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PROFESSOR WEI AN (Orcid ID : 0000-0002-9648-4497)

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Augmenter of liver regeneration-mediated mitophagy protects against hepatic ischemia/reperfusion injury

Wei-ning Kong[1], Wen Li[1], Chun Bai[2], Yuan Dong[1], Yuan Wu[1], Wei An[1]¶

[1]*Department of Cell Biology and Municipal Laboratory of Liver Protection and Regulation of Regeneration, Capital Medical University, Beijing China*

[2]*Department of Hepatobiliary and Pancreaticosplenic Surgery, Beijing Chao-Yang Hospital, Capital Medical University, Beijing China*

Wei-ning Kong and Wen Li contributed equally to this paper.

¶Correspondence

Wei An, Professor, Department of Cell Biology, Capital Medical University, You An Men Wai, Beijing, China

Email: anwei@ccmu.edu.cn.

Abbreviations: Ad, adenoviral; ALR, augmenter of liver regeneration; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; Bafi A1, bafilomycin A1; DHE, dihydroethidium; Drp1,dynamin-related protein 1; GSH, reduced glutathione; GSSG, oxidized glutathione; HPF, high-power field; H/R, hypoxia/ reoxygenation; I/RI, ischemia/reperfusion injury; KO, knockout;

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LAMP1, lysosome-associated membrane glycoprotein 1; LC3, microtubule-associated protein 1 light chain 3; LDH, lactate dehydrogenase; Mfn1, Mitofusin 1; Mfn2, Mitofusin 2; MDA, Malondialdehyde; MMP, mitochondrial membrane potential; MPO, myeloperoxidase; NC, Negative control; N.S, normal saline; Opa1, optic atrophy 1; OLT, orthotopic liver transplantation; PBS, phosphate buffered-saline; PGC-1α, peroxisome proliferator activated receptor γ coactivator-1α; PINK1, PTEN-induced putative kinase 1; Rapa, rapamycin; ROS, reactive oxygen species; SEC, sinusoidal endothelial cell; TEM, transmission electron microscopy; TOM20, translocase of outer mitochondrial membrane 20; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; WT, wild-type.

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Abstract

Augmenter of liver regeneration (ALR) is an anti-apoptotic protein found mainly in mitochondria. It protects hepatocytes from ischemia-reperfusion (I/R) injury, but the underlying mechanism is not clear. We found that in rats, delivery of the *ALR* gene alleviated hepatic I/R injury during orthotopic liver transplantation as evidenced by reduced serum aminotransferase, oxidative stress and apoptosis, and increased expression of autophagy markers. In an *in-vitro* hypoxia/reoxygenation (H/R) model, overexpression of the *ALR* gene activated autophagy and relieved defective mitophagy via the PINK1/Parkin pathway. Mechanistically, *ALR* transfection induced the expression of mitofusin 2 (Mfn2) in the H/R model, which led to PINK1 accumulation and mitochondrial translocation of Parkin. Deletion of *Mfn*2 abolished mitophagy activation induced by *ALR* transfection, promoted mitochondrial dysfunction, and eventually inhibited cell apoptosis. *Mfn*2 administration prevented the inhibition of mitophagy in *ALR*-knockout (KO) cells, thus attenuated mitochondrial dysfunction and cell apoptosis. In heterozygous *ALR*-knockout mice treated with a warm I/R injury, marked aggravation of liver injury was associated with mitophagy inhibition and reduction in Mfn2 expression. Taken together, our results confirm that ALR accelerated Parkin translocation and mitophagy via Mfn2, and protected hepatocytes from I/R-induced injury. Our findings provide a novel rationale for the treatment of hepatic I/R injury.

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Introduction

A variety of hepatic surgery procedures include temporary occlusion of the blood supply to the liver. Restoration of blood flow and reoxygenation of ischemic organs often lead to ischemia/reperfusion (I/R) injury. Hepatic I/R injury is one of the serious complications of liver resection, transplantation, and hemorrhagic shock.[1] Hepatic I/R injury accounts for up to 10% of early liver transplantation failures and increasing graft rejections.[2] In the context of the shortage of donor organs, I/R injury remains a significant clinical obstacle for liver resection and liver transplantation during procurement, organ preservation, and operative procedures.[3]

Despite its profound clinical importance, few of the available strategies effectively reduce the effects of hepatic I/R because the causes of that severe syndrome are extremely complicated and far from fully understood. Mitochondria comprise an extensive tubular network that is maintained by an equilibrium between fusion and fission. Loss of this equilibrium can degrade mitochondrial integrity and impair mitochondrial function.[4] The term mitochondrial autophagy or "mitophagy" refers to the selective removal of excess or damaged mitochondria so that mitochondrial homeostasis is maintained. It has been recently reported that mitophagy may be impaired during the pathogenesis of I/R injury.[5] Growing evidence indicates that reperfusion impairs mitophagy and that the demand for mitophagy to remove swollen and injured mitochondria exceeds the autophagic capacity in reperfused hepatocytes.[6] Deficient mitophagy in hepatic I/R injury is related to mitochondrial dysfunction, oxidation-phosphorylation uncoupling, and ultimate cell death.[7] Parkin, the gene product of PARK2 (Parkinson protein-2), is a primarily cytosolic ubiquitin E3 ligase that contains a ubiquitin-like domain. Parkin-related signaling is known as a key regulator of mitophagy.[8] Upon mitochondrial depolarization, PTEN-induced putative kinase 1 (PINK1) accumulates on the outer mitochondrial membrane (OMM), where it recruits Parkin from the cytosol and activates its E3 ligase activity. Parkin then ubiquitinates various outer-membrane proteins leading to the initiation of selective mitophagy.[9]

Augmenter of liver regeneration (ALR, genetic name *gfer*, or growth factor ERV1) was initially reported as a mitogenic factor that promoted liver regeneration after partial hepatectomy in rats.[10] ALR is primarily expressed in parenchymal hepatocytes and predominantly resides within the mitochondrial intermembrane space. ALR is believed to maintain hepatocyte survival by suppressing the onset of

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mitochondrial permeability transition and endoplasmic reticulum (ER) stress, thus protecting hepatocytes from various clinically significant injuries. We previously reported that ALR protected the liver from I/R injury by alleviating mitochondrial damage, oxidative stress, and death.[11] Recently, we reported that ALR protected hepatocytes from ethanol-induced injury by promoting autophagy through inhibiting mTOR signaling pathway.[12] We also reported that ALR inhibited mitochondrial fission and reduced hepatocyte apoptosis during liver I/R injury by inhibiting mitochondrial recruitment and phosphorylation of dynamin-related protein 1 (Drp1), a key promoter of mitochondrial fission.[13] These data imply that ALR protects against hepatic injury via its effects on mitophagy. However, the protective mechanism during I/R injury requires further elucidation.

In this study, we found that ALR promoted the recruitment of the mitophagy-related proteins PINK1 and Parkin to mitochondria, which enhanced mitophagy and helped maintain mitochondrial homeostasis in hepatic I/R injury. ALR protection seems to be closely associated with upregulation of mitofusin 2 (Mfn2).

Material and Methods

Rats and orthotopic liver transplantation surgery

Eight-week-old male Brown-Norway rats were purchased from the Beijing Vital River Laboratory Animal Technology (Beijing, China). All animals were treated humanely following the Guidelines for Animal Care and Use to Medical Research approved by Capital Medical University (Beijing, China). The rats were randomly divided into four groups of six each, a sham-operation group, control treatment, vector treatment, and ALR treatment. Except for the sham-operation group, rats in other three groups received orthotopic liver transplantation (OLT). Five days before OLT, rats were given normal saline, Ad-vector (Ad-Null), or Ad-*ALR* gene (3×10^9 pfu/100 μl) by injection via the dorsal penis vein. The *ALR* gene was inserted into an adenovirus (Ad) vector by Beijing Hesheng Gene Technology as previously described.[11] The OLT was performed using a modified two-cuff method as described previously.[14] Briefly, the rats were anesthetized by intraperitoneal injection of 1% ketamine, 40 mg/kg. After laparotomy, the donor livers were harvested and stored in Lactated-Ringer's solution at 4°C for 2 h before transplantation. The liver grafts were implanted by connecting the suprahepatic vena cava (SHVC) with a running 8-0 Prolene suture. The portal vein and infrahepatic vena cava (IHVC) were anastomosed using a cuff technique. The bile duct was

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anastomosed with a stent, and the hepatic artery was ligated. Sham-operated rats were laparotomized and the wound was closed without I/R. The rats were sacrificed 24 h after liver transplantation, and the liver tissue and blood were collected.

Mouse model of I/R injury

C57BL/6J wild-type (WT) *Gfer*$^{+/+}$ mice and heterozygous *ALR*-knockout (*ALR*-KO) *Gfer*$^{+/-}$ mice were bred under pathogen-free conditions at the animal care facility of Capital Medical University (Beijing, China). The methods used to generate and identify the heterozygous mice have been described previously.[15] Six to 8-week-old male mice, weighing 18–22 g were selected for the study procedures (n = 6). To induce a warm, hepatic I/R injury model, the mice were anesthetized by intraperitoneal injection of 4% chloral hydrate, 100 μl/10 g. The hepatic circulation to the left and middle liver lobes via the hepatic artery and hepatic portal vein was temporarily interrupted for 1 h, and then restored for 3 h.[16] The animals were sacrificed, and blood and liver samples were collected for further analysis.

Cell culture, transfection and H/R procedure

HepG2 human hepatoma cells were cultured in DMEM containing 10% fetal bovine serum in a 37°C incubator with 5% CO_2 and 95% air. HepG2 cells were stably transfected with either a pCDNA 3.0-Flag-*ALR* plasmid or a pCDNA 3.0 vector as a control. To induce hypoxia-reoxygenation (H/R) injury, the cells were incubated in serum- and glucose-free DMEM in a 37°C incubator with 1% O_2, 5% CO_2, and 94% N_2 for 6 h. Then culture medium was then replaced with normal DMEM and the cells were returned to a normoxic environment for an additional 2–6 h. During reoxygenation, 25 nmol/L rapamycin (Sigma Aldrich, St. Louis, MO, USA) was added to induce autophagy or 10 mmol/L bafilomycin A1 (Selleck Chemicals, Houston, TX, USA) was added to inhibit autophagy.

Statistical analysis

The results of multiple observations were reported as means ± standard deviation of at least three independent assays. The data were analyzed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Student's two-tailed *t*-test were used to compare between-group differences. Multiple group

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comparisons were performed by one-way analysis of variance followed by Bonferroni's post hoc test. *P*-values < 0.05 was considered statistically significant.

Results

Protection from liver I/R injury by *ALR* transfection in OLT-rats was associated with enhanced autophagy

The protective effect of ALR on liver I/R injury was tested in rats with OLT that were grouped and treated as described above. The livers of donor rats underwent cold-ischemia for 2 h before transplantation to the recipient rats. The liver allograft was removed for analysis 24 h later. A detailed description of rat OLT is provided in Supplemental Fig. 1 and the figure legend. The results showed that the levels of serum ALT and AST in the Ad-*ALR* rats were significantly reduced compared with those of rats given injections of normal saline or control vectors (Ad-Null, Fig 1A). Histological examination indicated that the structure of the hepatic lobules was preserved, and that cytoplasmic vacuolization and necrosis of parenchymal cells was significantly reduced in the livers of Ad-*ALR*-transfected rats, as shown by Suzuki histological scoring (Fig 1B).[17] In addition to the histology results, CD68- and MPO-positive cells, which are markers of recruited monocytes and neutrophils, were weakly stained in liver tissue from Ad-*ALR* rats compared with tissue from Ad-Null and normal saline groups after reperfusion for 24 h (Supplemental Fig. 2), and fewer TUNEL-positive cells were seen in the Ad-*ALR* group (Fig 1C). Hepatic ATP levels in Ad-*ALR* mice were increased, reflecting that the electron transfer activity required for mitochondrial oxidation-phosphorylation reactions was preserved (Fig 1D). MDA, a byproduct of lipid peroxidation, was decreased in the Ad-*ALR* group (Fig 1E). The GSH/GSSG ratio, reflecting hepatic antioxidant capacity, was well preserved in Ad-*ALR* group (Fig 1F). Injury of hepatic sinusoid endothelial cells is characteristic of cold I/R injury following liver transplantation. The sinusoidal endothelium and Disse's space could been seen in the sham-operated group, but damage of the sinusoidal endothelium was visible in the I/R-group rats. In contrast, in the Ad-*ALR* group, the sinusoidal endothelial lining was well preserved (Fig 1G). The coincidence of I/R-related damage of the sinusoidal epithelium injury and autophagy was assessed by TEM. As shown in Fig 1I, the number of autolysosomes was significantly reduced in liver tissue from rats in the normal saline group and in Ad-Null rats. In contrast, number of hepatic autophagosomes in liver tissue

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from *ALR*-transfected rats was increased. After the expression of Ad-*ALR* in rat liver tissue was verified in Western blots (Fig. 1H, left panel), the effects of *ALR* transfection on autophagy were determined. The right panel of Fig. 1H indicates that LC3-II/I, an autophagy-associated protein, was significantly upregulated and that p62 expression was decreased in liver allografts from Ad-*ALR* rats.

ALR promoted autophagic flux in cellular H/R injury

The involvement of autophagy was investigated in an *in-vitro* cell model of H/R injury. Cells were cultured in hypoxic conditions for 6 h with reoxygenation for 2, 4, 6 h. At 2 h, autophagy was significantly inhibited in vector-transfected (Tx) cells compared with control cells, as indicated by the reduced expression of LC3-II/I, Beclin1 and increased expression of p62 (Fig 2A). Autophagy activity increased with prolongation of reoxygenation to 4–6 h. The optical densities of autophagy-related proteins LC3-II/I, p62, and Beclin 1 were assayed by Image J at 2–6 h after reoxygenation. Fig. 2B shows that LC3II/I and Beclin 1 expression increased in *ALR*-Tx cells compared with vector-Tx cells and the expression of p62 decreased. The differences shown in Fig. 2B are significant, confirming that *ALR*-Tx promoted autophagy. ALR activation of the dynamic flux of autophagy is shown in Supplemental Fig. 3 by an increase in the number of green LC3 puncta aggregates after 6H/6R injury (Supplemental Fig 3A, B). Promotion of the autophagy index was strengthened by bafilomycin A1 (Bafi A1), an inhibitor that inactivates H^+-ATPase. After Bafi A1 treatment, expression of LC3-II/I was elevated in *ALR*-Tx cells compared with vector-Tx cells (Supplemental Fig 3C, D). The p62 level was also elevated but without statistical difference.

ALR-enhanced mitophagy via promoting Parkin recruitment to mitochondria in H/R injury

Mitophagy contributes to mitochondrial homeostasis because it selectively removes dysfunctional or damaged mitochondria in H/R injury. As shown in Fig 2C-D, the expression of the mitophagy markers mito-LC3-II and p62 was elevated in H/R injury, and relatively more elevated in *ALR*-Tx compared with vector-Tx cells. The immunofluorescence assay of mitophagy found significantly greater co-localization of GFP-LC3 with mitochondria after H/R injury in *ALR*-Tx cells than in vector-Tx cells as shown by merged fluorescence signals (Fig 2E, G). To determine whether degraded mitochondria were engulfed by lysosomes, mitochondria were labeled by TOM20 and lysosomes by lysotracker. As shown in Fig. 2F and

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2H, yellow dots, indicative of merged fluorescence of the two organelles, were significantly more abundant in *ALR*-Tx than in vector-Tx cells. Recent studies have described the PINK1/Parkin interaction as a key mediator of mitophagy in mammalian cells. In isolated mitochondria, PINK1 expression and Parkin recruitment were significantly increased in *ALR*-Tx compared with vector-Tx cells (Fig 2C, D). As shown in Fig. 2I, merged mitochondrial and Parkin fluorescence, indicating Parkin translocation to mitochondria, was increased in *ALR*-Tx cells after H/R compared with vector-Tx cells after H/R. The data indicate that mitophagy was significantly activated in *ALR*-transfected cells following H/R, inducing Parkin translocation to mitochondria.

ALR-enhanced mitophagy preserved mitochondrial function and protected cells from H/R injury

Hepatic I/R injury is characterized by mitochondrial dysfunction and cell death. To investigate whether ALR-enhanced mitophagy protected against liver I/R injury, vector-Tx cells were treated with rapamycin, an autophagy activator,[18] and *ALR*-Tx cells were treated with Bafi A1, a mitophagy inhibitor.[19] After H/R treatment, the viability and the ATP content of vector-Tx cells were both decreased (Fig 3A, B), ROS production was increased (Fig 3C), and MMP value was decreased (Fig 3D). As result, the number of apoptotic cells was significantly increased (Fig. 3E). The presence of rapamycin during reoxygenation not only increased the viability and the ATP content of vector-Tx cells, but it also maintained MMP value. In contrast, the viability and ATP content of *ALR*-Tx cells were maintained during H/R treatment, and both significantly decreased in the presence of Bafi A1. Similarly, the mitochondrial parameters and cell apoptosis deteriorated further with addition of Bafi A1. The results indicate that the ALR protected hepatocytes against H/R injury via enhanced mitophagy and its protective activity could be abolished by an inhibitor of mitophagy such as Bafi A1.

ALR-enhanced mitophagy was closely linked to *Mfn*2 expression

Mitophagy is closely related to mitochondrial fission and fusion. It is known that ALR inhibits mitochondrial fission in hepatic I/R injury by restricting Drp1 activation and mitochondrial translocation. We analyzed changes of Mfn2/1 and optic atrophy 1 (Opa1) and the regulatory role of ALR. As shown in Fig 4A-C, both Mfn2 mRNA and protein expression were significantly increased in *ALR*-Tx compared with

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vector-Tx cells under H/R conditions. The changes of Mfn1and Opa1expression were not statically significant (Supplemental Fig 4). We then focused on Mfn2. Previous studies indicated that Mfn2 is a substrate of E3 ubiquitin ligase Parkin, which regulates mitophagy by promoting Parkin translocation and phosphorylation.[20] We speculated whether Mfn2 was involved in mitophagy activation in the presence of *ALR*. siRNA was used to knockdown *Mfn*2 in HepG2 cells. Fig. 4D shows that the inhibition caused by si2-*Mfn*2 and si3-*Mfn*2 was more effective than that caused by the other si-*Mfn2s*, hence those two fragments were combined and used for *Mfn*2 knockdown. The mitochondria were isolated to analyze Parkin-mediated mitophagy. As shown in Fig. 4E, F, expression of the mitophagy markers LC3-II/I, Beclin 1, PINK1, Parkin, and Mfn2 was enhanced in *ALR*-Tx compared with vector-Tx cells after H/R. However, *Mfn*2 knockdown in *ALR*-Tx cells decreased the expression of those markers. Mitophagy, as shown by yellow dots of fluorescence overlay, was greatly increased in *ALR*-Tx cells after H/R. The overlay decreased in *ALR*-Tx cells after *Mfn*2 knockdown (Fig. 4G). The difference in the number of mitochondria engulfed by lysosomes was statistically significant (Fig 4H).

We attempted to rescue mitophagy by transfecting *Mfn*2 into *ALR*-siRNA cells. siRNA knockdown of *ALR* decreased mitophagy after H/R compared with scramble-cells negative controls (NC), with decreased expression of LC3-II/I, PINK1, and Mfn2 (Fig 5A). *Mfn*2-Tx rescued mitophagy activity as shown by the increased expression of LC3-II/I and PINK1 compared with *ALR*-siRNA control cells without *Mfn*2-Tx (Fig. 5B). Immunofluorescence showed a significant increase in mitochondria and lysosome overlay in *ALR*-siRNA cells after *Mfn*2-Tx (Fig. 5C, D). The co-localization of Parkin with mitochondria was significantly increased in NCs compared with *ALR*-siRNA cells. *Mfn*2 knockdown inhibited Parkin accumulation into mitochondria after H/R, but that was reversed after *Mfn*2-Tx (Fig. 5E), indicating that *Mfn*2 regulation by ALR was actually involved in Parkin-mediated mitophagy during the H/R process.

_Mfn_2 partially rescued mitochondrial function in the absence of ALR during in-vitro I/R injury

As shown above, *Mfn*2 was essential for *ALR*-enhanced mitophagy during H/R-injury. We speculated whether mitochondrial dysfunction would proceed if *Mfn*2 expression were inhibited, and performed *Mfn2*-knockdown in-vitro. As shown in Fig. 6, *ALR*-associated protection against H/R injury was reversed when *Mfn*2 was knocked down, as indicated by decreased cell survival, enhanced cell apoptosis, and lactate

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dehydrogenase (LDH) leakage (Fig. 6A–C). Simultaneously, reduction of ATP content, increased ROS production, and reduce MMP indicated significant impairment of mitochondrial membrane permeability (Fig. 6D–F). It is reasonable that the contribution of *Mfn*2 to mitophagy would be maintained despite *ALR* knockdown. Fig. 6 shows that cell survival, apoptosis, ATP production, and ROS generation appeared to be worse with siRNA-*ALR* treatment compared than in the NC cells and that *Mfn*2 overexpression in the *ALR*-siRNA cells partially rescued mitochondrial function and the effect on apoptosis caused by *ALR* deletion (Fig. 6A–F).

Aggravation of liver injury in heterozygous *ALR*-KO mice was associated with reduced mitophagy in a warm hepatic I/R injury

After confirming the beneficial effect of *Mfn*2 overexpression on I/R injury in-vitro, we used heterozygous *ALR*-KO *Gfer*$^{+/-}$ mice to validate *Mfn2* protection against warm hepatic I/R injury. After 1 h of ischemia followed by 3 h of reperfusion in mice, mild lobular edema, sinusoidal congestion, focal hepatocellular necrosis and inflammatory cell infiltration appeared in liver tissue from WT animals (Fig. 7A). In contrast, liver tissue from *ALR*-KO mice had more severe lobular edema, widespread sinusoidal congestion, and extensive hepatocellular necrosis and inflammatory cell infiltration. The severity of liver injury was confirmed by analysis of the Suzuki scores (Fig. 7C). Serum ALT and AST were significantly increased in the *ALR*-KO compared to WT mice after I/R injury (Fig. 7B). Compared with WT mice, apoptosis and oxidative stress were significantly increased and ATP content was significantly reduced, in *ALR*-KO mice after I/R injury (Fig. 7D, E, F). The western blot results indicated a decrease in mitophagy because the LC3-II/I ratio decreased and p62 expression increased (Fig. 7G). PINK1 accumulation in mitochondria was increased in WT mice, and the decrease of PINK1 expression in *ALR*-KO mice reflected a decrease in mitophagy activity (Fig. 7H). The results of immunohistochemical staining were consistent with those obtained by western blotting (Fig. 7I, Supplemental Fig. 5A). The TEM results found that autophagosome formation during I/R injury was significantly increased in WT compared with *ALR*-KO mice (Fig. 7J, Supplemental Fig. 5B). Although hepatic *Mfn*2 expression was significantly reduced in WT mice during I/R, the decrease in the livers of *ALR*-KO mice was significantly greater than that seen in the WT mice (Fig. 7H, I).

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Discussion

Previous studies have found that ALR protected hepatocytes from apoptosis during hepatic I/R, but the mechanism is not known. Autophagy is a cellular process beneficial to cellular survival and homeostasis, and numerous investigations have provided evidence that hepatic autophagy, especially mitophagy, is cytoprotective against I/R injury.[21,22] By selectively removing dysfunctional or damaged mitochondria, mitophagy preserves mitochondrial morphology and function. We recently reported that ALR-enhanced mitophagy in ethanol-damaged hepatocytes.[12] Whether ALR can protect liver against I/R injury in OLT remains unpublished. This study investigated the role and regulatory mechanism of ALR in the promotion of mitophagy in hepatic I/R injury. We found that ALR protected hepatocytes against liver cold I/R injury in rat model, and against liver warm I/R injury in a mouse model. The results are supportive and robust. Here, in our OLT model, the hepatic artery (HA) was ligated without arterial reconstruction, although the hepatic artery supplies 20%-30% blood and more than 50% O_2 to the liver. Our OLT methods were derived from the Kamada Technique, a standard surgical method for rat OLT, in which the arterial reconstruction is believed unnecessary. While the reconstruction of the HA is considered an important step in liver transplantation in humans and large animals. However, in rats, the OLT recipient can tolerate the operation without arterialization.[23] Subsequently, we found that the hepatic protection during liver transplantation is associated with activation of mitophagy via enhanced Parkin mitochondrial translocation and inhibition of cell apoptosis. Mechanistically, *ALR*-enhanced mitophagy of I/R injured hepatocytes is probably coordinated with *Mfn*2 expression. Up- or downregulation of *Mfn*2 controlled *ALR*-induced mitophagy and influenced mitochondrial functionality. To our knowledge, this is the first investigation to explore the effects of ALR on mitophagy in hepatic I/R injury. Moreover, Mfn2, a mitochondrial fusion protein, is thought to be a key effector responsible for *ALR*-induced mitophagy. ALR is a liver growth-promoting factor and protects against I/R injury, which makes it an ideal candidate for liver transplantation, especially in some specific surgical settings such as marginal liver donors (steatosis liver, old liver graft), or as an additive component in organ preservation solutions. However, continuing investigation is absolutely required.

Currently, a mitophagy mechanism driven by a PINK1 and Parkin interaction is widely accepted and is

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thought to be active during I/R injury. During mitochondrial depolarization, PINK1is stabilized on the OMM, leading to Parkin phosphorylation and recruitment to damaged mitochondria. Parkin is an evolutionarily conserved E3 ubiquitin ligase that promotes substrate ubiquitination and autophagosome formation. Activation of Parkin-mediated mitophagy has been shown to improve I/R outcomes. In aging mice, aggravated hepatic I/R injury has been associated with decreased Parkin expression and mitophagy inhibition, and increased mitophagy has been shown to result in significant protection against hepatic I/R injury. In human hepatic biopsy specimens, Parkin expression is negatively correlated with donor age and peak AST in the first week after liver transplantation,[24] suggesting that PINK1/Parkin-mediated mitophagy is likely to be a protective mechanism in I/R injury. Our results indicate that the recruitment of PINK1 and Parkin in mitochondria was enhanced in *ALR*-Tx cells after H/R and was coordinated with increased numbers mitophagy (i.e. lysosome engulfment). Rapamycin induced mitophagy and simulated the protective effect *ALR* against mitochondrial dysfunction and hepatocyte apoptosis during H/R injury, implying *ALR* function might be associated with Parkin-mediated mitophagy.

Mfn2 is located on the OMM and assists mitochondrial fusion and may also be involved in mitochondrial integrity, mitophagy/autophagy, and apoptosis.[25] The involvement of Mfn2 in might be related to its location at the mitochondrion-ER contact site, which is thought to be a source of autophagosome membranes during elongation.[26] Absence of mitochondria-ER interface proteins, such as Mfn2, prevents autophagosome formation in response to stimuli like starvation.[27] Alternatively, pathologically low levels of Mfn2 may adversely affect the delivery of autophagosomes to lysosomes, as Mfn2 may be involved in the fusion of autophagosome and lysosome through its interaction with the Ras-related protein Rab7.[28] A recent publication by Chen and Dorn described the role of Mfn2 in regulating mitophagy. When phosphorylated by PINK, Mfn2 enhanced Parkin recruitment by damaged mitochondria. Phosphorylated Mfn2 was a receptor for cytosolic Parkin. Once bound to depolarized mitochondria, Parkin provoked ubiquitination of mitochondrial proteins that target the organelle for autophagic elimination.[20] Ablation of Mfn2 in mouse cardiomyocytes prevented depolarization-induced Parkin translocation and suppressed mitophagy.[20] Morphologically and functionally abnormal mitochondria accumulated in Mfn2-deficient mouse embryonic fibroblasts and cardiomyocytes and induced respiratory dysfunction that led to dilated cardiomyopathy. Normally, aging skeletal muscle undergoes Mfn2 repression that cause

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impaired mitophagy, leads to the accumulation of dysfunctional mitochondria, and contributes to age-associated metabolic alterations and sarcopenia.[29] It has been reported that Parkin regulated mitochondria-ER interaction via ubiquitination of Mfn2 on lysine K416, which was a prerequisite for mitochondrial-ER physical and functional interaction. This also implied a parallel pathway in which Parkin-dependent ubiquitination of Mfn2 might promote mitophagy.[30]

The study results showed that the Mfn2 expression was reduced in H/R injury along with compromised Parkin expression and mitophagy. *ALR* transfection upregulated Mfn2 expression, enhanced Parkin translocation and mitophagy activation. To elucidate the interactive role of Mfn2 with ALR in promoting mitophagy, Mfn2 was deleted in *ALR*-Tx cells. It was surprisingly found that inhibition of Mfn2 expression suppress mitophagy independent of *ALR*, and that the effect was likely related to the accumulation of PINK1 and Parkin recruitment on mitochondria. As a result, the protection against to I/R injury by ALR was diminished. Likely, overexpression of Mfn2 in *ALR*-siRNA cells reversed mitophagy activation by promoting Parkin translocation to the mitochondria, preventing mitochondrial damage and cell injury to partially restore the protective effect of ALR. The evidence indicates that ALR translocated Parkin to depolarized mitochondria and subsequently triggered mitophagy by interacting with Mfn2.

The study has not unraveled the molecular mechanism by which *ALR* regulates *Mfn*2 expression in hepatic I/R injury. Our previous study found that *ALR* affected the expression of PGC-1α (peroxisome proliferator activated receptor γ coactivator-1α).[15] Whether that effect can be entirely considered as an *ALR*-PCG-1α-*Mfn*2 signaling cascade for regulating mitophagy during hepatic I/R injury,[31] requires additional study. Investigation of the *ALR*-promotion of mitophagy to protect hepatocytes against I/R injury is ongoing. In conclusion, mitophagy was activated by ALR via Mfn2 by promoting Parkin translocation to mitochondria, thus preventing mitochondrial dysfunction and hepatic IR injury. By identifying the Mfn2-medated activation of mitophagy by ALR, our findings provide a rationale for novel therapeutic measures to counteract I/R-triggered liver injury.

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ORCID

Wei An: 0000-0002-9648-4497

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Figure legends

Fig. 1. Protection from liver I/R injury by *ALR* transfection in OLT-rats was associated with enhanced autophagy. Donor rats were given equal volume of normal saline (N.S), Ad-vector (Ad-Null), Ad-*ALR* (3×10^9 pfu/100μl) 5 d before surgery. The donor livers were harvested and stored in Lactated-Ringer's solution at 4°C for ischemia of 2 h and were orthotopically transplanted into recipient rats (n=6) using a modified two-cuff method. Twenty-four hours after liver transplantation, rats were sacrificed and the liver graft tissue and blood were collected for analysis. A: Serum ALT and AST levels. B: Representative images of liver tissues stained with hematoxylin and eosin, liver damage and inflammation were evaluated by Suzuki histological score. C: Representative images and quantification of TUNEL staining of liver tissues. D: Hepatic malondialdehyde (MDA) levels. E: Hepatic ATP levels. F: Hepatic GSH/GSSH levels. G: Evaluation of hepatic sinusoid using Electronic microscope. Although the sham-operated livers showed an intact sinusoidal structure, the sinusoidal endothelium linings were destroyed and detached into the sinusoidal space in the Ad-Null group after the cold I/R Injury (arrows). In contrast, the sinusoidal endothelial lining was well preserved in the Ad-*ALR* group. E: endothelial cell; S: hepatic sinusoid. TEM magnification ×8000, ×15000 respectively. H: After the expression of Ad-*ALR* in rat liver tissue was verified in Western blot using both anti-Flag and anti-ALR antibody (left panel), the effects of *ALR* transfection on autophagy were determined by Western blot assay from the indicated groups (right panel). I: Representative TEM images and quantification of autophagosomes in hepatocytes. Number of autophagosomes (black arrow) was significantly reduced in liver tissue from rats in the normal saline and in Ad-Null group post-OLT. In contrast, number of hepatic autophagosomes in liver tissue from *ALR*-transfected rats was increased. TEM magnification ×1000, ×3000, ×6000 respectively. Data are expressed as means±SD. Ad, adenoviral; ALR, augmenter of liver regeneration; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; GSH, reduced glutathione; GSSG, oxidized glutathione; H&E, hematoxylin and eosin; I/R, ischemia/reperfusion; OLT, orthotopic liver transplantation; TEM, transmission electron microscopy; *$P < 0.05$, **$P < 0.01$.

Fig. 2. ALR promoted autophagy and Parkin-mediated mitophagy in cellular H/R injury. HepG2 cell was subjected to 6 h hypoxia followed by different times of reoxygenation (2, 4, 6 h). A: Western blot analysis

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of autophagy related protein LC3-II/I, p62, Beclin 1. At 2 h of reoxygenation, autophagy was significantly inhibited in vector-Tx cells compared with control cells. Autophagy activity increased with prolongation of reoxygenation to 4–6 h in both vector-Tx cells and *ALR*-Tx cells. B: The optical densities of autophagy-related protein LC3-II/I, p62 and Beclin 1 were assayed by Image J at 2–6 h after reoxygenation. Vector-Tx cells without H/R were used as controls. The expression of LC3II/I and Beclin 1 increased in *ALR*-Tx cells compared with vector-Tx cells and the expression of p62 decreased. C: *ALR*-Tx and Vector-Tx cells underwent H/R (6h/6h) before mitochondria were extracted and Western Blot was used to analyze the proteins related to mitophagy. D: Quantitative analysis of the relative expressions of mito-LC3-II, p62, PINK1and Parkin. Increased expression of mito-LC3-II, PINK1, Parkin and reduced expression of p62 were observed in *ALR*-Tx cells compared with vector-Tx cells after H/R. E, G: The immunofluorescence assay and quantitative analysis of mitochondria and LC3, the yellow fluorescence is indicative of mitophagy. F, H: The immunofluorescence assay and quantitative analysis of mitochondria and lysosome. The yellow fluorescence is indicative of mitophagy. I: The immunofluorescence assay of Parkin and mitochondria. Data are expressed as means ± SD. H/R, hypoxia/reoxygenation; LC3, microtubule-associated protein 1 light chain 3; PINK1, PTEN-induced putative kinase 1; TOM20, translocase of outer mitochondrial membrane 20; *P < 0.05, **P < 0.01.

Fig. 3. ALR-enhanced mitophagy preserved mitochondrial function and protected cells from H/R injury. Vector-Tx cells and *ALR*-Tx cells underwent H/R (6h/6h) with or without mitophagy activator and inhibitor. Rapamycin (50 μM) was added in vector-Tx cells and bafilomycin A1 (10 nM) was added in *ALR*-Tx cells during the reoxygenation. A: Cellular viability was detected by CCK8. B: ATP contents. C: ROS level was analyzed by flow cytometry. D: Alteration of mitochondrial membrane potential as evaluated by JC-1 staining. E: TUNEL assay for cellular apoptosis. Data are expressed as means±SD. ATP, adenosine triphosphate; H/R, hypoxia/ reoxygenation; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; *P < 0.05, **P < 0.01.

Fig. 4. ALR-enhanced mitophagy was closely linked to *Mfn*2 expression. A: *ALR*-Tx and vector-Tx cells underwent H/R (6h/6h) before the expression of mitochondria fusion protein was detected by Western Blot.

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B: Quantitative analysis of the relative expression of Mfn2. C: mRNA expression of Mfn2. D: Four *Mfn*2-siRNA fragments (si1-*Mfn*2 to si4-*Mfn*2) were used to knockdown *Mfn*2 expression and western blot analysis was used to confirm the efficiency of Mfn2 knockdown. As the inhibition caused by si2-*Mfn*2 and si3-*Mfn*2 was more effective than that caused by the other si-*Mfn2*s, those two fragments were combined and used for *Mfn*2 knockdown in the following experiments. *ALR*-Tx cells were transfected with control siRNA or *Mfn2*-siRNA before subjected to H/R. E: Western Blot was used to analyze the proteins related to mitophagy. F: Quantitative analysis of the relative levels of mitophagy related proteins. *Mfn*2 knockdown in *ALR*-Tx reversed the enhanced expression of the mitophagy markers LC3-II/I, Beclin 1, PINK1, Parkin in *ALR*-Tx cells. G: The immunofluorescence assay of mitochondria and lysosome. The fluorescence overlay (yellow dots, arrowheads) between mitochondria and lysosomes increased in *ALR*-Tx cells as compared with vector-Tx cells after H/R, while the overlay decreased in *ALR*-Tx cells after *Mfn2* knockdown. H. Quantitative analysis of mitophagy. Data are expressed as means±SD. H/R, hypoxia/ reoxygenation; LAMP1, lysosome-associated membrane glycoprotein 1; Mfn1, Mitofusin 1; Mfn2, Mitofusin 2; Opa1, optic atrophy 1; TOM20, translocase of outer mitochondrial membrane 20; *P < 0.05, **P < 0.01.

Fig. 5. Mfn2 administration in si*ALR* cells rescued mitophagy activity. si*ALR* cells were transfected with control plasmid or pcDNA-*Mfn2* plasmid before subjected to H/R. A: Western blot was used to analyze the mitophagy related proteins. B: Quantitative analysis of the relative levels of Mfn2, LC3-II/I, Tom20 and PINK1. *Mfn*2-Tx rescued mitophagy activity as shown by the increased expression of LC3-II/I, PINK1 and reduced expression of Tom20 compared with si*ALR* cells. C: The immunofluorescence assay of mitochondria and lysosome. The yellow dots were indicative of mitophagy. D: Quantitative analysis of the mitophagy. E: The immunofluorescence assay of Parkin and mitochondria. Data are expressed as means± SD. H/R, hypoxia/ reoxygenation; Mfn2, Mitofusin 2; LAMP1, lysosome-associated membrane glycoprotein 1; PINK1, PTEN-induced putative kinase 1; Tom20, translocase of outer mitochondrial membrane 20; *P < 0.05, **P < 0.01.

Fig. 6. *Mfn*2 partially rescued mitochondrial function in the absence of ALR during in-vitro I/R injury. A:

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Cell viability was detected by CCK8. B: LDH levels in supernatant. C: TUNEL assay for cellular apoptosis. D: ATP relative levels. E: ROS relative levels were analyzed. F. The mitochondrial membrane potential was detected via JC-1 staining. Data are expressed as means ± SD. ATP, adenosine triphosphate; LDH, lactate dehydrogenase; Mfn2, Mitofusin 2; ROS, reactive oxygen species; *P < 0.05, **P < 0.01.

Fig. 7. Aggravation of liver injury in heterozygous *ALR*-KO mice was associated with reduced mitophagy in a warm hepatic IR injury. WT mice (*Gfer*$^{+/+}$) and heterozygous *ALR*-KO (*Gfer*$^{+/-}$) mice were given an ischemia of 1h followed by 3h of reperfusion (n = 6). A: Representative images of liver tissues stained with H&E, TUNEL, DHE respectively. B: Serum ALT and AST levels. C: Liver damage was evaluated by Suzuki score. D: Quantification of TUNEL staining of liver tissues. E: Quantification of ROS levels by DHE staining. F: Hepatic ATP levels. G: The expression of mitophagy related proteins were determined by western blot from the indicated groups. H: Quantitative analysis of the relative levels of Mfn2, LC3-II/I, p62 and PINK1. The decreased expression of LC3-II/I, PINK1, Mfn2 and increased expression of p62 were observed in *ALR*-KO mice compared with WT mice after I/R. I: Representative images of immunohistochemistry staining of mitophagy related proteins, and quantitative analysis of mitophagy related proteins staining was shown in Supplemental Figure 5A. J: Representative TEM images of autophagosomes in hepatocytes, and quantification analysis of autophagosomes was shown in Supplemental Figure 5B. Numbers of autophagosomes (arrows) were significantly increased in liver tissue from WT mice compared with *ALR*-KO mice after I/R injury. Data are expressed as means±SD. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; DHE, dihydroethidium; H&E, hematoxylin and eosin; KO, knockout; Mfn2, Mitofusin 2; ROS, reactive oxygen species; TEM, transmission electron microscopy; WT, wild-type; *P < 0.05, **P < 0.01.

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Supporting Information

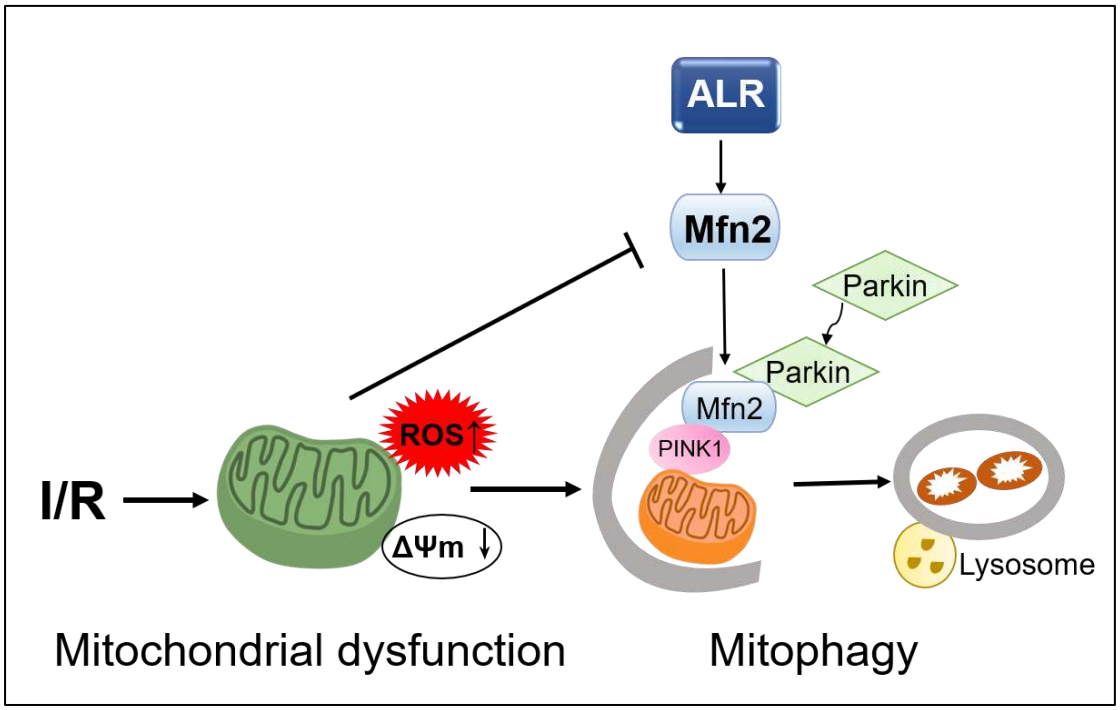
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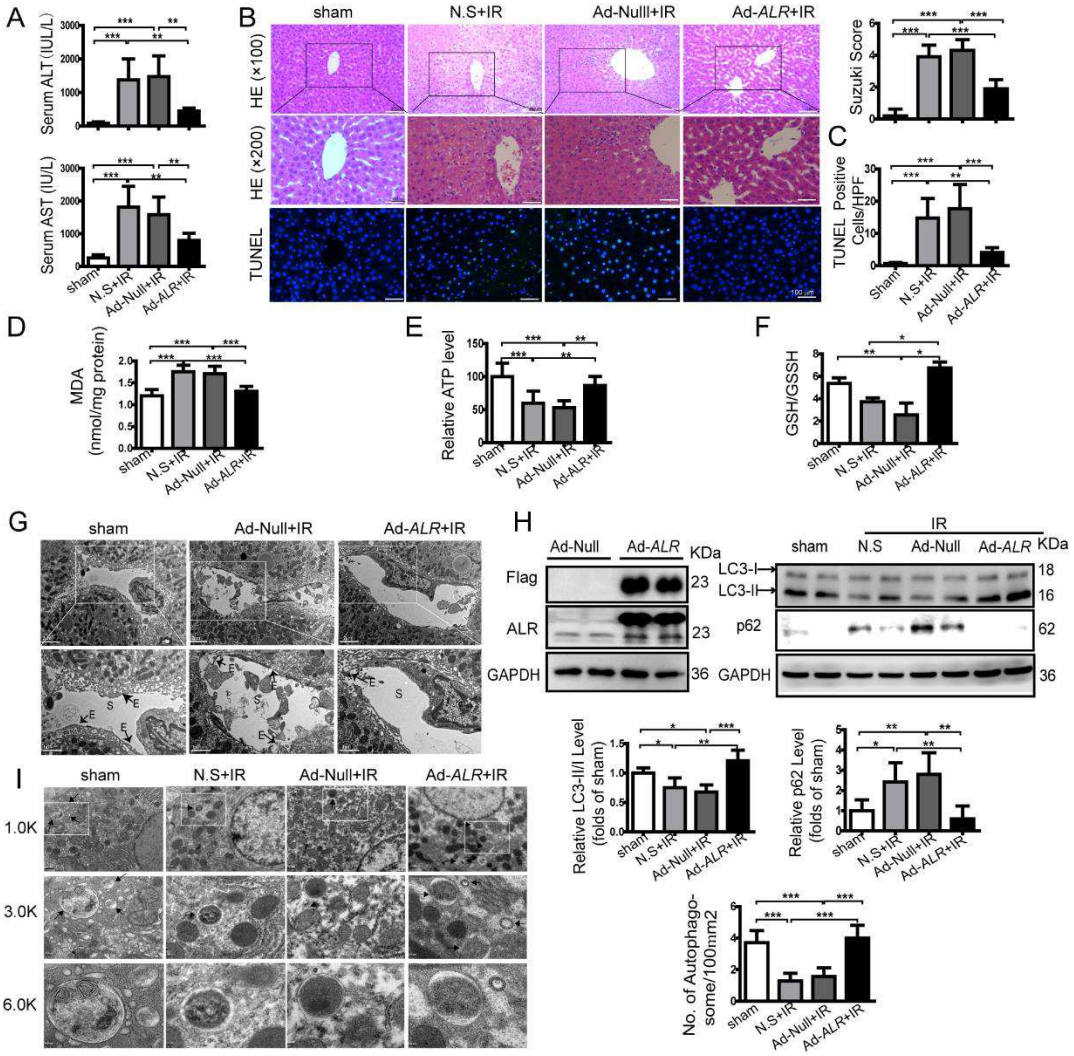
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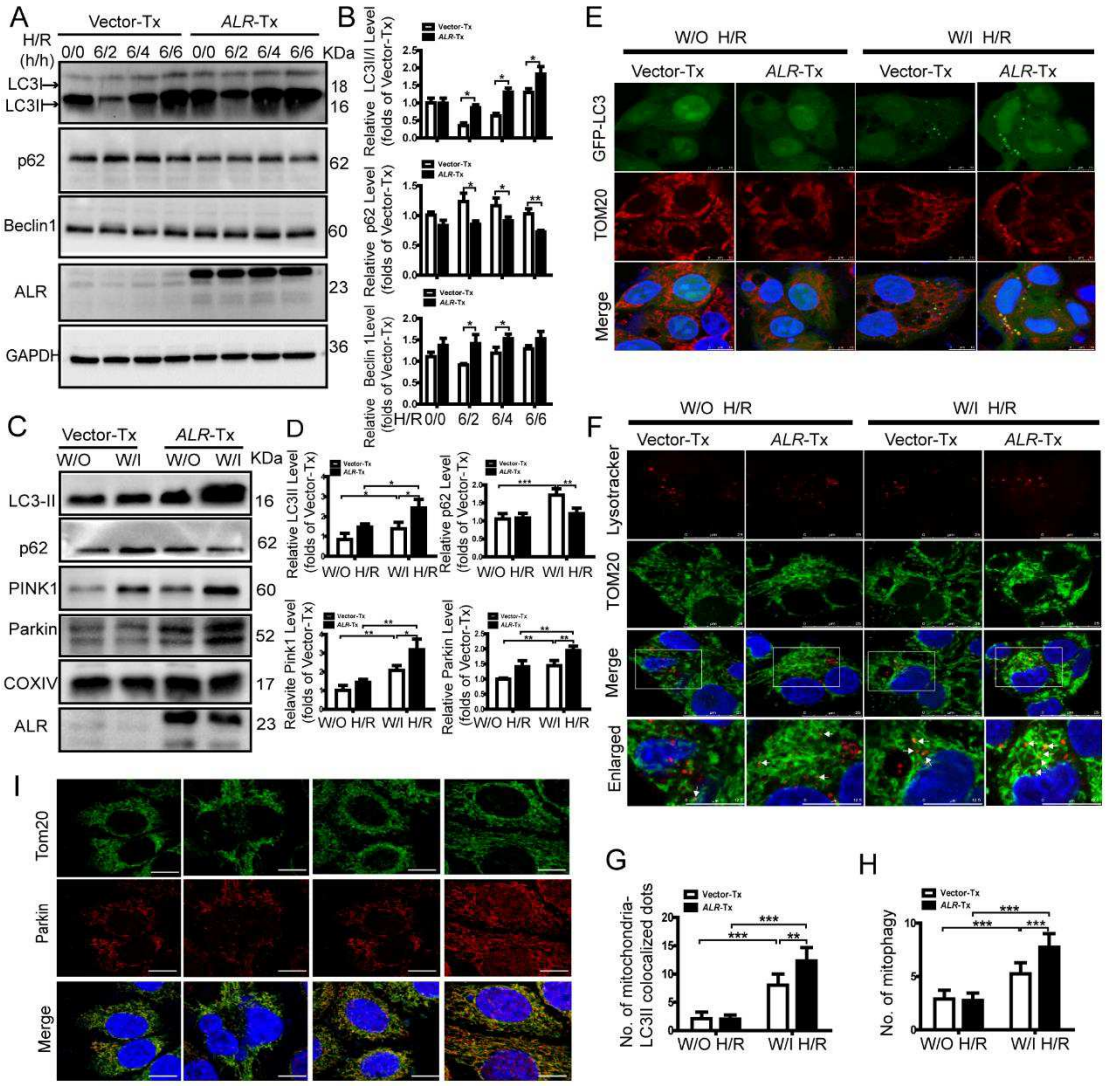
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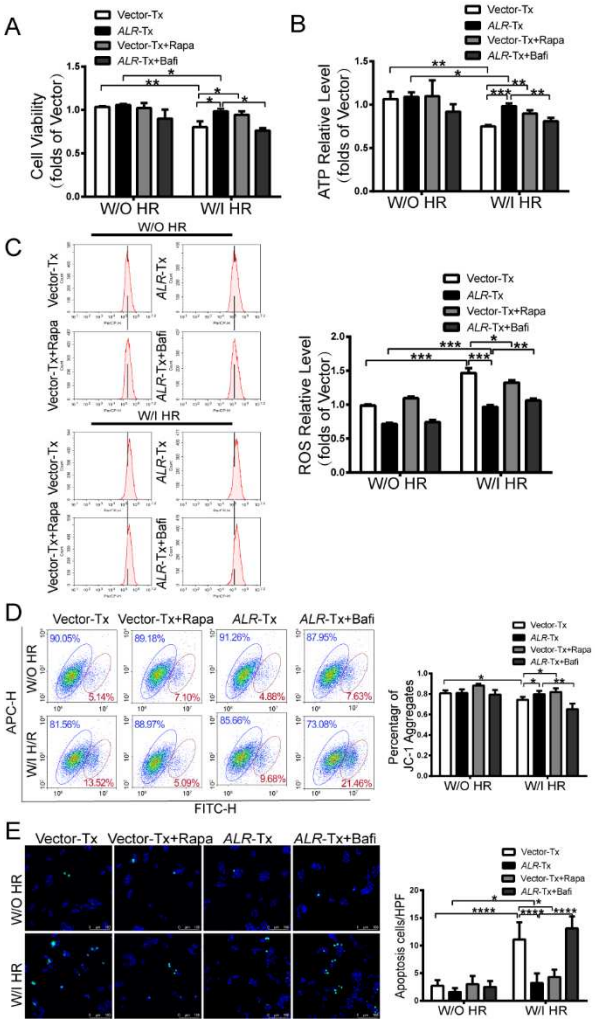
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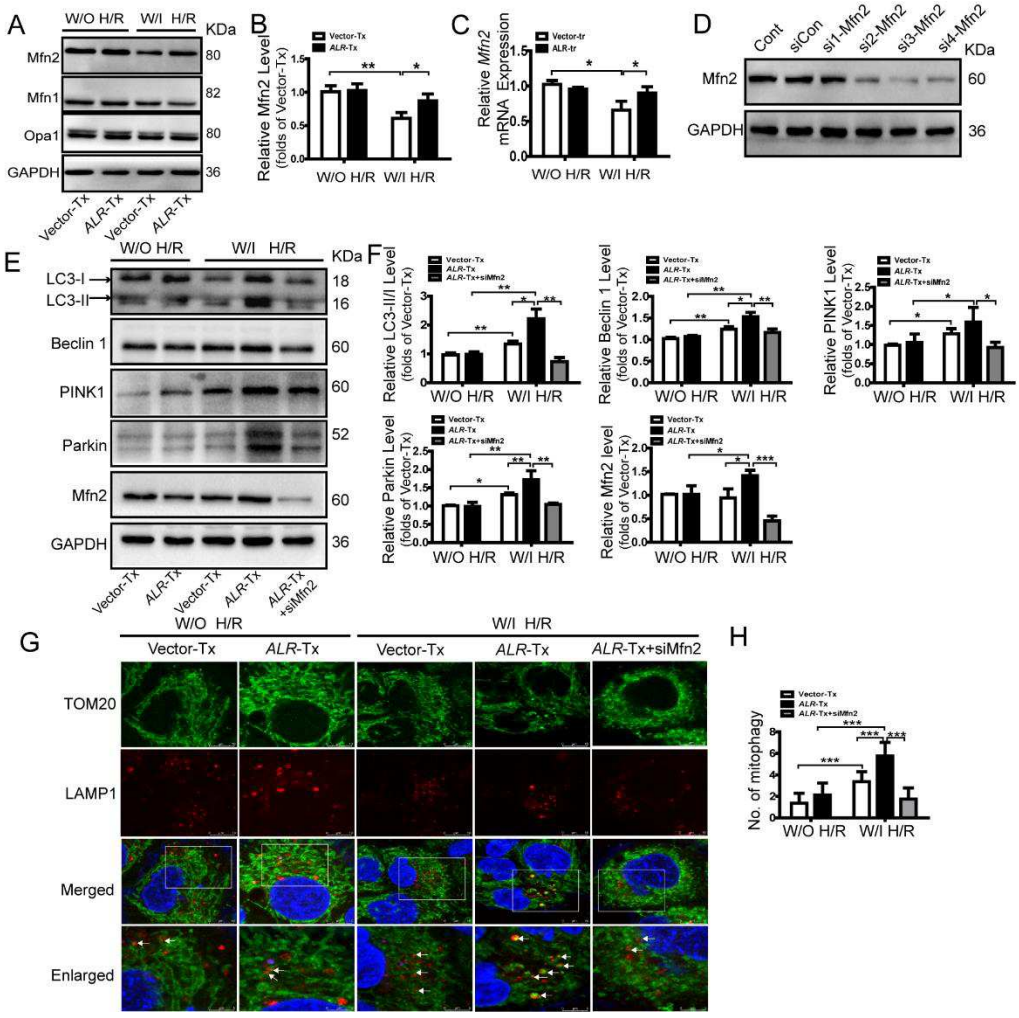
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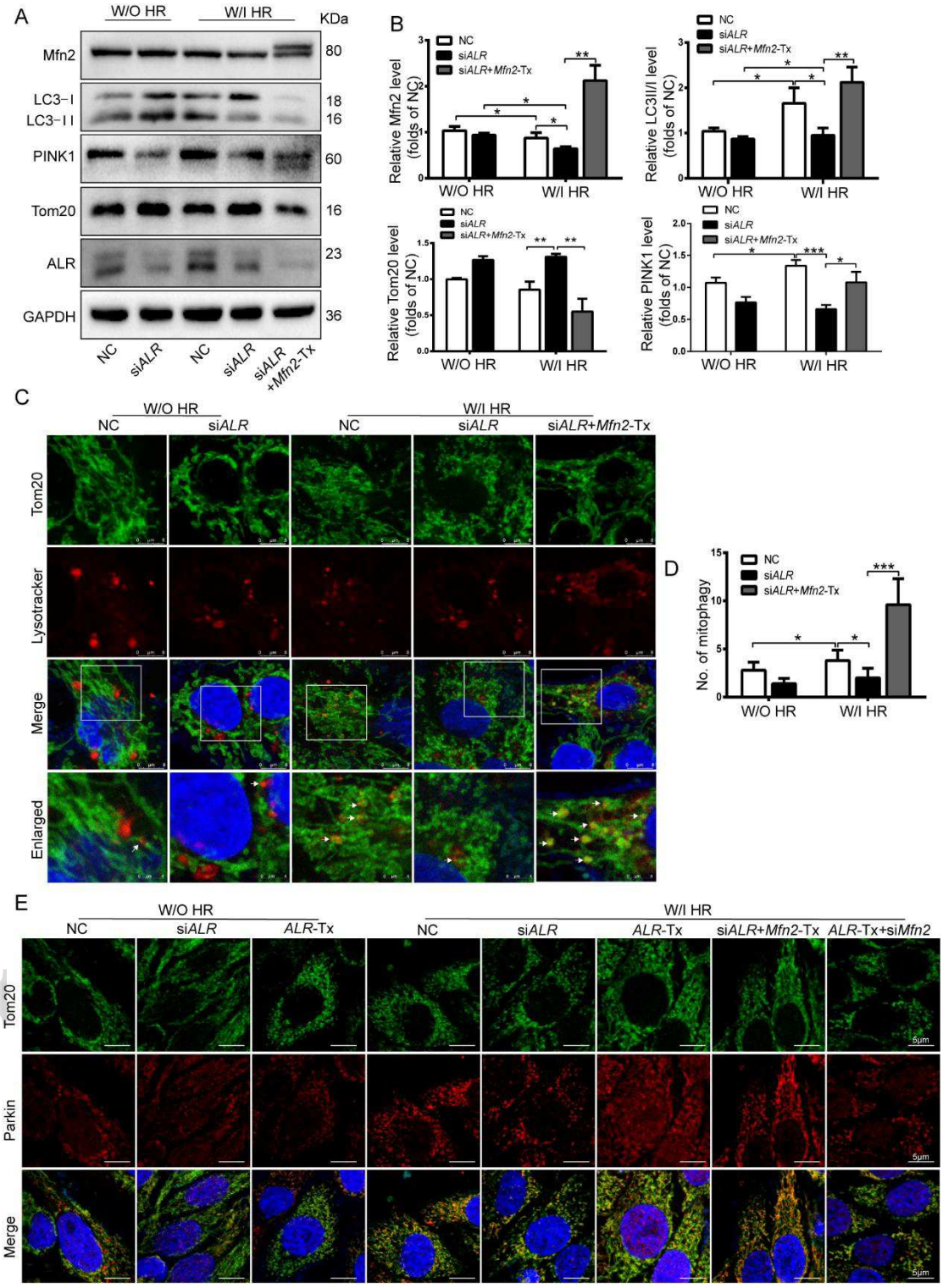
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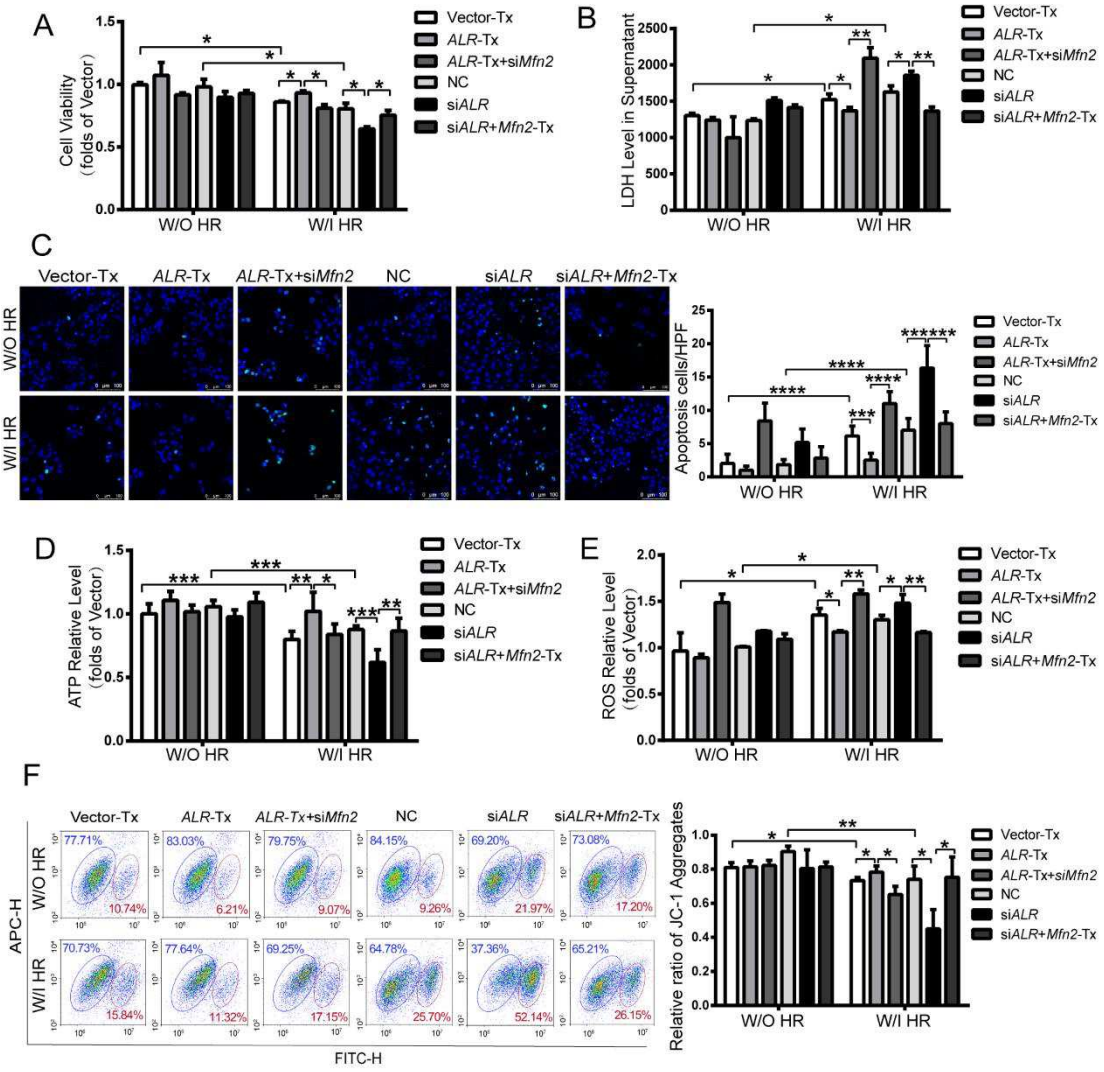
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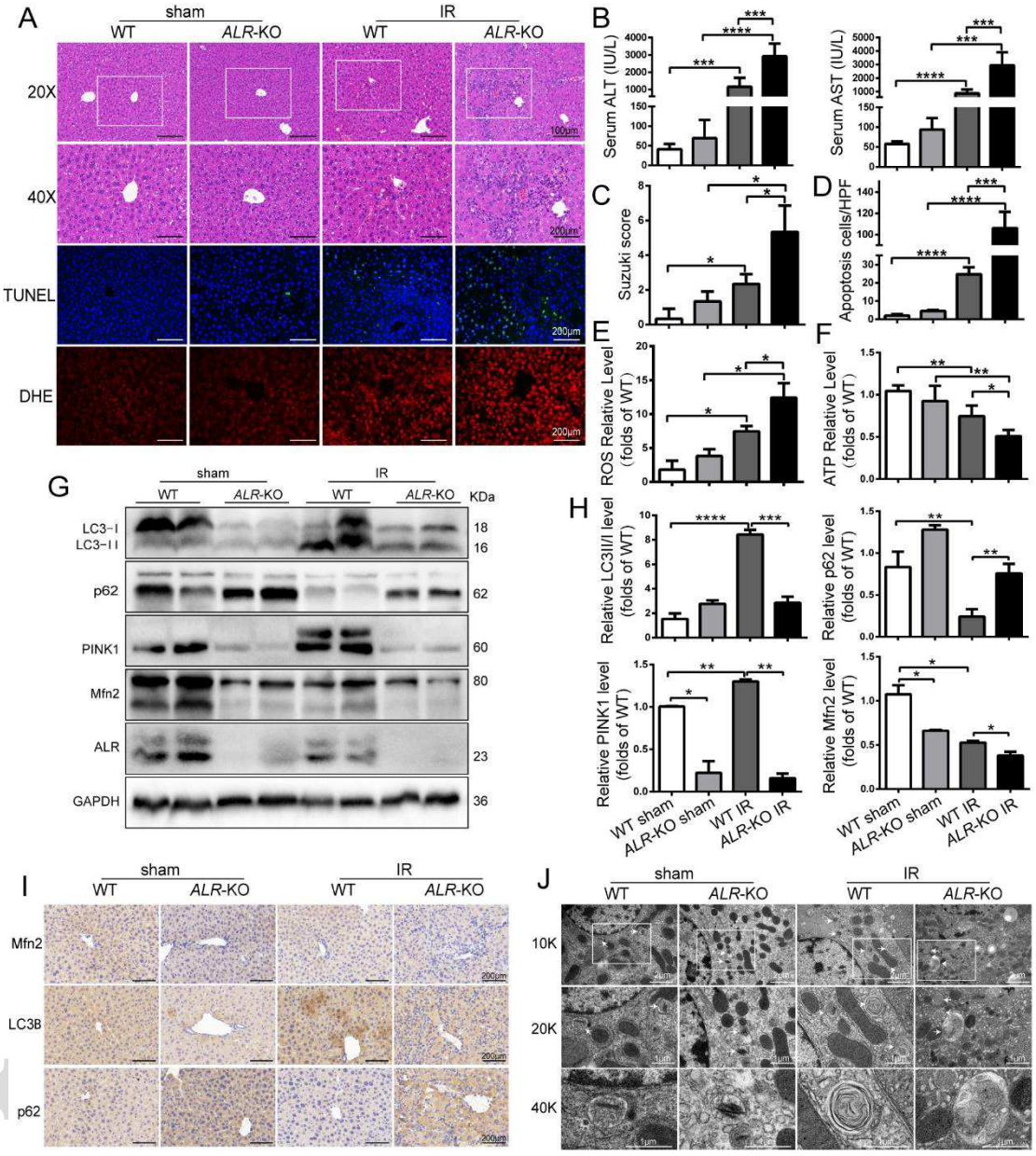
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Figure 7



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