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ORIGINAL ARTICLE

USP5-induced deubiquitination of P4HB alleviates ER stress-mediated apoptosis in intestinal ischemia/reperfusion

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Abstract

Intestinal ischemia/reperfusion (I/R) is a severe pathophysiological process that occurs in a variety of clinical conditions and can trigger multiple life-threatening syndromes. Intestinal I/R is associated endoplasmic reticulum (ER) stress. Prolyl 4-hydroxylase subunit beta (P4HB) contributes significantly to maintaining ER redox homeostasis, which is affected by I/R injury. Nevertheless, the molecular mechanism of P4HB expression and function in intestinal I/R is still unknown. In our study, we discovered that the expression of P4HB was clearly downregulated in the intestine of mice at the reperfusion stage and in Caco2 cells at the reoxygenation stage. In addition, P4HB-knockdown mice exhibited clearly enhanced ER stress-mediated apoptosis of intestinal tissue under intestinal I/R, whereas P4HB overexpression in Caco2 cells alleviated ER stress-mediated apoptosis under HR. Furthermore, via bioinformatics screening of proteins that interact with P4HB, ubiquitin-specific protease 5 (USP5) was identified as a critical factor in the abnormal expression of P4HB. USP5 interacts with P4HB and remains stable by removing ubiquitin. In vivo, P4HB knockdown counteracted the effect of USP5 overexpression on alleviating ER stress-mediated apoptosis in response to intestinal I/R. In summary, this study revealed that P4HB plays a crucial role in regulating ER stress-mediated apoptosis and identified USP5, which is a novel mediator of P4HB, as a prospective target for the treatment of intestinal I/R.

Keywords Ischemia/reperfusion · P4HB · ER-stress · Apoptosis · Deubiquitination

Introduction

Intestinal ischemia/reperfusion (I/R) injury is a dangerous condition that can arise in various settings, such as mesenteric arterial thrombotic disease, shock, and organ transplantation [1, 2]. Eventually, intestinal I/R can lead to systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS), both of which have high mortality rates [3, 4]. Therefore, it is important to explore the pathogenesis of intestinal I/R injury and find countermeasures to intervene according to its mechanism to reduce the mortality rate and improve the prognosis of patients.

Endoplasmic reticulum stress (ER stress) refers to a complex state, for various reasons, including internal environmental disorders, that occurs when the ability of cells to properly fold and modify proteins after translation in the ER is impaired, resulting in the accumulation of unfolded, misfolded and newly synthesized proteins in the ER and the disruption of protein homeostasis. To restore protein homeostasis, the unfolded protein response (UPR) is triggered. Moreover, excessively severe or chronic ER stress can cause multiple consequences including apoptosis through the terminal UPR program [5–7]. Previous studies have demonstrated that ER stress and the UPR play vital roles in multiple disease processes, such as neurodegeneration, cancer, diabetes, and vascular diseases [8–11]. ER stress has also been reported in a variety of organ models of I/R injury, including intestinal I/R injury, and the inhibition

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of excessive ER stress can reduce tissue damage [12, 13]. As an important member of the protein disulfide isomerase (PDI) family [14], prolyl 4-hydroxylase subunit beta (P4HB) is crucial for maintaining ER redox homeostasis, which is impacted by I/R injury [15, 16]. However, the functional role of P4HB in intestinal I/R has not been studied. Ubiquitination, which is considered one of the most important protein post-translational modifications, is essential for protein degradation [17] and participates in organ I/R processes [18, 19]. However, whether ubiquitination has a role in controlling P4HB stability is unclear. Ubiquitin specific protease 5 (USP5) is a deubiquitinase (DUB) that has been shown to be involved in apoptosis, DNA repair, inflammation, and colorectal cancer through the regulation of specific substrates [20, 21]. Using a database of interacting proteins, BioGRID (https://thebiogrid.org) [22], combined with the transcriptomics of intestinal I/R, we identified the interaction between USP5 and P4HB.

In this study, we found that P4HB expression is downregulated at different stages during intestinal I/R. Our data indicate that P4HB contributes to ER stress-mediated apoptosis. USP5 can maintain P4HB stabilization by deubiquitinating P4HB, thus reducing intestinal I/R injury. Therefore, the USP5–P4HB axis may be useful as a therapeutic target for intestinal I/R injury.

Materials and methods

Animals

As described previously [23], a mouse intestinal I/R model was established using eight-week-old male C57BL/6 mice weighing between 18 and 22 g. All mice were provided water and standard laboratory chow. Anesthetized mice underwent 45 min of noninvasive microvascular clamping of the superior mesenteric artery to simulate ischemia, and then followed by 1, 2, 4, or 8 h of reperfusion [12]. An identical procedure was used on sham mice, but vascular clamping was not performed. Intestinal tissue and plasma were collected for further analysis at each reperfusion time point. All experimental procedures were authorized by the Ethics Committee of Dalian Medical University and performed in accordance with the corresponding guidelines. Lentiviruses (LVs) were obtained from GenePharma (Shanghai, China). Briefly, vector plasmid and packaging plasmid were prepared after vector design, and cells were co-transfected and cultured. The virus was prepared by concentrating and screening, and the virus titer was more than 10^9 TU/mL. One week prior to intestinal I/R, the mice received a single intravenous injection of LV3-sh-P4HB or LV5-USP5. The shRNA sequences (GenePharma) used are listed in Table S1.

Cell culture, treatment and transfection

Previous studies have shown that the hypoxia/reoxygenation (H/R) model has characteristics analogous to those of in vivo I/R condition [24]. We rigorously matched the time of reoxygenation to the time of reperfusion, an approach that was consistent with our previous study [25]. Caco-2 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were used to establish a hypoxia-reoxygenation model. The cells were cultured in MEM (Gibco, Carlsbad, USA) supplemented with fetal bovine serum and a variety of nutrients as needed. To simulate a hypoxic environment in vitro, the cells were incubated in a microaerophilic environment consisting of 94% N2, 5% CO2, and 1% O2 for 15 h, and then incubated in a normoxic environment for reoxygenation for 1, 2, 4–8 h. Using Lipofectamine 3000 (Invitrogen, Shanghai, China), several siRNAs or overexpression plasmids (GenePharma) were transfected into Caco-2 cells for a duration of 48 h. Table S1 contains a list of the siRNA sequences. Negative controls were used for the above transfection procedures. Furthermore, after USP5 overexpression plasmids were transfected into Caco2 cells, 20 μM MG132 (Selleck, USA) was treated for 3 h, and 100 μM cycloheximide (CHX, Sigma, USA) was applied for 0, 1, 2, and 4 h after USP5 siRNA was transfected.

Western blotting

Total protein was obtained from small intestinal tissue and Caco2 cells. Briefly, the extracted proteins were separated via SDS–PAGE (10–12%) in a suitable electric field and transblotted onto PVDF membranes, after which the membranes were incubated with various primary antibodies. The primary antibodies used are listed in Table S2. BeyoECL Plus was used to visualize protein bands following incubation with HRP-conjugated secondary antibodies (Beyotime, Shanghai, China). The Gel-Pro Analyzer version 4.0 (Media Cybernetics, USA) was used to quantify proteins.

Co-IP

Protein A/G magnetic beads (Bimake, USA) were treated with specific antibodies for four hours at room temperature. Then, the proteins extracted from Caco2 cells were added to the antibody and bead mixture, followed by incubation for a full hour at room temperature. Following isolation and purification, western blotting was performed for the proteins attached to the beads.

RNA extraction and qRT–PCR

TRIzol (Invitrogen) was used to isolate total RNA, and a reverse transcription kit (Accurate Biotechnology, Changsha, China) was used to synthesize cDNA. qRT–PCR analysis was subsequently performed using a qPCR Kit (Accurate Biotechnology). β-actin mRNA expression was used as a standard. Table S3 displays the PCR primers used (Sangon Biotech, Shanghai, China).

TUNEL staining

A TUNEL Cell Apoptosis Kit (TransGen, Beijing, China) was used to quantify the degree of apoptosis caused by H/R. Terminal deoxynucleotidyl transferase (TdT) was used to label apoptotic cells for 60 min at 37 °C. Nuclei were stained with DAPI (Beyotime).

Biochemical analysis and pathological observation

Common serologic markers following intestinal I/R, including serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, were measured using appropriate detection kits (Jiancheng, Nanjing, China).

Hematoxylin–eosin (H&E) staining was applied to intestinal tissue sections, which were collected and then fixed overnight in 4% paraformaldehyde solution. These samples were subsequently observed using an orthogonal microscope.

Statistics

GraphPad Prism software (La Jolla, USA) was used to analyze the data, and the results are presented as means ± SDs for all the data. Statistical significance was verified by analyzing the data from several groups. The threshold for significance was $P < 0.05$.

Results

Downregulation of P4HB expression at the reperfusion stage in intestinal I/R

To investigate the changes in P4HB expression in intestinal I/R, we used western blotting to assess the expression of P4HB in the intestinal tissue samples of C57BL/6 mice. The expression level of P4HB decreased progressively during reperfusion, reached the lowest value at 4 h after reperfusion and then gradually increased from 4 h to 8 h of reperfusion (Fig. 1A). Small intestine tissue samples were collected after the sham operation and at 4 h after reperfusion, and H&E staining revealed severe pathological injury to the small intestine, including villi collapse, rupture, apical stripping and significant expansion of the intestinal mucosal epithelium space (Fig. 1C). When Caco-2 cells were exposed to H/R damage in vitro, the outcomes matched those observed in vivo (Fig. 1B).

P4HB knockdown aggravates ER stress-mediated apoptosis in intestinal I/R

To investigate the role of P4HB in intestinal I/R, we used a lentivirus (LV3-sh-P4HB) to knockdown P4HB expression. As expected, P4HB knockdown in vivo clearly aggravated intestinal mucosal epithelial damage (Fig. 2A). Additionally, the levels of serologic markers (serum ALT and AST) increased after P4HB knockdown (Fig. 2B). Significantly increased levels of CHOP, GrP78, Bax, and C-cas-3 as well as decreased levels of Bcl2 (anti-apoptotic factor) were

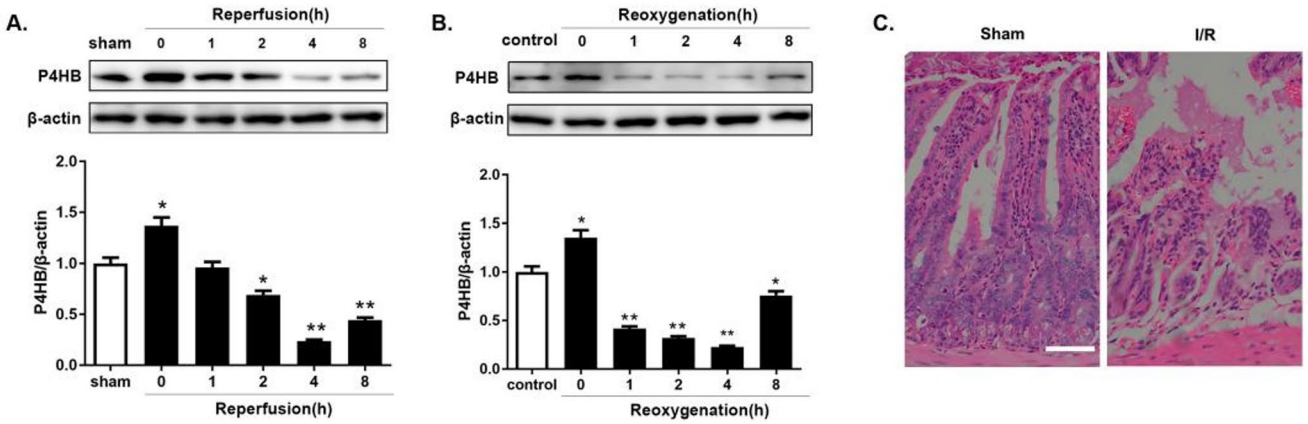

Fig. 1 Downregulation of P4HB expression at the reperfusion stage in intestinal I/R. **A**. Intestinal P4HB protein expression in mice subjected to 45 min of ischemia, followed by reperfusion for varying durations, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the sham group. **B**. The protein expression of P4HB in Caco2 cells exposed to hypoxia followed by reoxygenation for various durations, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group. **C**. H&E staining. Scale bar, 100 μm

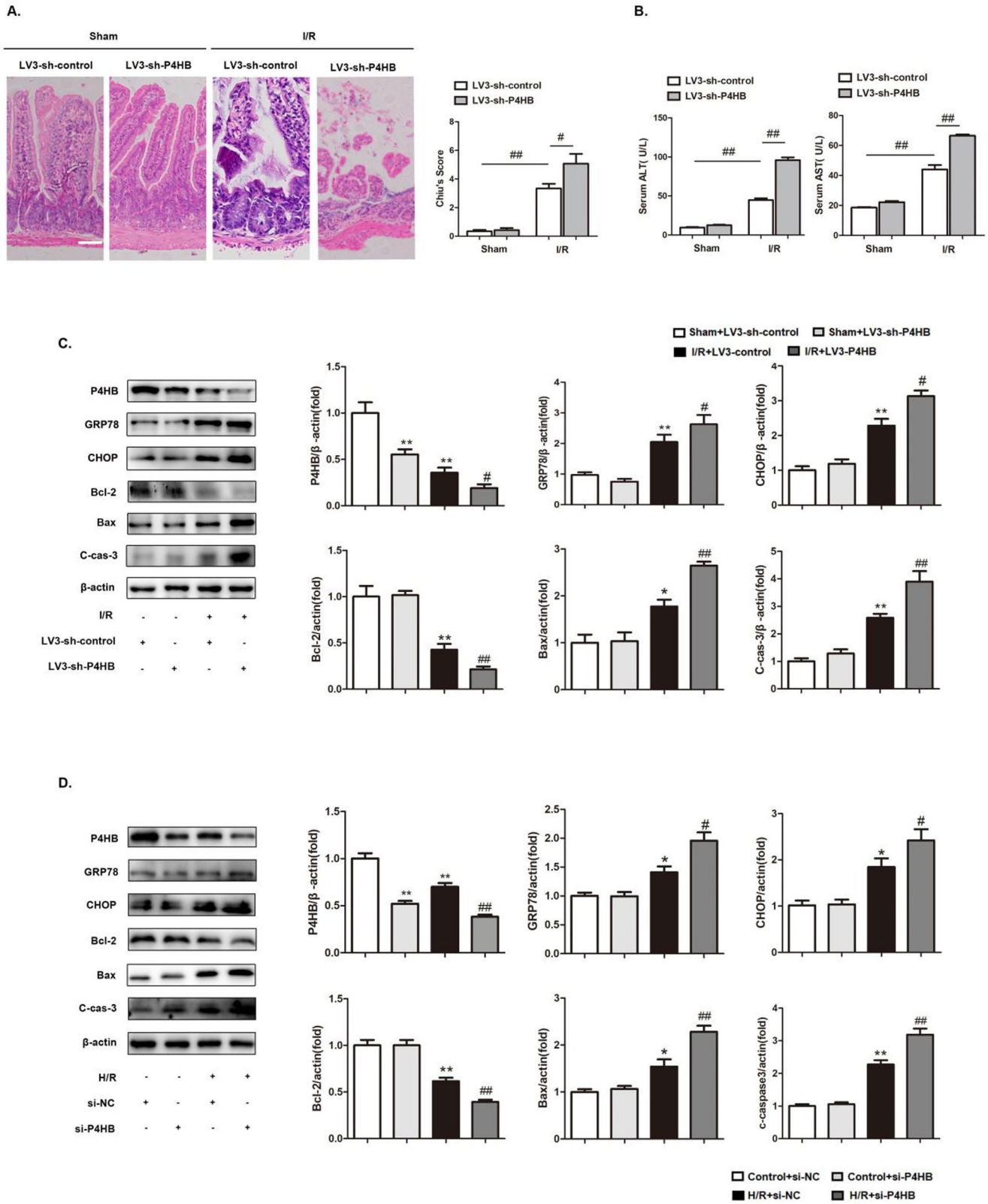

Fig. 2 P4HB knockdown aggravates ER stress-mediated apoptosis in intestinal I/R in mice. LV3-sh-P4HB was injected into mice exposed to intestinal I/R. **A**. H&E staining. Scale bar, 100 μm. **B**. Serum ALT and AST levels, $n = 6$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. **C**. Expression of P4HB and other indicator proteins, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the sham +LV3-sh-control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the I/R +LV3-sh-control group. **D**. P4HB siRNA was transfected into Caco2 cells before H/R. The expression of P4HB and other indicator proteins, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control +si-NC group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the H/R +si-NC group

observed following P4HB knockdown (Fig. 2C). The in vitro and in vivo outcomes were consistent. P4HB siRNA exacerbated the ER stress-mediated apoptosis in Caco2 cells after H/R (Fig. 2D).

P4HB overexpression alleviates ER stress-mediated apoptosis in intestinal I/R injury

We transfected a P4HB overexpression plasmid into Caco2 cells, and western blot results showed that GRP78, CHOP, Bax, and C-cas-3 expression decreased significantly and Bcl-2 expression increased (Fig. 3A). The quantity of apoptotic cells (TUNEL+) significantly decreased after P4HB overexpression (Fig. 3B). Collectively, these findings indicate that ER stress-mediated apoptosis was alleviated by P4HB overexpression in vitro.

USP5 interacts with P4HB

We assessed the molecular mechanism through which the expression of P4HB is downregulated in intestinal I/R injury during the reperfusion stage. The results of qRT–PCR experiments revealed that the P4HB mRNA level did not change in the intestinal I/R model but that the P4HB protein level significantly decreased both in vitro and in vivo (Figs. 1A and 4A). In addition, the ubiquitination level of P4HB increased obviously in the context of intestinal I/R (Fig. 4B). Thus, the lower stability of P4HB could be

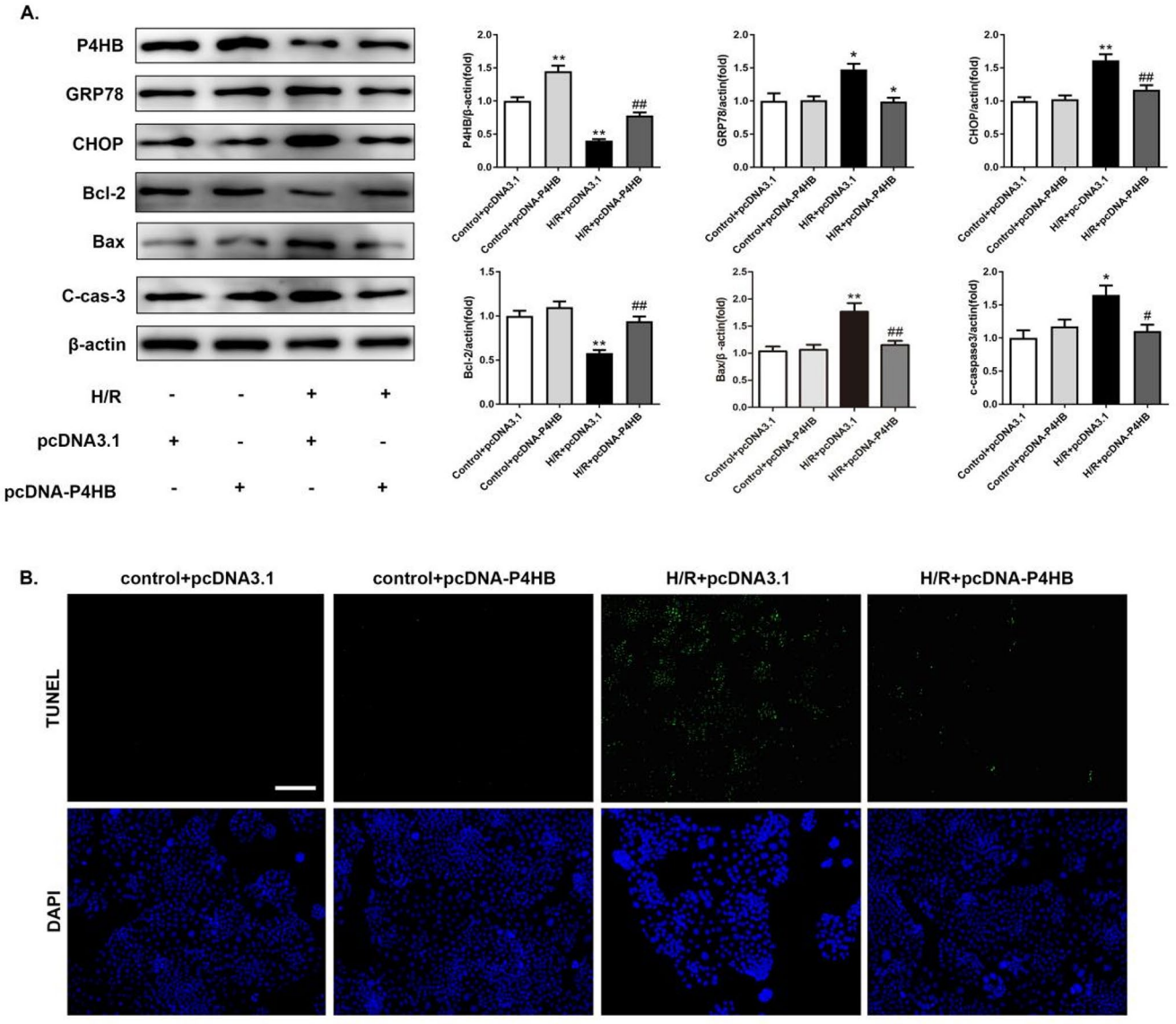

Fig. 3 P4HB overexpression alleviates ER stress-mediated apoptosis in intestinal I/R in vitro. P4HB overexpression plasmid was transfected into Caco2 cells, followed by H/R. **A**. Expression of P4HB and other indicator proteins, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control + pcDNA3.1 group; $n = 3$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the H/R + pcDNA3.1 group. **B**. TUNEL staining. Scale bar, 100 μm

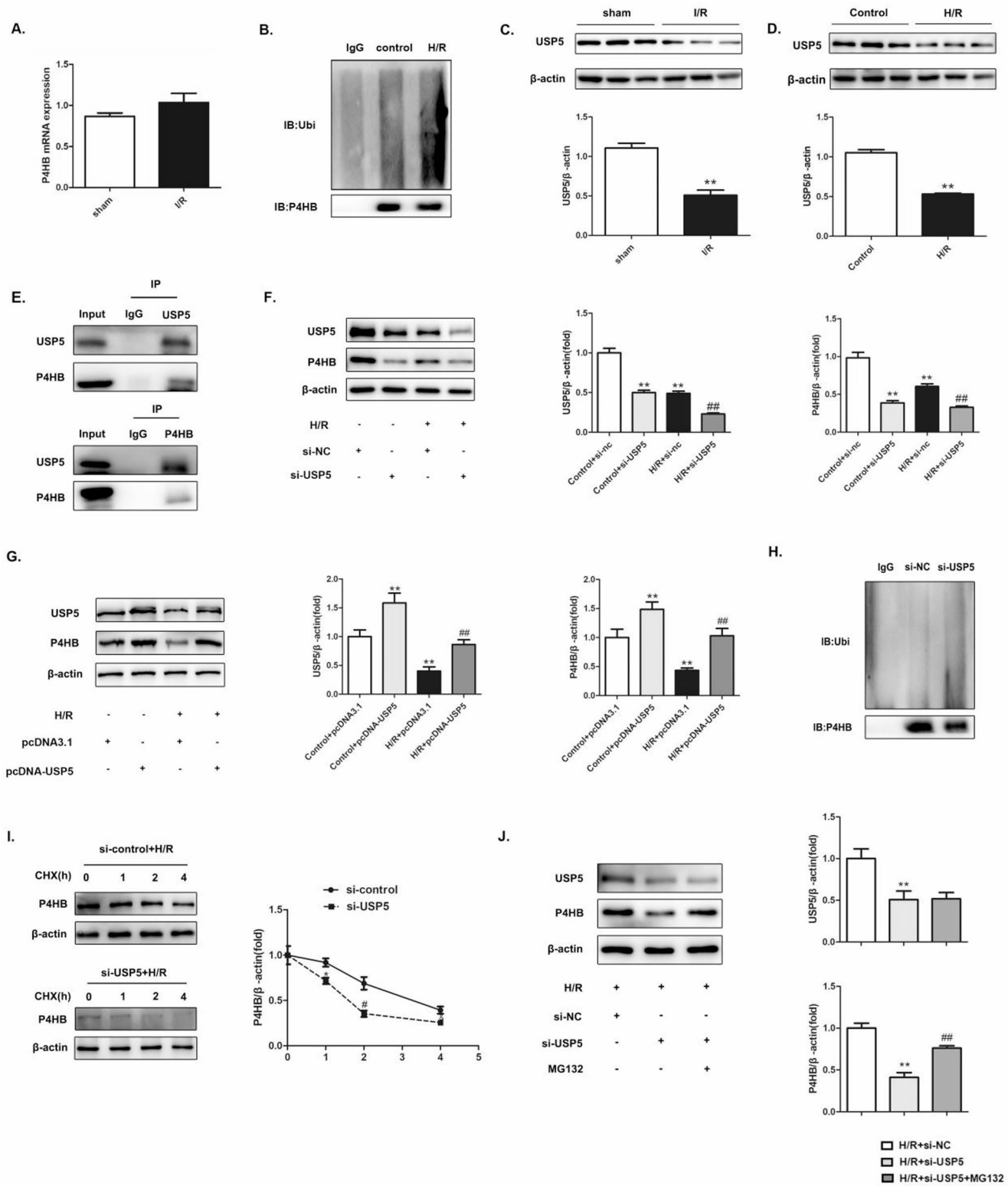

Fig. 4 USP5 interacts with P4HB and promotes the deubiquitination and stability of P4HB. **A**. P4HB mRNA levels. $n = 6$. **B**. P4HB ubiquitination levels were assessed by an IP assay. **C**. USP5 protein expression in vivo. $n = 3$. $^{**}P < 0.01$ vs. the sham group. **D**. USP5 protein expression in vitro. $n = 3$. $^{**}P < 0.01$ vs. the control group. **E**. The interaction between USP5 and P4HB. **F**, **G**. Caco2 cells were transfected with USP5 siRNA or pcDNA-USP5 and then subjected to H/R. USP5 and P4HB proteins. $n = 3$. **H-J**. USP5 siRNA was transfected into Caco2 cells exposed to H/R, which were then treated with CHX or MG132, as shown in panels I and J, respectively. **H**. P4HB ubiquitination levels were measured. **I**. Evaluation of P4HB protein expression. $^{*}P < 0.05$, $^{\#}P < 0.05$, $^{\&}P < 0.01$. **J**. P4HB protein expression. $^{**}P < 0.01$ vs. the H/R + si-NC group; $^{\#\#}P < 0.01$ vs. the H/R + si-USP5 group

explained by ubiquitination. Previous studies have shown that ubiquitin proteasome-associated proteins (USPs) family may play an important role in regulating endoplasmic reticulum stress and apoptosis. Therefore, we focused on the USPs family molecules that may interact with P4HB in the online protein interaction database Biogrid (https://thebiogrid.org), and combined with the transcriptomics of intestinal I/R (GSE233710).The Biogrid database showed that USP5 may have a strong interaction with P4HB. Transcriptome results showed that the mRNA level of USP5 in the control group was significantly lower than that in the model group (Supplementary Table 4). Based on this, we initially identified USP5 as a candidate factor. Western Blot results showed that USP5 protein expression was decreased in intestinal I/R (Fig. 4C, D). Co-IP assays revealed that endogenous USP5 strongly interacted with P4HB (Fig. 4E).

USP5 promotes the deubiquitination and stability of P4HB

Considering that USP5 interacts with P4HB and has deubiquitination activity, it is possible that USP5 influences the deubiquitination of P4HB. P4HB protein expression significantly increased after USP5 overexpression but markedly decreased upon USP5 knockdown (Fig. 4F, G). Following our discovery that USP5 regulates P4HB protein expression, we next investigated the possibility that USP5 catalyzes P4HB deubiquitination. After transfecting USP5 siRNA into Caco2 cells, the level of ubiquitinated P4HB significantly increased (Fig. 4H), and the protein expression of P4HB observably decreased (Fig. 4F). Furthermore, USP5 inhibition led to a markedly quicker breakdown of P4HB after CHX treatment. USP5 knockdown shortened the P4HB protein half-life (Fig. 4I). Additionally, P4HB degradation induced by USP15 siRNA was inhibited by the proteasome-specific inhibitor MG132 (Fig. 4J), suggesting that a proteasomal pathway is involved in P4HB degradation. As a result, USP5 inhibits P4HB ubiquitination and degradation.

P4HB knockdown attenuates USP5 overexpression-ameliorated ER stress-mediated apoptosis in intestinal I/R

To determine whether the effect of USP5 on intestinal I/R depends on the expression of P4HB, we used LV3-sh-P4HB, LV5-USP5 or the corresponding controls to generate mice with USP5 overexpression combined with P4HB knockdown. USP5 overexpression alleviated intestinal mucosal epithelial injury and ER stress-induced apoptosis in the intestine, as expected (Fig. 5A, B). These favorable effects of USP5 overexpression were significantly inhibited by P4HB knockdown in mice. These above results were further confirmed in vitro. P4HB siRNA inhibited USP5 overexpression-ameliorated ER stress-mediated apoptosis in Caco2 cells after H/R (Fig. 5C). In summary, these data indicate that P4HB knockdown attenuates USP5 overexpression-ameliorated ER stress-mediated apoptosis in intestinal I/R.

Discussion

Excessive ER stress is linked to intestinal I/R and ultimately results in apoptosis, and the inhibition of ER stress-mediated apoptosis promotes the repair of the intestinal mucosal barrier [12, 26]. In this study, we revealed the critical role of P4HB in intestinal I/R by knocking down or overexpressing P4HB. P4HB knockdown aggravated intestinal I/R injury, disrupted ER redox homeostasis, and promoted excessive ER stress-mediated apoptosis; in contrast, P4HB overexpression had the opposite effects. Thus, P4HB is pivotal for intestinal I/R injury.

In previous studies, P4HB was thought to interact with ERO1, synthesize disulfide bonds, and produce H_2O_2 [27–29]. Those processes are important prerequisites for the correct folding and modification of proteins in the ER, and the maintenance of redox homeostasis in the ER is critical during those processes [30–33]. In this study, we found that P4HB expression was increased in mice during ischemia (and increased in Caco2 cells during hypoxia). Interestingly, ERO1 expression has been shown to be similar to P4HB expression during ischemia or hypoxia in a variety of diseases [34, 35]. This may be due to the functional interdependence of ERO1 and P4HB to maintain normal disulfide bond formation and ER redox homeostasis. During the subsequent reperfusion stage, P4HB expression gradually decreased with time, reaching its lowest level at 4 h of reperfusion. P4HB knockdown aggravated ER stress-mediated apoptosis. P4HB overexpression alleviated these effects. This finding is similar to the conclusion of some past studies that P4HB may mitigate injury through multiple pathways [36, 37]. Notably, in contrast to some tumor models [38, 39], PDI plays a protective role in ischemic and hypoxic diseases such as myocardial infarction and stroke [37, 40]. In this respect, P4HB may play a different role in different disease models, or it may be that during the reperfusion stage, when the absolute expression of P4HB is reduced, the function of correctly folded proteins in the ER does not completely recover during the reperfusion stage, after which unfolded proteins accumulate and unfolded protein-mediated cytotoxicity persists, resulting in excessive and chronic

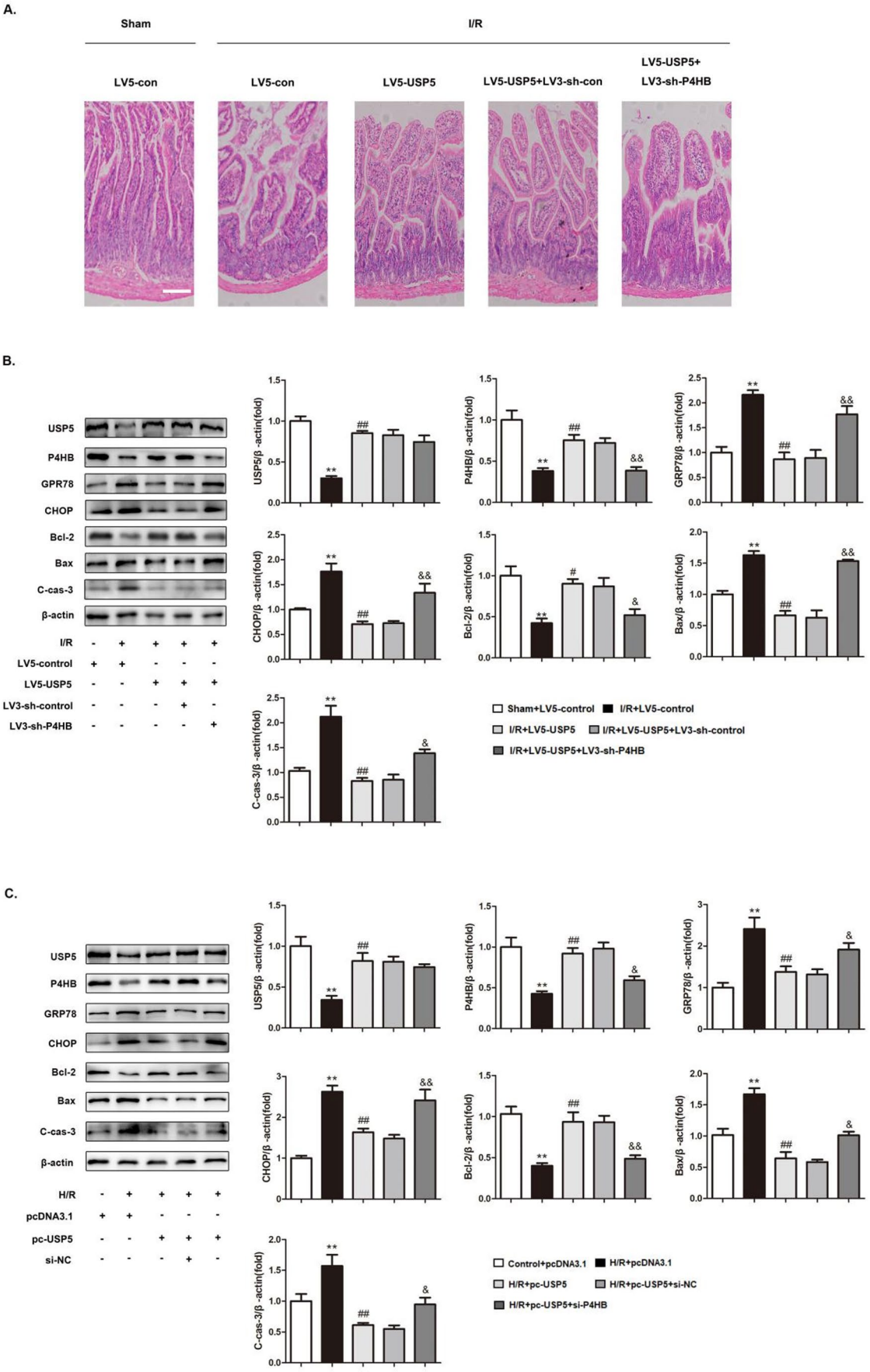
A.
Sham
I/R
LV5-con
LV5-con
LV5-USP5
LV5-USP5+LV3-sh-con
LV5-USP5+ LV3-sh-P4HB
B.
USP5
P4HB
GPR78
CHOP
Bcl-2
Bax
C-cas-3
β-actin
I/R
LV5-control
LV5-USP5
LV3-sh-control
LV3-sh-P4HB
USP5/β -actin(fold)
P4HB/β -actin(fold)
GRP78/β -actin(fold)
CHOP/β -actin(fold)
Bcl-2/β -actin(fold)
Bax/β -actin(fold)
C-cas-3/β -actin(fold)
Sham+LV5-control
I/R+LV5-control
I/R+LV5-USP5
I/R+LV5-USP5+LV3-sh-control
I/R+LV5-USP5+LV3-sh-P4HB
C.
USP5
P4HB
GRP78
CHOP
Bcl-2
Bax
C-cas-3
β-actin
H/R
pcDNA3.1
pc-USP5
si-NC
USP5/β -actin(fold)
P4HB/β -actin(fold)
GRP78/β -actin(fold)
CHOP/β -actin(fold)
Bcl-2/β -actin(fold)
Bax/β -actin(fold)
C-cas-3/β -actin(fold)
Control+pcDNA3.1
H/R+pcDNA3.1
H/R+pc-USP5
H/R+pc-USP5+si-NC
H/R+pc-USP5+si-P4HB

Fig. 5 P4HB knockdown attenuates USP5 overexpression-ameliorated ER stress-mediated apoptosis in intestinal I/R. LV3-sh-P4HB and LV5-USP5 were injected into mice subjected to intestinal I/R. **A**. H&E staining. Scale bar, 100 μm. **B**. Expression of P4HB and other indicator proteins. $^{*}P< 0.05$, $^{**}P< 0.01$ vs. the sham + LV5-control group. $^{\#}P< 0.05$, $^{\#\#}P< 0.01$ vs. the I/R + LV5-control group. $^{\&}P< 0.05$, $^{\&\&}P< 0.01$ vs. the I/R + LV5-USP5 + LV3-sh-control group. **C**. P4HB siRNA and USP5 overexpression plasmids were transfected into Caco2 cells subjected to H/R. Expression of P4HB and other indicator proteins. $^{*}P< 0.05$, $^{**}P< 0.01$ vs. the control + pcDNA3.1 group. $^{\#}P< 0.05$, $^{\#\#}P< 0.01$ vs. the H/R + pcDNA3.1 group. $^{\&}P< 0.05$, $^{\&\&}P< 0.01$ vs. the H/R + pc-USP5 + si-NC

ER stress. Moreover, ROS from various sources completely disrupt redox homeostasis in the ER due to the burst of ROS during reperfusion, leading to a cascade reaction involving both oxidative stress and ER stress, which ultimately leads to apoptosis [15, 41, 42]. In summary, how P4HB (PDI) affects ER redox homeostasis and cell fate during intestinal I/R requires further study, and P4HB may become a vital molecular target for the treatment of intestinal I/R injury.

Despite the growing awareness of P4HB function, little attention has been given to the aberrant expression of P4HB. During the reperfusion phase, intestinal I/R caused a steady decline in the P4HB protein level, but there was no difference in P4HB mRNA levels between I/R and normal samples. Accordingly, we assume that P4HB expression in intestinal I/R is regulated by ubiquitination, which is considered a significant pathway of protein degradation and may be essential for regulating P4HB expression in intestinal I/R. In this study, USP5, a novel DUB that affects specific substrates through deubiquitination [20, 21], stabilized P4HB and prevented its degradation. Upon intestinal I/R, the protein level of USP5 decreased. Strikingly, we detected a decreasing trend in the mRNA level of USP5 in the transcriptome of intestinal I/R samples, and the bioinformatics results suggested that USP5 and P4HB might interact [22]. This finding was confirmed by Co-IP assays. Moreover, USP5 knockdown enhanced the ubiquitination of P4HB, which lowered P4HB protein levels. The deubiquitination of P4HB by USP5 may be the cause of the interaction between P4HB and USP5. Thus, USP5 interacts with P4HB and stabilizes P4HB expression via its deubiquitinating function.

Previous studies have confirmed the critical functions of DUBs in controlling cell fate and apoptotic pathogenesis [18, 19, 43]. According to mounting data, USP5 is essential for regulating apoptosis [44]. Myeloma cell apoptosis is caused by the inhibition of the deubiquitinase USP5 [45]. Our study demonstrated that USP5 overexpression attenuated intestinal I/R-mediated apoptosis. The protective effect of USP5 overexpression on intestinal I/R was found to be due to an increase in P4HB expression mediated by USP5, with opposite effects observed when P4HB was silenced. Taken together, our findings point to a unique mechanism by which USP5 attenuates ER stress-mediated apoptosis in intestinal epithelial cells by maintaining the stability of P4HB. USPs play an important role by regulating the stability of substrates [18, 19, 43, 46], a property that makes them potential drug targets. In recent years, the development and application of inhibitors targeting USPs have confirmed this view [47]. Notably, unlike USPs inhibitors, which kill tumor cells and pathogenic cells [48, 49], the activation of USPs and even other classes of deubiquitinating enzymes may become an important therapeutic means for the treatment of acute diseases such as ischemia and hypoxia [50–52].

In summary, P4HB plays a protective role in intestinal I/R by regulating ER stress-mediated apoptosis, and fluctuations in P4HB expression can be regulated by deubiquitination, which is mediated by USP5. Our study focused on the changes in and possible roles of P4HB i*n* intestinal I/R and highlighted the importance of P4HB post-translational modification by USP5 in intestinal I/R. We believe that the essence of ER redox homeostasis during I/R can be understood by developing a more large-scale model of I/R at different stages and using more advanced technology to test ER stress. Although our study reveals a critical role of USP5-P4HB axis in intestinal ischemia-reperfusion injury, the specific binding site of USP5 to P4HB remains unknown. In addition, existing studies may not reflect the regulatory network in spatial dimensions and the role of different cell types in intestinal I/R. Moreover, our experimental design, in which only male mice were included as experimental animals, may limit the potential for clinical translation of the findings. Subsequent investigations will employ a multifaceted approach to systematically address the aforementioned research inquiries. Even so, targeting the USP5-P4HB axis may be an important means to treat intestinal I/R injury.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00018-025-05752-3.

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Author contributions SN, PZ, JY and XT designed the study. SN, PZ, XZ, YZ and FY performed the experiments. SN, ZC, FZ contributed experimental materials. PZ, SG and YW analyzed the data. SN, PZ, JY and XT drafted the manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the.
corresponding author on reasonable request. The RNA-seq data have been deposited.
at GEO (https://www.ncbi.nlm.nih.gov/geo/): GSE233710.

Declarations

Ethics approval and consent to participate All experimental procedures were authorized by the Ethics Committee of Dalian Medical University.

Competing interests The authors declare that there are no conflicts of interest.

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