detect signals. To demonstrate the quantity of total RNA, a solution of 0.02 % methylene blue in 0.3 M sodium acetate (pH 5.2) was employed.

2.9. Cell culture

The HK2 cell line, derived from human kidney, was acquired from Shanghai Cell Bank in Shanghai, China. It was cultured in high glucose HyClone Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % fetal bovine serum (FBS) in a 95 % air and 5 % CO2 atmosphere. The HK2 cells were subjected to hypoxia/reoxygenation (H/R) injury. After digestion, the cells were pelleted by centrifugation and counted. They were then diluted to a concentration of 1 million cells per milliliter and cultured in 60-mm dishes at a density of 4 thousand cells per square centimeter. The cells were incubated overnight and observed under a microscope. When the cells reached 80 % confluence, they were subjected to hypoxic conditions (94 % N2, 1 % O2, and 5 % CO2) for 6 h. Afterward, the cells were washed with PBS and cultured under regular conditions for 24 h.

2.10. Cell viability assessment

After establishing the H/R HK2 cellular model in 96-well plates, CCK-8 reagents from Beyotime in Shanghai, China were added. After incubation, the optical density at 450 nm was measured using a microplate reader (ELx 800, Bio-Tek Instrument).

2.11. RNA stability

To evaluate the mRNA stability of NCOA4, cells were treated with actinomycin to halt transcription. Specimens were gathered at 0, 3, and 6 h following cessation, and real-time PCR was used to determine the expression of TAB3 after extracting the total RNA.

2.12. Assessment of ferroptosis

Ferroptosis is characterized by lipid-reactive oxygen species (ROS) buildup, lipid peroxidation, depletion of glutathione (GSH), and iron accumulation. Intracellular ROS levels were assessed using the DCFH-DA dye. GSH levels were assessed using a Glutathione Assay

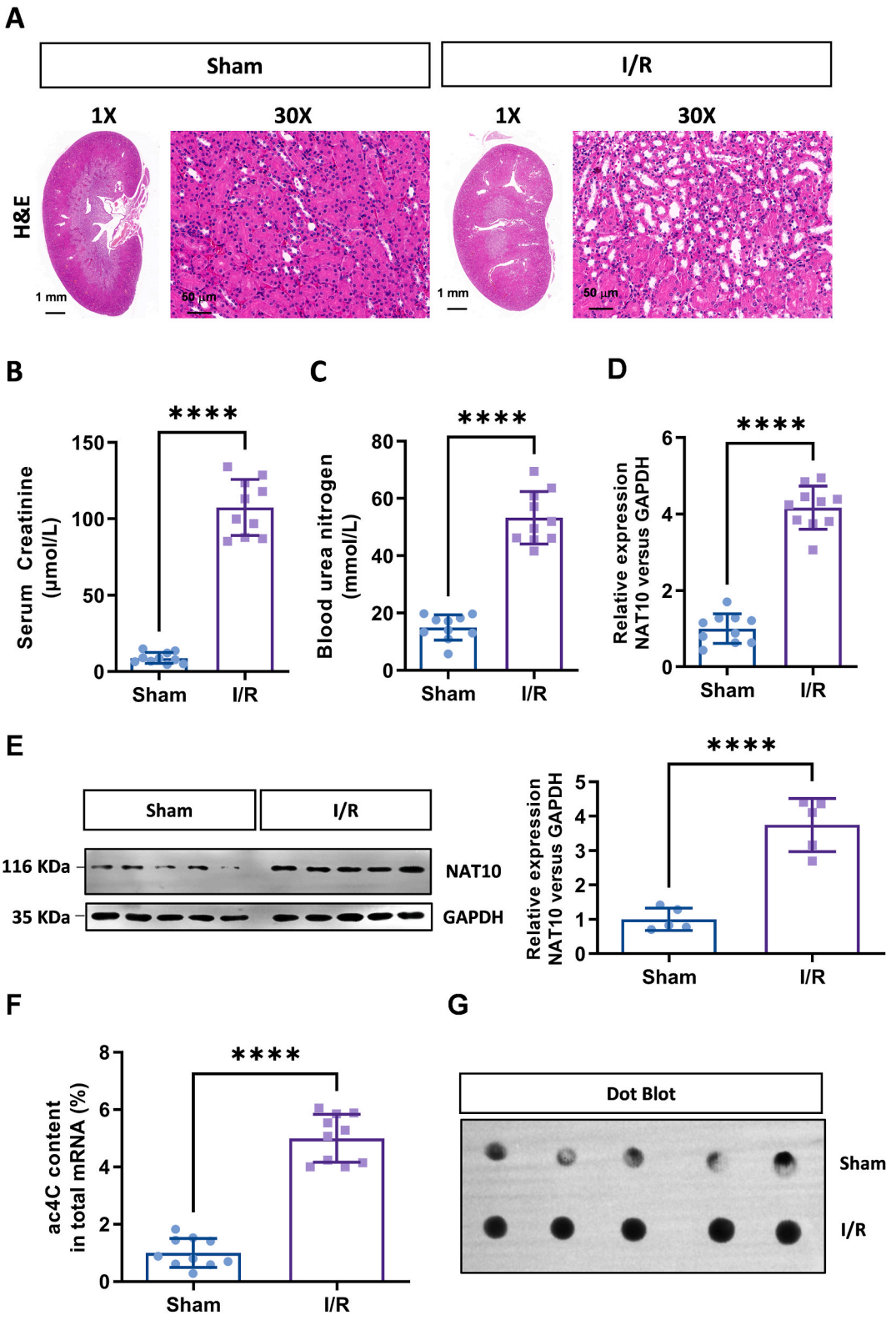

Fig. 1. The levels of NAT10 and its mediated ac4C RNA modification is elevated in the IRI kidneys. (A) Hematoxylin and eosin (H&E) staining was performed to observe the kidney tissue structure. (B) Then the serum was collected to determine levels of urea and creatinine by kits. (C) The real-time PCR and (D) Western blot were performed to analyze the expression level of NAT10. (E) The global ac4C RNA quantification analysis kit was used to detect RNA ac4C modifications. (F) The change in global ac4C RNA levels was confirmed by Dot Blot.

Kit. The concentration of malondialdehyde (MDA) was measured using a TBA method Kit. Iron concentration in cells was measured using an Iron Assay Kit. Serum LDH activity was assessed using an LDH Activity Kit.

2.13. ac4C-RIP-qPCR

The ac4C-RIP protocol was described previously. The Dynabeads mRNA Purification Kit was used to isolate poly(A) RNA, with a fraction retained as the input control. Pierce Protein A/G Magnetic Beads were prewashed and then exposed to anti-ac4C antibody or rabbit immunoglobulin G (IgG). After three washes, the beads conjugated with antibodies were combined with purified poly(A) RNA and 1 × IP buffer enriched with RNase inhibitors. Methylated mRNAs were collected and subjected to qPCR analysis to calculate enrichment.

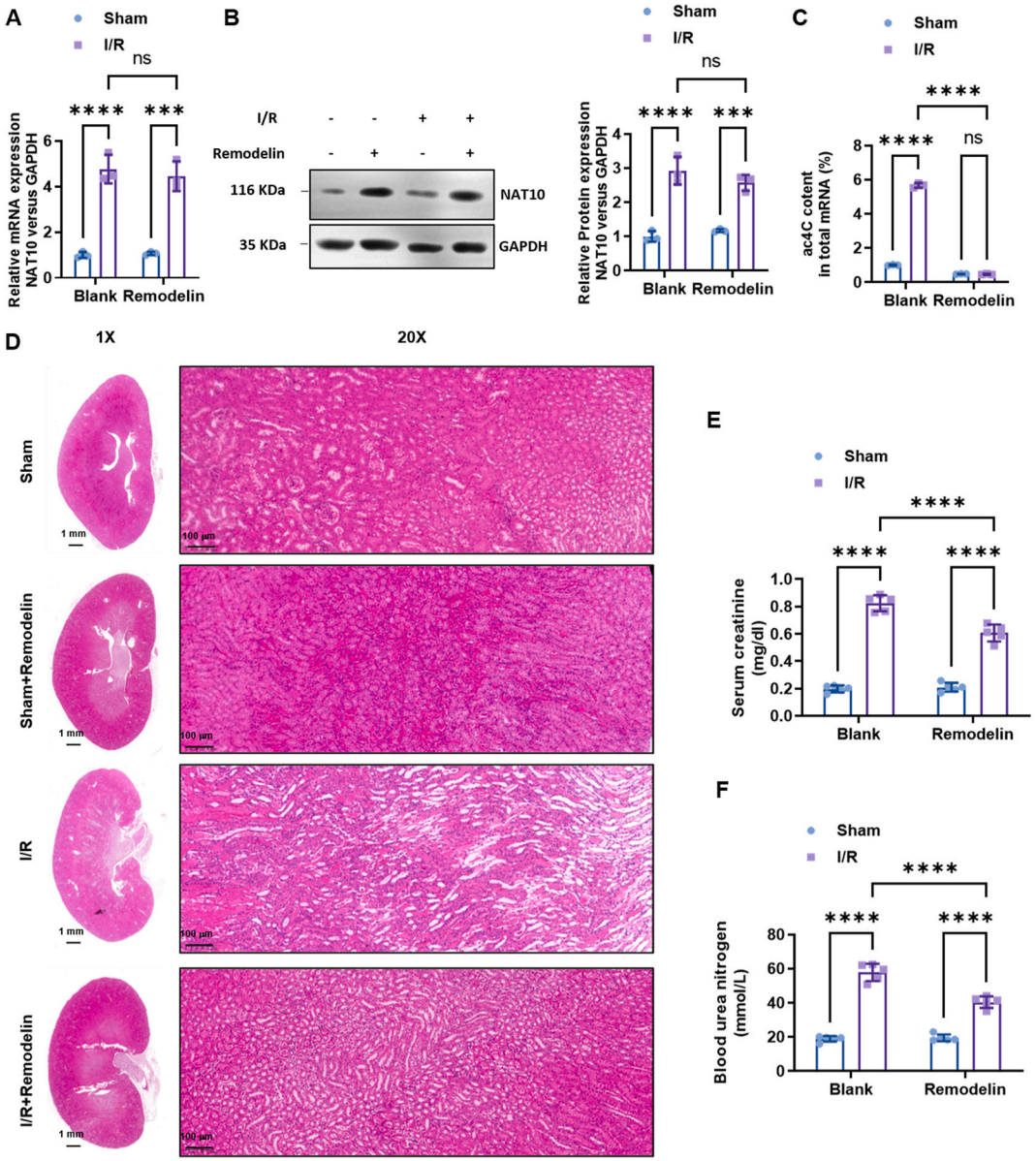

Fig. 2. NAT10 inhibition attenuates the IRI-induced renal injury. (A) The real-time PCR and (B) Western blot were performed to analyze the expression level of NAT10. (C)The global ac4C RNA quantification analysis kit was used to detect RNA ac4C modifications. (D) Hematoxylin and eosin (H&E) staining was performed to observe the kidney tissue structure. (E)Then the serum was collected to determine levels of urea and creatinine by kits.

2.14. Statistical analysis

Statistical analyses were performed using SPSS Inc. version 23.0. Graphs were generated using GraphPad Prism 9.0. The Shapiro-Wilk method was used to test the normal distribution of the results, and means ± SEM were reported for normally distributed quantitative data. Independent sample t-tests and one-way ANOVA followed by Tukey's post hoc test were used to investigate differences among groups. A p-value less than 0.05 was considered statistically significant.

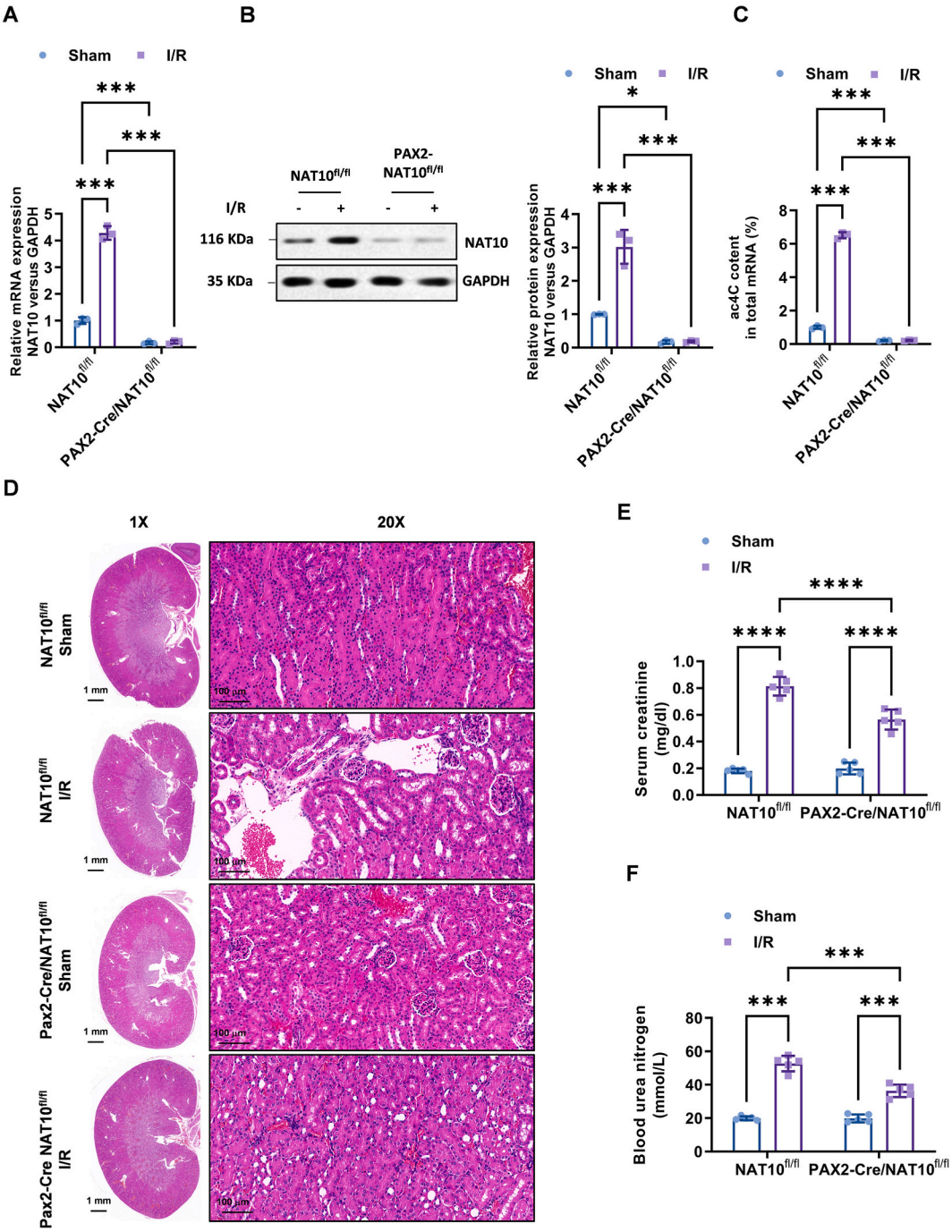

Fig. 3. Conditional knockout of NAT10 from mouse kidneys attenuated attenuates the IRI-induced renal injury. (A) The real-time PCR and (B) Western blot were performed to analyze the expression level of NAT10. (C) The global ac4C RNA quantification analysis kit was used to detect RNA ac4C modifications. (D) Hematoxylin and eosin (H&E) staining was performed to observe the kidney tissue structure. (E) Then the serum was collected to determine levels of urea and creatinine by kits.

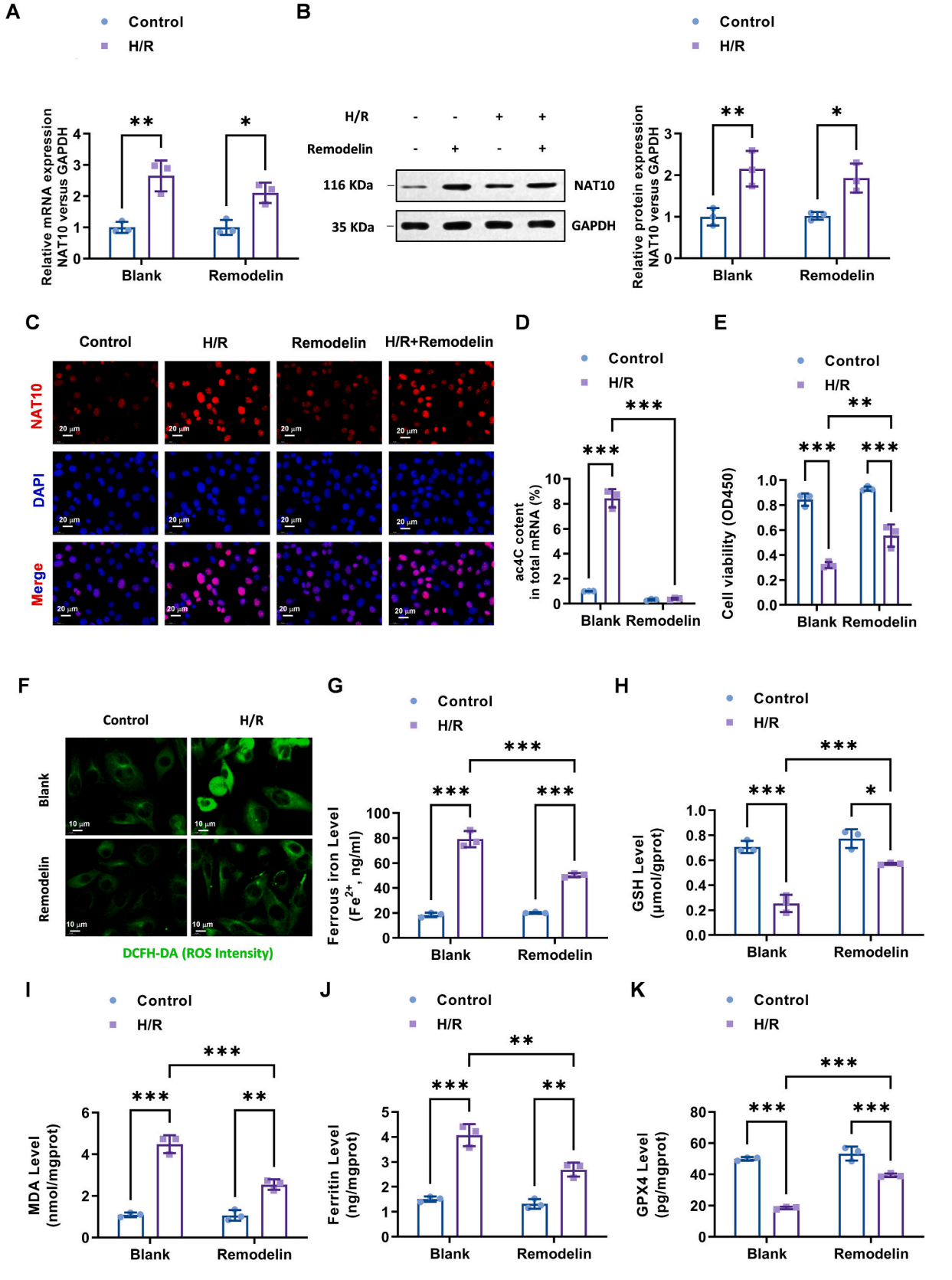

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Fig. 4. NAT10 inhibition decreases H/R-induced TECs injury and ferroptosis. (A) The real-time PCR, (B) Western blot and (C) immunofluorescence were performed to analyze the expression level of NAT10. (D)The global ac4C RNA quantification analysis kit was used to detect RNA ac4C modifications. (E) The cell viability of TECs was analyzed by CCK-8 assay. (F–I) The level of oxidative stress markers, including intracellular ROS, GSH, and malondialdehyde (MDA) were quantified by colorimetric kits. (J–K) The level of ferroptosis markers, including ferritin and GPX4 were analyzed by ELISA kits.

3. Results

3.1. Increased levels of NAT10 and ac4C RNA modification in IRI kidneys

An ischemia-reperfusion injury (IRI) mouse model was established to investigate the impact of IRI on NAT10 expression and RNA ac4C levels. After euthanizing rats with IRI-induced acute kidney injury (AKI), we examined the structure of the left kidney tissue using hematoxylin and eosin (H&E) staining. Fig. 1A shows significant pathological changes, including disrupted renal tissue structure, increased glomerular mesangial matrix, capillary compression, focal glomerular wall adhesion, renal tubular atrophy, loss of the brush border of proximal tubular epithelial cells, localized renal interstitial edema, and partial exfoliation of tubular epithelial cells.

Next, we collected serum to measure urea and creatinine concentrations. Compared to the sham group, the IRI group exhibited a notable increase in serum urea (Fig. 1B) and creatinine (Fig. 1C) levels. To assess NAT10 protein levels, we isolated total RNA from left kidney tissues and performed real-time PCR (Fig. 1D) and Western blot (Fig. 1E) analyses. The results indicated significantly elevated levels of NAT10 mRNA and protein in the IRI group compared to the sham group. Furthermore, we quantified global RNA ac4C levels to identify RNA ac4C modifications. Our findings revealed a significant increase in RNA ac4C levels in the IRI group compared to the sham group (Fig. 1F), which was further confirmed through Dot Blot analysis (Fig. 1G). Taken together, these findings suggest that IRI has the potential to increase NAT10 levels and promote RNA ac4C modification.

3.2. NAT10 inhibition attenuates IRI-induced renal injury

To investigate the role of NAT10 in IRI-induced renal injury, we inhibited NAT10's acetylation activity with Remodelin, which did not affect the elevated mRNA (Fig. 2A) and protein (Fig. 2B) expressions of NAT10 in IRI mice. However, Remodelin significantly suppressed global ac4C RNA modification (Fig. 2C). H&E staining results showed that pathogenic structural changes caused by IRI were significantly reduced by inhibiting NAT10 with Remodelin (Fig. 2D). Similarly, the elevated serum urea (Fig. 2E) and creatinine (Fig. 2F) levels in the IRI group were significantly decreased with NAT10 inhibition. These results further support the conclusion that NAT10 inhibition attenuates IRI-induced renal injury.

3.3. Conditional knockout of NAT10 from mouse kidneys attenuated attenuates the IRI-induced renal injury

The conditional knockout of NAT10 from mouse kidneys attenuates IRI-induced renal injury. To further confirm the crucial role of NAT10 in vivo, we generated NAT10 cKO mice (NAT10 fl/fl, PAX2-Cre). The absence of NAT10 from TECs was confirmed by real-time PCR (Fig. 3A) and Western blot (Fig. 3B). Additionally, NAT10 cKO markedly inhibited the overall ac4C RNA alteration (Fig. 3C). The results of H&E staining indicated that NAT10 cKO significantly reduced the pathological changes induced by IRI in renal tissue structure. These changes included disruption of renal tissue structure, elevated glomerular mesangial matrix, capillary compression, glomerular wall adhesion, renal tubular atrophy, reduction or loss of proximal tubular epithelial cell brush border, local renal interstitial edema, and partial shedding of tubular epithelial cells (Fig. 3D). Consistently, the IRI-elevated serum urea (Fig. 3E) and creatinine (Fig. 3F) levels were significantly decreased in the NAT10 cKO IRI group. These results further support the critical role of NAT10 in IRI-induced renal injury.

3.4. NAT10 inhibition decreases H/R-induced TECs injury and ferroptosis

To investigate the functions of NAT10 in H/R injury and its correlation with ferroptosis, we inhibited the acetylation activity of NAT10 with Remodelin [21] in the TECs. As shown in Fig. 4A, H/R significantly increased the mRNA level of NAT10 mRNA, which remained unchanged with Remodelin treatment. Similar changes were observed in the protein level of NAT10, which was analyzed by Western Blot (Fig. 4B) and immunofluorescence analysis (Fig. 4C). Notably, Remodelin dramatically suppressed the H/R-induced global ac4C RNA level (Fig. 4D). H/R significantly decreased the cell viability of TECs. Remodelin did not affect the cell viability of TECs under normoxia conditions, but dramatically increased it in the H/R-injured TECs (Fig. 4E). Next, we assessed the concentrations of intracellular reactive oxygen species (ROS), glutathione (GSH), and malondialdehyde (MDA), which serve as indicators of oxidative stress. Fig. 4F showed a notable rise in lipid ROS levels in H/R-injured TECs compared to normoxia TECs, as evidenced by the intensity of DCFH-DA. Furthermore, the levels of ferrous iron (Fig. 4G), GSH depletion (Fig. 4H), MDA (Fig. 4I), and ferritin (Fig. 4J) exhibited a significant increase following H/R injury. These increased ferroptosis markers were unchanged with Remodelin in the normoxia group but were significantly suppressed in H/R-injured TECs. On the other hand, the GPX4 level (Fig. 4K), a critical ferroptosis suppressor, was significantly increased by Remodelin in H/R-injured TECs. These results demonstrate that NAT10 inhibition decreases H/R-induced TECs injury and ferroptosis.

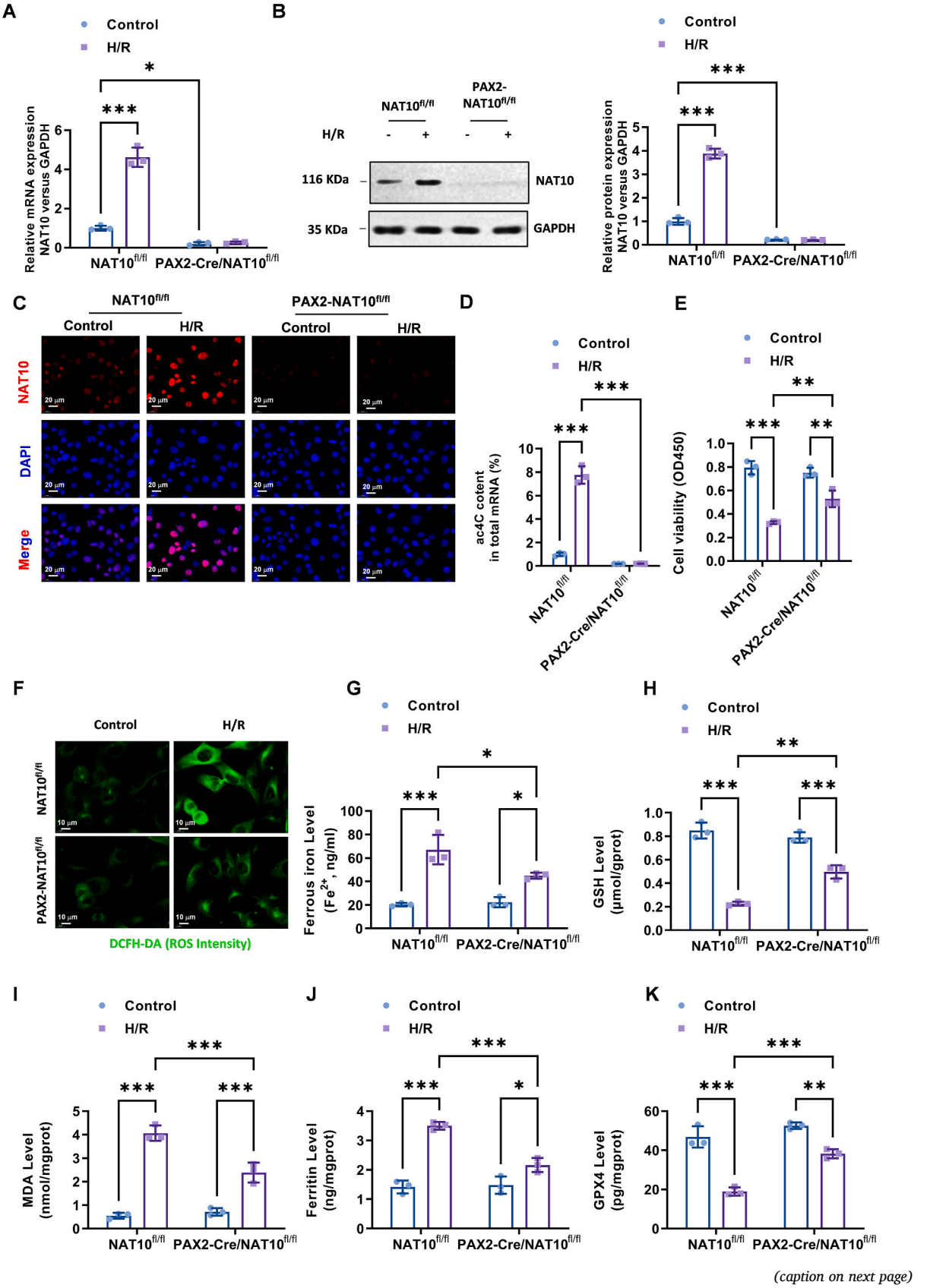

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Fig. 5. NAT10 cKO decreases H/R-induced TECs injury and ferroptosis. The TECs were isolated from normal or NAT10 cKO mice, and subjected to H/R treatment. (A) The real-time PCR, (B) Western blot and (C) immunofluorescence were performed to analyze the expression level of NAT10. (D) The global ac4C RNA quantification analysis kit was used to detect RNA ac4C modifications. (E) The cell viability of TECs was analyzed by CCK-8 assay. (F–I) The level of oxidative stress markers, including intracellular ROS, GSH, and malondialdehyde (MDA) were quantified by colorimetric kits. (J–K) The level of ferroptosis markers, including ferritin and GPX4 were analyzed by ELISA kits.

3.5. Conditional knockout of NAT10 decreases H/R-induced TECs injury and ferroptosis

To investigate the functions of NAT10 in H/R injury and its correlation with ferroptosis, we isolated TECs from normal and NAT10 cKO mice. We confirmed the absence of NAT10 from TECs using real-time PCR (Fig. 5A), Western blot (Fig. 5B) and immunofluorescence analysis (Fig. 5C). Similarly, the global ac4C RNA modification was significantly reduced by NAT10 cKO (Fig. 5D). In line with NAT10 inhibition, we observed that H/R significantly decreased the cell viability of TECs. While NAT10 cKO did not affect the cell viability of TECs under normal conditions, it dramatically increased viability in H/R-injured TECs (Fig. 5E). We also assessed the concentrations of intracellular reactive oxygen species (ROS), glutathione (GSH), and malondialdehyde (MDA) as indicators of oxidative stress. Fig. 5F showed a notable increase in lipid ROS levels in H/R-injured TECs compared to those under normal conditions, as evidenced by the heightened intensity of DCFH-DA. Additionally, the levels of ferrous iron (Fig. 5G), GSH depletion (Fig. 5H), MDA (Fig. 5I), and ferritin (Fig. 5J) exhibited a significant increase following H/R injury. These elevated ferroptosis markers were unchanged with NAT10 cKO in the normal group, but were significantly suppressed in H/R-injured TECs. Moreover, the GPX4 level (Fig. 5K), a critical ferroptosis suppressor, was significantly increased by NAT10 cKO in H/R-injured TECs. These results demonstrate

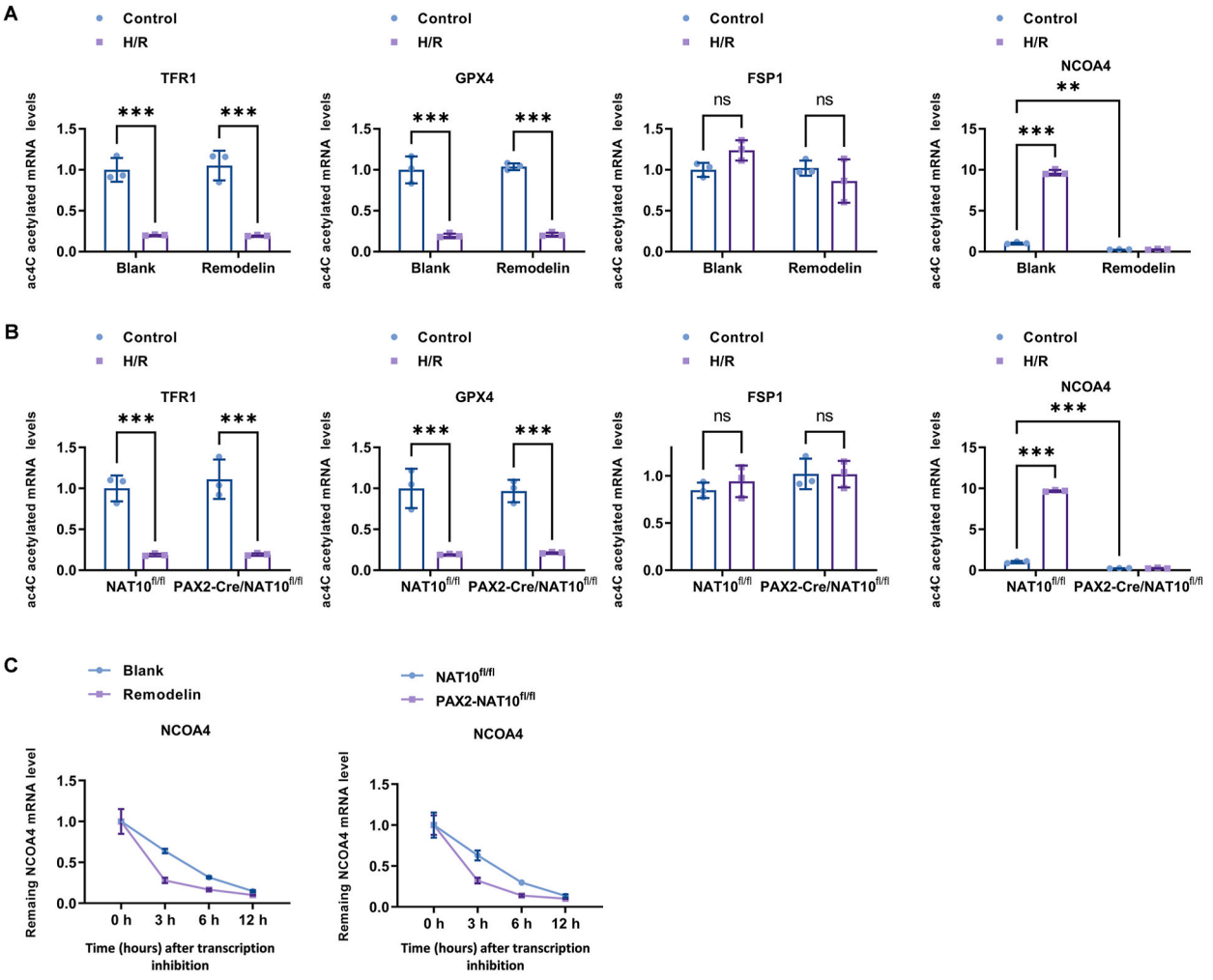

Fig. 6. NAT10 promotes the ac4C RNA modification of NCOA4 mRNA and enhances its stability. (A) The effect of NAT10 inhibition with Remodelin treatment and (B) NAT10 cKO on the ac4C acetylated mRNA levels of serval ferroptosis-related genes, including Tfr1, GPX4, FSP1, and NCOA4, in TECs was analyzed by acetylated RNA immunoprecipitation and real-time PCR. (B) The decay rate of NCOA4 mRNA after actinomycin D (5 μg/ml) administration in NAT10 inhibited and NAT10 cKO TECs was evaluated by real-time PCR.

that NAT10 plays a critical role in H/R-induced TECs injury and ferroptosis.

3.6. NAT10 promotes the ac4C RNA modification of NCOA4 mRNA and enhances its stability

To further investigate the mechanism by which NAT10 promotes IRI injury, we conducted methylated RNA immunoprecipitation and quantified the levels of ac4C acetylated mRNAs of several ferroptosis-related genes, including Tfr1, GPX4, FSP1, and NCOA4. Fig. 6A shows that H/R injury significantly reduced the ac4C acetylation levels of Tfr1 and GPX4 mRNAs, which remained unchanged with Remodelin treatment. Neither H/R injury nor Remodelin treatment affected the ac4C acetylation level of FSP1 mRNA. However, H/R injury significantly increased the ac4C acetylation level of NCOA4 mRNA, which was suppressed by Remodelin treatment. Consistently, Fig. 6B demonstrates that H/R injury led to a decrease in the ac4C acetylation levels of Tfr1 and GPX4 mRNAs, which were unaffected by NAT10 cKO. Similarly, neither H/R injury nor NAT10 cKO influenced the ac4C acetylation level of FSP1 mRNA. H/R injury resulted in an increase in the ac4C acetylation level of NCOA4 mRNA, which was suppressed by NAT10 cKO. Furthermore, we observed a shortened mRNA half-life of NCOA4 mRNA in TECs treated with Remodelin or subjected to NAT10 cKO (Fig. 6C). These findings suggest that NAT10 promotes the ac4C RNA modification of NCOA4 mRNA and enhances its stability, which represents a critical mechanism mediating IRI-induced ferroptosis in TECs.

4. Discussions

Ischemia-reperfusion injury (IRI) is a common cause of acute kidney injury (AKI) and is linked to significant morbidity and mortality [22–24]]. In this study, we aimed to explore the involvement of NAT10 and its mediated ac4C RNA modification in IRI-induced renal injury. Our findings revealed elevated levels of NAT10 expression and RNA ac4C in the kidneys of IRI mice. Additionally, inhibiting NAT10 with Remodelin or employing conditional knockout of NAT10 resulted in the mitigation of IRI-induced renal injury, underscoring the critical role of NAT10 in this process.

Alterations in ac4C RNA represent the most common modifications of human mRNA and carry significant implications in the pathogenesis of various illnesses [17]. However, limited research has focused on the impact of ac4C alterations on renal disorders, including AKI. In this study, we established a connection between ac4C alterations and AKI. The observed pathological changes in the IRI kidneys, such as disruption of renal tissue organization, heightened glomerular mesangial matrix, and tubular degeneration, were consistent with prior research. Additionally, the increase in serum urea and creatinine levels provided further confirmation of the renal damage caused by IRI. Notably, significantly elevated levels of NAT10 mRNA and protein were found in the IRI group compared to the sham group, representing an important finding. This suggests that NAT10 may play a role in the progression of kidney damage following IRI. Furthermore, our examination of RNA ac4C modification levels in the kidneys affected by IRI revealed a substantial increase in RNA ac4C levels in the IRI group compared to the sham group. This discovery implies that the involvement of NAT10-mediated ac4C RNA modification in IRI-induced renal injury may contribute to its pathogenesis. The confirmation of increased RNA ac4C levels by Dot Blot further bolstered our findings.

In order to determine the functional significance of NAT10 in IRI-induced renal injury, we utilized Remodelin to inhibit the acetylation activity of NAT10. Surprisingly, Remodelin treatment did not impact the elevated mRNA and protein expressions of NAT10 in IRI mice. However, it did significantly suppress global ac4C RNA modification and mitigate the pathological changes observed in the IRI kidneys. The reduction in serum urea and creatinine levels further supported the protective effect of NAT10 inhibition in IRI-induced renal injury. To further validate the role of NAT10 in renal injury, we generated NAT10 conditional knockout (cKO) mice. We confirmed the absence of NAT10 from tubular epithelial cells (TECs) and assessed the effects on RNA ac4C modification and renal injury. In line with the results from Remodelin treatment, NAT10 cKO also significantly suppressed global ac4C RNA modification and attenuated the pathological changes in the IRI kidneys. Once again, the decrease in serum urea and creatinine levels provided further support for the protective effect of NAT10 cKO in IRI-induced renal injury.

The development of acute kidney injury (AKI) is believed to involve nephrotoxicity, inflammatory reactions, acute tubular hypoxia and necrosis, pericyte damage, and microvascular injury/dysfunction. Recently, the role of ferroptosis in the progression and treatment of AKI has come to light [25]. Ferroptosis, distinct from other types of programmed cell death (PCD) in terms of morphology and biochemistry, involves iron-dependent lipid peroxidation and reactive oxygen species (ROS) [26]. The process of ferroptosis initiates an initial phase of cell death, which subsequently triggers an inflammatory reaction that further contributes to the decline in kidney function. Several research studies have demonstrated that approaches aimed at inhibiting ferroptosis can effectively slow down the advancement of AKI [27]. To investigate the role of NAT10 in hypoxia/reoxygenation (H/R)-induced TEC injury and ferroptosis, we inhibited NAT10 acetylation activity with Remodelin or performed NAT10 conditional knockout (cKO) in TECs. H/R injury significantly increased NAT10 mRNA and protein levels, which remained unchanged with Remodelin treatment or NAT10 cKO. However, both Remodelin treatment and NAT10 cKO significantly suppressed the H/R-induced global ac4C RNA modification. Furthermore, cell viability was significantly decreased in H/R-injured TECs, and this decrease was reversed by Remodelin treatment or NAT10 cKO. Moreover, the increased levels of intracellular reactive oxygen species (ROS), malondialdehyde (MDA), and ferrous iron, as well as the decreased levels of glutathione (GSH) and ferritin observed in H/R-injured TECs, were all suppressed by Remodelin treatment or NAT10 cKO. Additionally, the expression of GPX4, a critical ferroptosis suppressor, was significantly increased by Remodelin treatment or NAT10 cKO in H/R-injured TECs. These findings suggest that NAT10 inhibition attenuates H/R-induced TEC injury and ferroptosis.

Ferritinophagy is a process in which Nuclear receptor coactivator 4 (NCOA4) acts as a specific cargo receptor to facilitate the autophagic breakdown of ferritin, a complex responsible for storing iron in the cytosol l [28–30]. The maintenance of intracellular and

systemic iron balance, as well as iron-dependent physiological processes like erythropoiesis, depends on NCOA4-facilitated ferritinophagy [26,31]. By modulating the ferritinophagic flux mediated by NCOA4, the sensitivity to ferroptosis can be altered, which plays a crucial role in maintaining iron homeostasis [27,32–35]. Ferroptosis, an iron-dependent form of oxidative cell death, presents a promising target for preventing and treating ischemic reperfusion diseases. Research has shown that nuclear receptor coactivator 4 (NCOA4), a cargo receptor for ferritinophagy, is the focus of compound 9a. Compound 9a functions by reducing the availability of intracellular ferrous iron, thereby inhibiting ferroptosis through disruption of the NCOA4-FTH1 protein-protein interaction. Further investigations have revealed that 9a directly binds to the recombinant protein NCOA4383-522, effectively blocking the NCOA4383-522-FTH1 interaction. This action significantly mitigates ischemic-reperfusion injury [36]. To investigate the underlying mechanism of NAT10-mediated renal injury, we focused on NCOA4 mRNA, which is a gene associated with ferroptosis. Methylated RNA immunoprecipitation analysis revealed that H/R injury significantly increased the acetylation level of NCOA4 mRNA at the ac4C site, and this increase was suppressed by treatment with Remodelin or NAT10 conditional knockout (cKO). Additionally, the half-life of NCOA4 mRNA was shortened in TECs treated with Remodelin or subjected to NAT10 cKO. These findings suggest that NAT10 promotes the ac4C RNA modification of NCOA4 mRNA and enhances its stability, thereby contributing to ferroptosis induced by ischemia-reperfusion injury (IRI) in TECs.

In summary, our study offers evidence supporting the involvement of NAT10 and its mediated ac4C RNA modification in IRI-induced renal injury. Inhibition of NAT10 or conditional knockout of NAT10 demonstrated a reduction in renal injury in IRI mice and H/R-induced TEC injury. These protective effects were linked to the suppression of global ac4C RNA modification and the modulation of NCOA4 mRNA stability. These findings underscore the potential of NAT10 and ac4C RNA modification as therapeutic targets for AKI treatment, while also providing insights into the molecular mechanisms underlying IRI-induced renal injury and ferroptosis. Further research is necessary to comprehensively grasp the regulatory network and explore potential clinical applications of NAT10 and ac4C RNA modification in renal injury.

Funding

This work was supported by grants from the Youth Talent Technology Project of Changzhou Health Commission (QN201914) and the Young Talent Development Plan of Changzhou Health Commission (2020–045).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Jie Shen: Writing - original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yangyang Sun:** Software, Resources, Methodology, Formal analysis, Data curation. **Qianfeng Zhuang:** Visualization, Validation, Software, Resources, Methodology, Investigation. **Dong Xue:** Writing - original draft, Software, Resources, Project administration, Investigation. **Xiaozhou He:** Writing - review & editing, Supervision, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24573.

References

[1] M. Chen, M.C. Menon, W. Wang, J. Fu, Z. Yi, Z. Sun, J. Liu, Z. Li, L. Mou, K. Banu, S.W. Lee, Y. Dai, N. Anandakrishnan, E.U. Azeloglu, K. Lee, W. Zhang, B. Das, J.C. He, C. Wei, HCK induces macrophage activation to promote renal inflammation and fibrosis via suppression of autophagy, Nat. Commun. 14 (1) (2023) 4297.

[2] B. Deng, Y. Lin, Y. Chen, S. Ma, Q. Cai, W. Wang, B. Li, T. Liu, P. Zhou, R. He, F. Ding, Plasmacytoid dendritic cells promote acute kidney injury by producing interferon-alpha, Cell. Mol. Immunol. 18 (1) (2021) 219–229.

[3] R. Duan, Y. Li, R. Zhang, X. Hu, Y. Wang, J. Zeng, M. Gao, Reversing acute kidney injury through coordinated interplay of anti-inflammation and iron supplementation, Adv. Mater. 35 (28) (2023) e2301283.

[4] D. Golshayan, N. Schwotzer, F. Fakhouri, J. Zuber, Targeting the complement pathway in kidney transplantation, J. Am. Soc. Nephrol. 34 (11) (2023) 1776–1792.

[5] X. Li, X. Peng, X. Zhou, M. Li, G. Chen, W. Shi, H. Yu, C. Zhang, Y. Li, Z. Feng, J. Li, S. Liang, W. He, X. Gou, Small extracellular vesicles delivering lncRNA WAC-AS1 aggravate renal allograft ischemia–reperfusion injury by inducing ferroptosis propagation, Cell Death Differ. 30 (9) (Sep 2023) 2167–2186.

[6] G. Wang, B. Heijs, S. Kostidis, A. Mahfouz, R.G.J. Rietjens, R. Bijkerk, A. Koudijs, L.A.K. van der Pluijm, C.W. van den Berg, S.J. Dumas, P. Carmeliet, M. Giera, B.M. van den Berg, T.J. Rabelink, Analyzing cell-type-specific dynamics of metabolism in kidney repair, Nat. Metab. 4 (9) (2022) 1109–1118.
[7] K. Boulias, E.L. Greer, Biological roles of adenine methylation in RNA, Nat. Rev. Genet. 24 (3) (2023) 143–160.
[8] A. Gatsiou, K. Stellos, RNA modifications in cardiovascular health and disease, Nat. Rev. Cardiol. 20 (5) (2023) 325–346.
[9] D. Han, M.M. Xu, RNA modification in the immune system, Annu. Rev. Immunol. 41 (2023) 73–98.
[10] Y. Kong, E.A. Mead, G. Fang, Navigating the pitfalls of mapping DNA and RNA modifications, Nat. Rev. Genet. 24 (6) (2023) 363–381.
[11] J. Chen, C. Xu, K. Yang, R. Gao, Y. Cao, L. Liang, S. Chen, S. Xu, R. Rong, J. Wang, T. Zhu, Inhibition of ALKBH5 attenuates I/R-induced renal injury in male mice by promoting Ccl28 m6A modification and increasing Treg recruitment, Nat. Commun. 14 (1) (2023) 1161.
[12] F. Meng, Y. Liu, Q. Chen, Q. Ma, S. Gu, R. Cui, R. Cao, M. Zhao, METTL3 contributes to renal ischemia-reperfusion injury by regulating Foxd1 methylation, Am. J. Physiol. Ren. Physiol. 319 (5) (2020) F839–F847.
[13] J.N. Wang, F. Wang, J. Ke, Z. Li, C.H. Xu, Q. Yang, X. Chen, X.Y. He, Y. He, X.G. Suo, C. Li, J.T. Yu, L. Jiang, W.J. Ni, J. Jin, M.M. Liu, W. Shao, C. Yang, Q. Gong, H.Y. Chen, J. Li, Y.G. Wu, X.M. Meng, Inhibition of METTL3 attenuates renal injury and inflammation by alleviating TAB3 m6A modifications via IGF2BP2-dependent mechanisms, Sci. Transl. Med. 14 (640) (2022) eabk2709.
[14] D. Wiener, S. Schwartz, The epitranscriptome beyond m(6)A, Nat. Rev. Genet. 22 (2) (2021) 119–131.
[15] H. Sun, K. Li, C. Liu, C. Yi, Regulation and functions of non-m(6)A mRNA modifications, Nat. Rev. Mol. Cell Biol. 24 (10) (2023) 714–731.
[16] G. Jin, M. Xu, M. Zou, S. Duan, The processing, gene regulation, biological functions, and clinical relevance of N4-acetylcytidine on RNA: a systematic review, Mol. Ther. Nucleic Acids 20 (2020) 13–24.
[17] L. Xie, X. Zhong, W. Cao, J. Liu, X. Zu, L. Chen, Mechanisms of NAT10 as ac4C writer in diseases, Mol. Ther. Nucleic Acids 32 (2023) 359–368.
[18] D. Arango, D. Sturgill, N. Alhusaini, A.A. Dillman, T.J. Sweet, G. Hanson, M. Hosogane, W.R. Sinclair, K.K. Nanan, M.D. Mandler, S.D. Fox, T.T. Zengeya, T. Andresson, J.L. Meier, J. Coller, S. Oberdoerffer, Acetylation of cytidine in mRNA promotes translation efficiency, Cell 175 (7) (2018), 1872-1886 e1824.
[19] W. Wei, S. Zhang, H. Han, X. Wang, S. Zheng, Z. Wang, C. Yang, L. Wang, J. Ma, S. Guo, J. Wang, L. Liu, J. Choe, S. Lin, NAT10-mediated ac4C tRNA modification promotes EGFR mRNA translation and gefitinib resistance in cancer, Cell Rep. 42 (7) (2023) 112810.
[20] J. Luo, J. Cao, C. Chen, H. Xie, Emerging role of RNA acetylation modification ac4C in diseases: current advances and future challenges, Biochem. Pharmacol. 213 (2023) 115628.
[21] M.H. Dalhat, M.R.S. Mohammed, A. Ahmad, M.I. Khan, H. Choudhry, Remodelin, a N-acetyltransferase 10 (NAT10) inhibitor, alters mitochondrial lipid metabolism in cancer cells, J. Cell. Biochem. 122 (12) (2021) 1936–1945.
[22] C. Yang, H. Xu, D. Yang, Y. Xie, M. Xiong, Y. Fan, X. Liu, Y. Zhang, Y. Xiao, Y. Chen, Y. Zhou, L. Song, C. Wang, A. Peng, R.B. Petersen, H. Chen, K. Huang, L. Zheng, A renal YY1-KIM1-DR5 axis regulates the progression of acute kidney injury, Nat. Commun. 14 (1) (2023) 4261.
[23] L. Yang, B. Wang, F. Guo, R. Huang, Y. Liang, L. Li, S. Tao, T. Yin, P. Fu, L. Ma, FFAR4 improves the senescence of tubular epithelial cells by AMPK/SirT3 signaling in acute kidney injury, Signal Transduct. Targeted Ther. 7 (1) (2022) 384.
[24] H. Zhu, C. Cao, Z. Wu, H. Zhang, Z. Sun, M. Wang, H. Xu, Z. Zhao, Y. Wang, G. Pei, Q. Yang, F. Zhu, J. Yang, X. Deng, Y. Hong, Y. Li, J. Sun, F. Zhu, M. Shi, K. Qian, T. Ye, X. Zuo, F. Zhao, J. Guo, G. Xu, Y. Yao, R. Zeng, The probiotic L. casei Zhang slows the progression of acute and chronic kidney disease, Cell Metabol. 33 (10) (2021), 1926-1942 e1928.
[25] X. Gao, W. Hu, D. Qian, X. Bai, H. He, L. Li, S. Sun, The mechanisms of ferroptosis under hypoxia, Cell. Mol. Neurobiol. 43 (7) (Oct 2023) 3329–3341.
[26] X. Fang, H. Ardehali, J. Min, F. Wang, The molecular and metabolic landscape of iron and ferroptosis in cardiovascular disease, Nat. Rev. Cardiol. 20 (1) (2023) 7–23.
[27] A.B. Sanz, M.D. Sanchez-Nino, A.M. Ramos, A. Ortiz, Regulated cell death pathways in kidney disease, Nat. Rev. Nephrol. 19 (5) (2023) 281–299.
[28] M. Gryzik, M. Asperti, A. Denardo, P. Arosio, M. Poli, NCOA4-mediated ferritinophagy promotes ferroptosis induced by erastin, but not by RSL3 in HeLa cells, Biochim. Biophys. Acta Mol. Cell Res. 1868 (2) (2021) 118913.
[29] K. Hadian, B.R. Stockwell, The therapeutic potential of targeting regulated non-apoptotic cell death, Nat. Rev. Drug Discov. 22 (9) (Sep 2023) 723–742.
[30] X. Gao, W. Hu, D. Qian, X. Bai, H. He, L. Li, S. Sun, The mechanisms of ferroptosis under hypoxia, Cell. Mol. Neurobiol. 43 (7) (2023) 3329–3341.
[31] H. Bayir, S.J. Dixon, Y.Y. Tyurina, J.A. Kellum, V.E. Kagan, Ferroptotic mechanisms and therapeutic targeting of iron metabolism and lipid peroxidation in the kidney, Nat. Rev. Nephrol. 19 (5) (2023) 315–336.
[32] X. Jin, C. Jiang, Z. Zou, H. Huang, X. Li, S. Xu, R. Tan, Ferritinophagy in the etiopathogenic mechanism of related diseases, J. Nutr. Biochem. 117 (2023) 109339.
[33] M.Z. Liu, N. Kong, G.Y. Zhang, Q. Xu, Y. Xu, P. Ke, C. Liu, The critical role of ferritinophagy in human disease, Front. Pharmacol. 13 (2022) 933732.
[34] Y. Qin, Y. Qiao, D. Wang, C. Tang, G. Yan, Ferritinophagy and ferroptosis in cardiovascular disease: mechanisms and potential applications, Biomed. Pharmacother. 141 (2021) 111872.
[35] N. Santana-Codina, A. Gikandi, J.D. Mancias, The role of NCOA4-mediated ferritinophagy in ferroptosis, Adv. Exp. Med. Biol. 1301 (2021) 41–57.
[36] Y. Fang, X. Chen, Q. Tan, H. Zhou, J. Xu, Q. Gu, Inhibiting ferroptosis through disrupting the NCOA4-FTH1 interaction: a new mechanism of action, ACS Cent. Sci. 7 (6) (2021) 980–989.