

**Supplementary Information for**

**Autophagy of OTUD5 Destabilizes GPX4 to Confer Ferroptosis-  
Dependent Kidney Injury**

Likai Chu, *et al.*

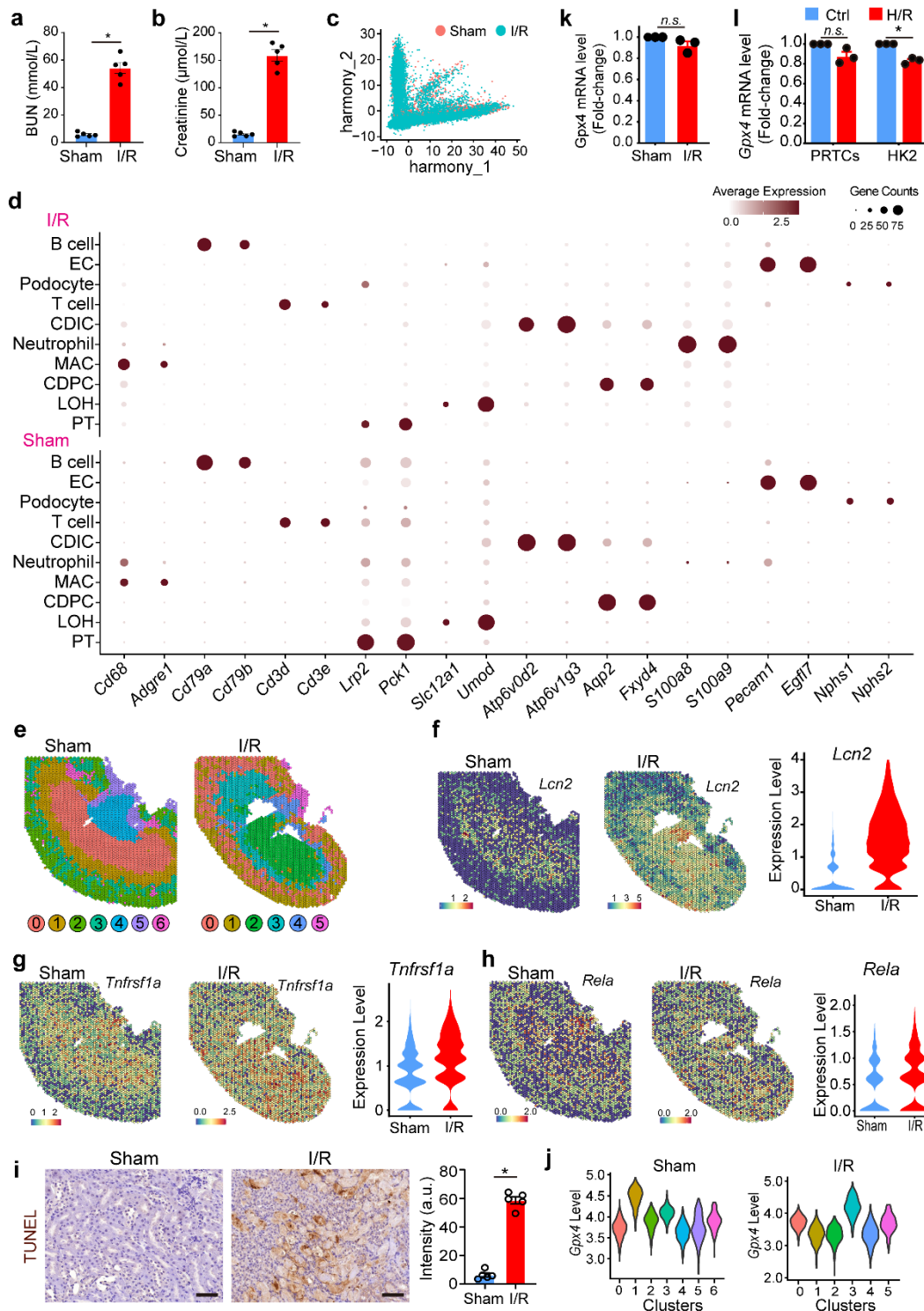
\*Correspondance to: junliu@suda.edu.cn

**This PDF file includes:**

Supplementary Figs. 1 to 7,

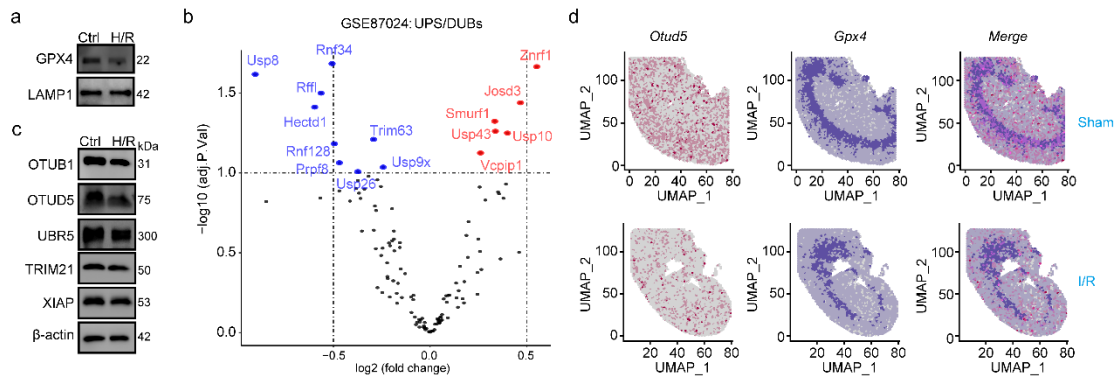
Supplementary Tables 1 and 2

Uncropped immunoblots gels for Supplementary Figures 2, 3, 5, 6, and 7

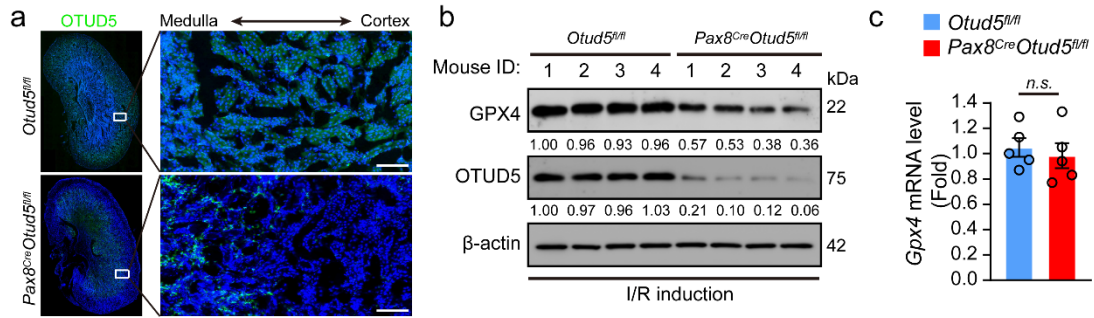


**Supplementary Fig. 1. scRNA sequencing and spatial transcriptomics reveal GPX4-mediated tubular cell ferroptosis in I/R-induced AKI.** **a, b** Serum BUN (**a**) and (**b**) creatinine levels of sham or I/R-treated mice. Data are presented as mean  $\pm$  s.e.m.; statistical significance was determined using an unpaired two-tailed Student's *t*-test, \**p* < 0.05. **c** The batch effect correction of the two samples. **d** The dot plot shows expressions of two representative marker genes of each cell type from scRNA data of Figure 1a. **e** Hematoxylin and eosin (H&E) staining of kidney sections and unbiased clustering of the spatial transcriptome (ST) spots of sham and I/R-treated kidneys. (**f-h**) Spatial feature plots and Violin plots of kidney injury marker gene *Lcn2* (**f**), *Tnfrsf1a* (**g**),

*Rela* (h) in ST spots of sham or I/R-treated kidneys. i IHC staining and quantification of TUNEL expression on kidney sections from sham or I/R-treated mice (n=5); scale bars, 50  $\mu$ m. j Violin plots of *Gpx4* expressions in clusters from sham or I/R-treated mouse kidneys. k, l mRNA level of GPX4 in mouse kidneys (n=5 per group), PRTC, and HK2 cells. Data are presented as mean  $\pm$  s.e.m.; statistical significance was determined using an unpaired two-tailed Student's *t*-test, \**p* < 0.05, n.s.: no significance.

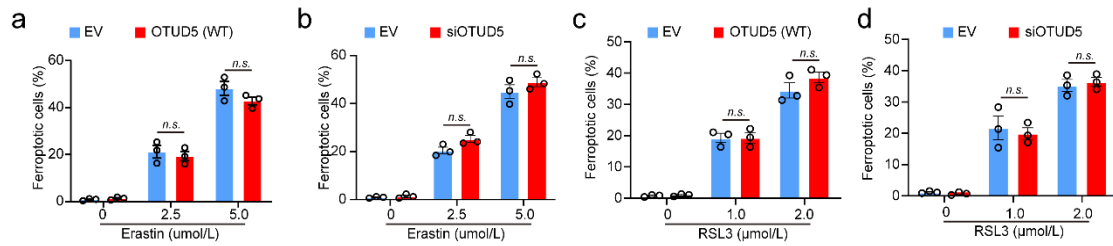


**Supplementary Fig. 2. OTUD5 is a GPX4 interacting protein for stabilization in response to I/R.** a Immunoblot analysis of GPX4 in the lysosome of cells treated with or without H/R. b The volcano plot shows the expression profile of UPS/DUBs-associated genes in the kidney before and after I/R induction (GSE87024), the most dysregulated UPS/DUBs genes were labeled in blue (downregulation) or red font (upregulation). c Immunoblot analysis of the indicated proteins in cells treated with or without H/R. d Feature plot of *Otud5* and *Gpx4* expression, and their colocalization in ST spots of the two groups.

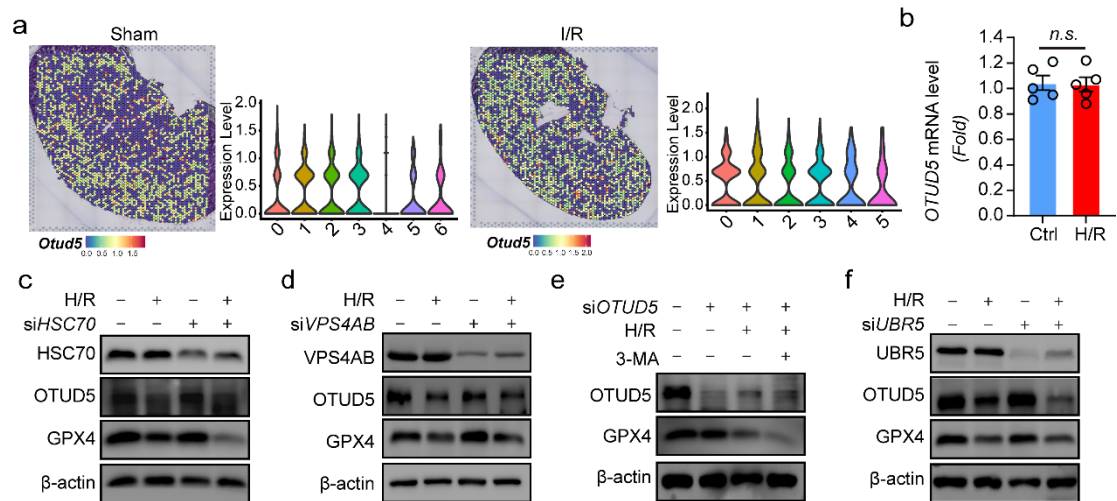


**Supplementary Fig. 3. *Otud5* deletion confers kidneys vulnerable to I/R.**

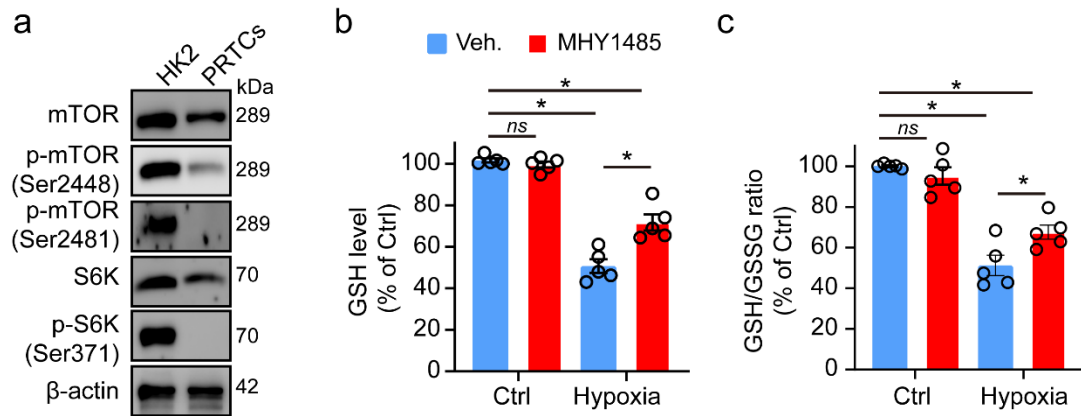
**a** Immunofluorescence analysis of OTUD5 expression in kidney samples from *Pax8<sup>Cre</sup>Otud5<sup>fl/fl</sup>* mice and their WT littermates (*Otud5<sup>fl/fl</sup>*). **b** 4 to 6-week-old *Pax8<sup>Cre</sup>Otud5<sup>fl/fl</sup>* ( $n = 4$ ) and their WT littermates ( $n = 4$ ) were subjected to kidney I/R surgery. After 48 hours, kidneys were collected and subjected to immunoblot analysis for OTUD5 and GPX4. **c** The mRNA level of *Gpx4* in *Pax8<sup>Cre</sup>Otud5<sup>fl/fl</sup>* mice and their WT littermates. All values are presented as mean  $\pm$  s.e.m.;  $p$  values were calculated by unpaired two-tailed student's  $t$ -test, \* $p < 0.05$ .



**Supplementary Fig. 4. OTUD5's minimal effect on erastin and RSL3-induced ferroptosis in renal tubular cells.** **a** Cell ferroptosis was measured in EV or WT OTUD5-transfected cells in the presence or absence of Erastin with the indicated doses for 24 hours. **b** Cell ferroptosis was measured in siCtrl or siOTUD5-transfected cells in the presence or absence of Erastin with the indicated doses for 24 hours. **c** Cell ferroptosis was measured in EV or WT OTUD5-transfected cells in the presence or absence of RSL3 with the indicated doses for 24 hours. **d** Cell ferroptosis was measured in siCtrl or siOTUD5-transfected cells in the presence or absence of RSL3 with the indicated doses for 24 hours. Data are from three repeated experiments, presented as mean  $\pm$  s.e.m.; statistical significance between groups as indicated was determined using an unpaired two-tailed Student's *t*-test, *n.s.*: no significance, *\*p* < 0.05.

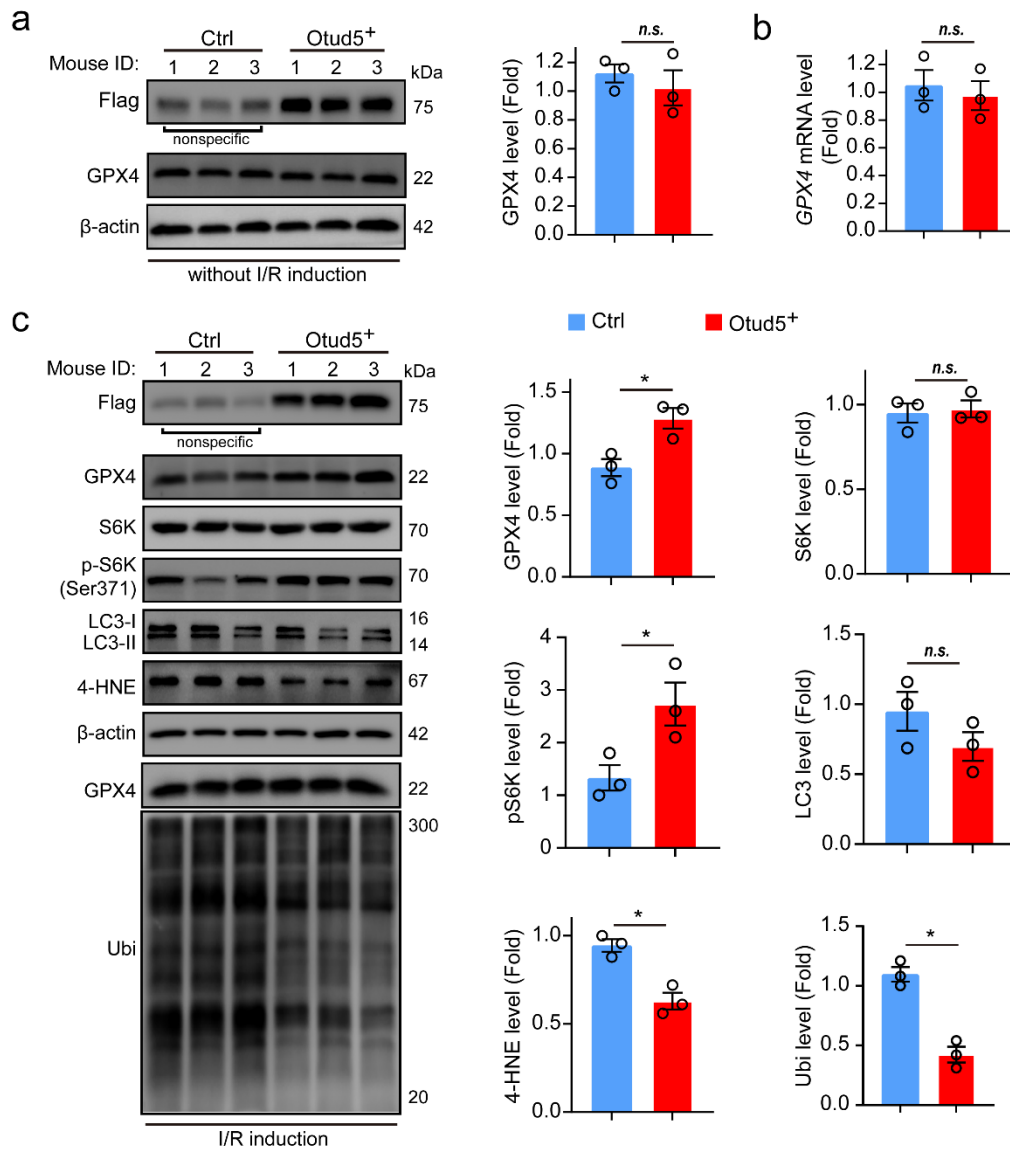


**Supplementary Fig. 5. Hypoxia activates autophagy degradation on OTUD5.** **a** Spatial expression pattern of *Otud5* in ST spots and clusters of sham or I/R-treated mice. **b** The expression level of *Otud5* was analyzed in PRTCs treated with or without H/R. **c-f** HK2 cells were transfected with siControl or siHSC70 (**c**), siVPS4AB (**d**) for 48 hours and treated with H/R condition. (**e**) OTUD5-depleted HK2 cells were treated with H/R conditions in the presence or absence of 3-MA. (**f**) HK2 cells were transfected with siControl or siUBR5 for 48 hours and treated with H/R condition.



**Supplementary Fig. 6. H/R reduces OTUD5 through repressing mTORC1 signaling.**

**a** The protein lysate of HK2 and PRTCs were subjected to immunoblot and the levels of proteins associated with mTOR signaling were analyzed. **b, c** The GSH level (**b**) and GSH/GSSG ratios (**c**) in HK2 cells were determined before and after hypoxia induction. Data are presented as mean  $\pm$  s.e.m.; Statistical significance was determined using an unpaired two-tailed Student's *t*-test, \*  $p < 0.05$ , *n.s.*: no significance.



**Supplementary Fig. 7. AAV-mediated OTUD5 therapy protects renal function against I/R injury.** **a** 4 to 6-week-old C57BL/6J mice were intravenously injected with a single dose of  $3 \times 10^{11}$  copies of *Otud5*-contained virus. 48 hours after injection, mice were sacrificed and the kidneys were collected for immunoblot. The levels of GPX4 and Flag (OTUD5) were analyzed. **b** The mRNA level of *Gpx4* in the *Otud5*-expressed mice and the control mice ( $n=3$ ). **c** 4 to 6-week-old C57BL/6J mice were intravenously injected with a single dose of  $3 \times 10^{11}$  copies of *Otud5*-contained virus. 48 hours after injection, mice were subjected to I/R surgery and lived for another 48 hours. After that, mice were sacrificed and the kidneys were collected for immunoblot ( $n=3$ ). Data are presented as mean  $\pm$  s.e.m.; Statistical significance was determined using an unpaired two-tailed Student's *t*-test, *n.s.*: no significance.



**Supplementary Table 1. Reagents and antibodies were used in this study.**

<b>Antibody</b>	<b>Supplier</b>	<b>Catalog number</b>	<b>Clone number</b>
Anti-Glutathione Peroxidase 4	Abcam	ab125066	EPNCIR144
Anti-Glutathione Peroxidase 4	Abcam	ab41787	
Anti-4 Hydroxynonenal	Abcam	ab48506	HNEJ-2
Anti-F4/80	Abcam	ab300421	EPR26545-166
Anti-OTUD5 Antibody	Polyclonal Invitrogen	PA5-20611	
Anti-OTUD5 Antibody	Polyclonal Proteintech	21002-1-AP	
Anti-OTUD5 Rabbit mAb	Cell Signaling	20087S	D8Y2U
Anti-GPX4 Antibody	Cell Signaling	52455S	
Anti-p70 S6 Kinase Antibody	Cell Signaling	9202S	
Anti-Phospho-p70 S6 Kinase (Ser371)	Cell Signaling	9208S	
Anti-mTOR Rabbit mAb	Cell Signaling	2983S	7C10
Anti-Phospho-mTOR (Ser2481)	Cell Signaling	2974S	
Anti-Phospho-mTOR (Ser2448) Rabbit mAb	Cell Signaling	5536S	D9C2
Anti-DYKDDDDK Tag Rabbit mAb	Cell Signaling	14793S	D6W5B
Anti-Ubiquitin Mouse mAb	Cell Signaling	3936S	P4D1
Anti-Ubiquitin Rabbit mAb	Cell Signaling	20326S	E6K4Y
Anti-LC3A/B Antibody	Cell Signaling	4108S	
Anti-Becclin-1 Antibody	Cell Signaling	3738S	
Anti-Atg5 Rabbit mAb	Cell Signaling	9980S	D5G3
Anti-Hamartin/TSC1	Cell Signaling	6935S	D43E2
Anti-LAMP1 Rabbit mAb	Cell Signaling	9091S	D2D11
Anti-OTUB1 Rabbit mAb	Cell Signaling	3783S	D8F7
Anti-UBR5 Rabbit mAb	Cell Signaling	65344S	D6O8Z
Anti-XIAP Antibody	Cell Signaling	2042S	
Anti-TRIM21 Rabbit mAb	Cell Signaling	92043S	D1O1D
Anti-mouse IgG HRP-linked Antibody	Cell Signaling	7076S	
Anti-rabbit IgG HRP-linked Antibody	Cell Signaling	7074S	
Anti- $\beta$ -actin Rabbit mAb	Cell Signaling	4970S	13E5
Anti-HSPA8/HSC70	Santa Cruz Biotechnology	sc-7298	sc-7298
Anti-VSP4	Santa Cruz Biotechnology	sc-133122	E-8
Anti-Glutathione Peroxidase 4	Santa Cruz Biotechnology	sc-166437	D-3
Anti-KIM-1 (HAVCR1)	Boster Biological Technology	BA3536	
Normal rabbit IgG	Santa Cruz Biotechnology	sc-2027	
Normal mouse IgG	Santa Cruz Biotechnology	sc-2025	
<b>Reagent</b>			

MG132	Selleck	S2619
Bafilomycin A1	Selleck	S1413
RSL3	Selleck	S8155
Cycloheximide	Selleck	S7418
Protein G Plus Agarose	Santa Cruz Biotechnology	sc-2002
Lipofectamine 3000	Invitrogen	L3000015
MHY1485	Selleck	S7811
7-AAD	Invitrogen	00-6993-50
BODIPY™ 581/591 C11	Thermo Fisher Scientific	D3861
CQ	Selleck	S6999
<b>Plasmid</b>		
Mouse_OTUD5	Generalbiol	N/A
Human_His-OTUD5	Generalbiol	N/A
Human_OTUD5 (C224S)	Generalbiol	N/A

**Supplementary Table 2. Information of primers used for quantitative real-time PCR analyses in this study.**

Gene	Forward (5' to 3')	Reverse (5' to 3')
Mouse_ <i>Gpx4</i>	CCTCTGCTGCAAGAGCCTCCC	CTTATCCAGGCAGACCATGTGC
Human_ <i>GPX4</i>	ACAAGAACGGCTGCGTGGTGAA	GCCACACACTTGTGGAGCTAGA
Mouse_ <i>Otud5</i>	G TTCATGACATTCGGCTT	CACAAGCAAAAAGTACCCTA
Mouse_ <i>Lcn2</i>	TCAAGGACGACAACATCATCTTCT	CTCCAGATGCTCCTTGGTATGG
Mouse_ <i>Havcr1</i>	TACCTCCACTCCTCCAACATCTA	TGCCAACATAGAAGCCCTTAGTA
Mouse_ <i>Il6</i>	CCAGAAACCGCTATGAAGTTCCT	TGTGTAATTAGCCTCCGACTTGT
Mouse_ <i>Tnf</i>	GACCCTCACACTCAGATCATCTT	CCTTGAAGAGAACCTGGGAGTAG
Mouse_ <i>GAPDH</i>	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAG
Human_ <i>GAPDH</i>	ACCCACTCCTCCACCTTTGA	CTGTTGCTGAGCCAAATTCGT

## Uncropped immunoblot gels for Supplementary Figure 2

Figure S2A-GPX4

Antibody information: Abcam, ab41787, 1:1000

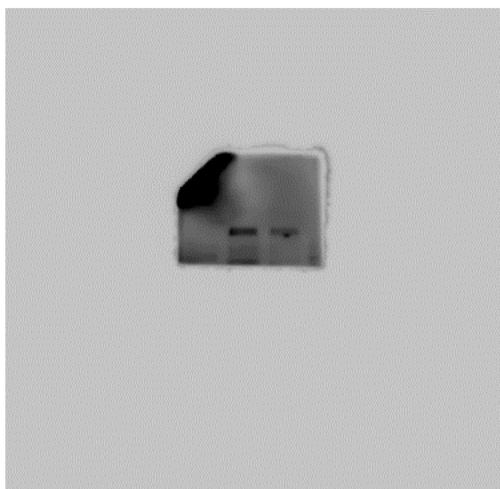


Figure S2C- $\beta$ -actin

Antibody information: Cell Signaling, 4970S, 1:1000

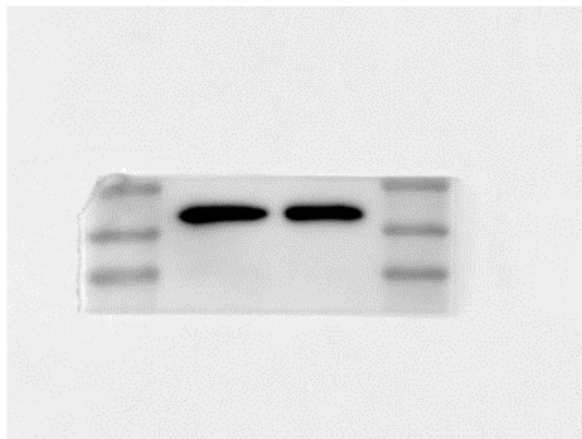


Figure S2A-LAMP1

Antibody information: Cell signaling, 9091S, 1:1000

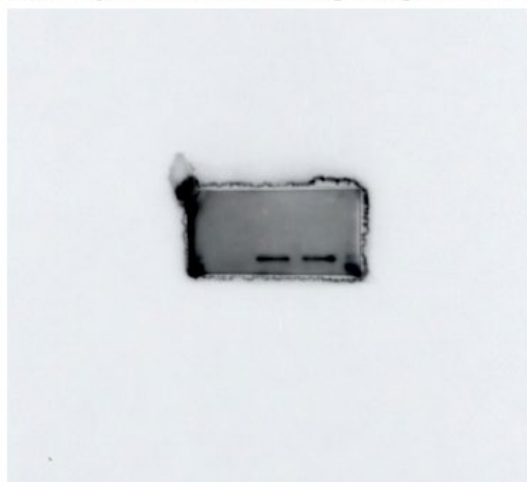


Figure S2C-OTUB1

Antibody information: Cell signaling, 3783S, 1:1000

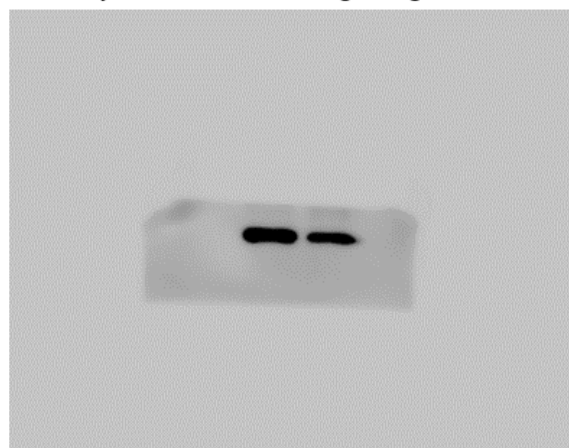


Figure S2C-UBR5

Antibody information: Cell signaling, 65344S, 1:500

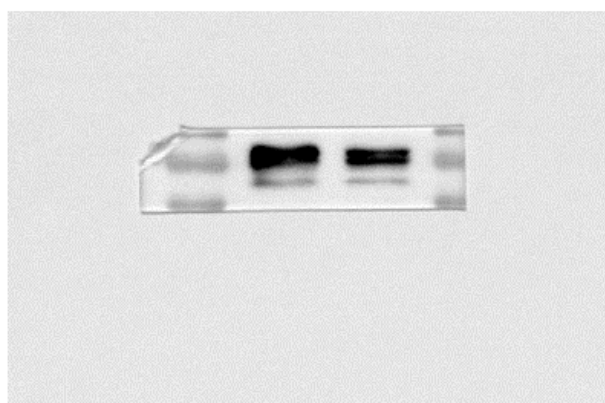
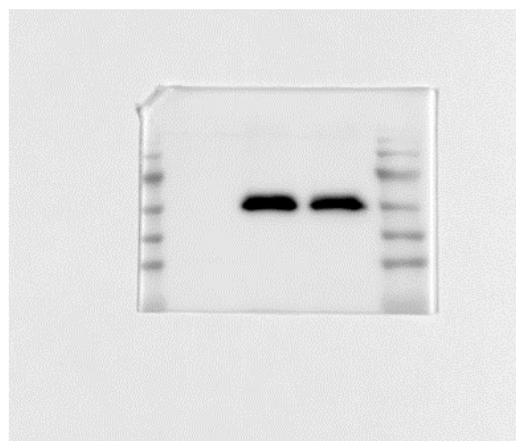


Figure S2C-XIAP

Antibody information: Cell signaling, 2042S, 1:1000



### Uncropped immunoblot gels for Supplementary Figure 3

Figure S3B-Actin

Antibody information: Cell Signaling, 4970S, 1:1000

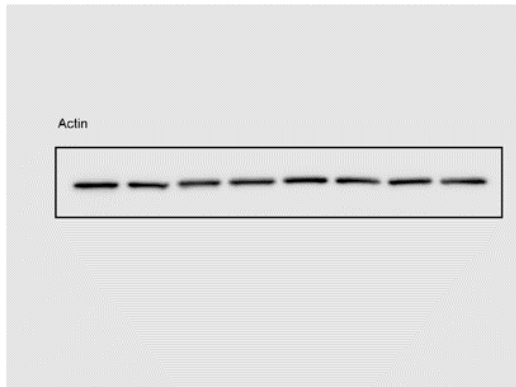


Figure S3B-GPX4

Antibody information: Abcam, ab41787, 1:1000

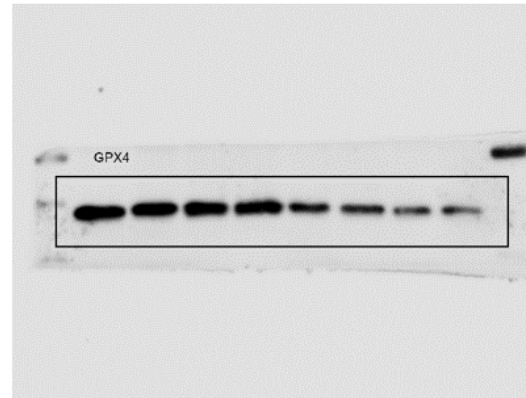
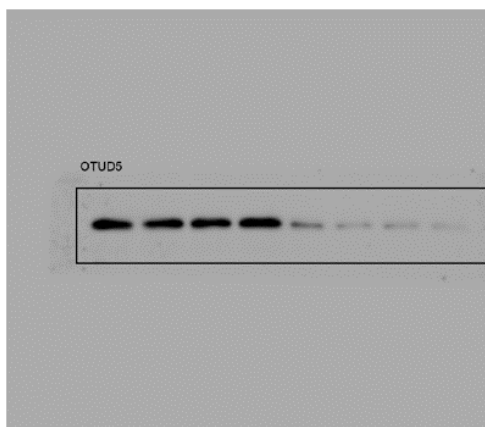


Figure S3B-OTUD5

Antibody information: Cell signaling, 20087S, 1:1000



## Uncropped immunoblot gels for Supplementary Figure 5

Figure S5E-Actin  
Antibody information: Cell Signaling, 4970S, 1:1000

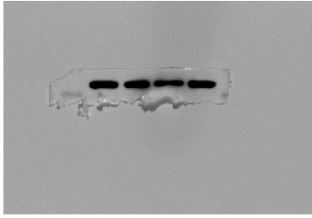


Figure S5C-GPX4  
Antibody information: Abcam, ab41787, 1:1000

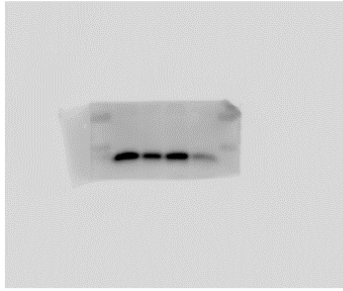


Figure S5C-OTUD5  
Antibody information: Cell signaling, 20087S, 1:1000

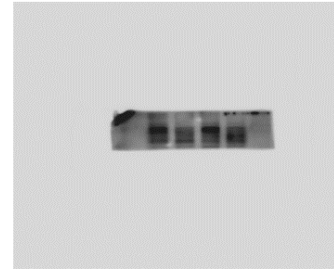


Figure S5C- Actin  
Antibody information: Cell Signaling, 4970S, 1:1000

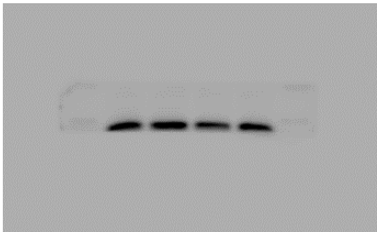


Figure S5D-VPS4AB  
Antibody information: Santa Cruz Biotechnology, sc-133122, 1:500

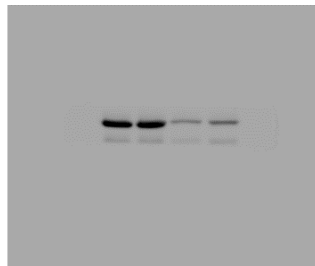


Figure S5D-GPX4  
Antibody information: Abcam, ab41787, 1:1000

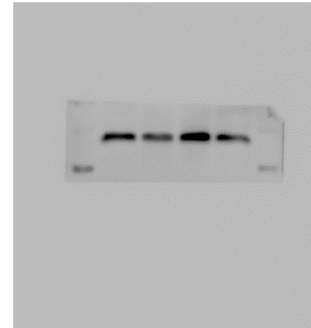


Figure S5D-OTUD5  
Antibody information: Cell signaling, 20087S, 1:1000

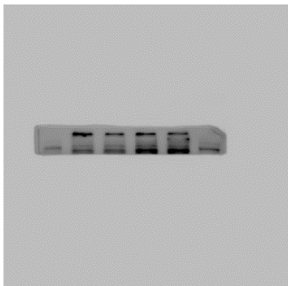


Figure S5D-Actin  
Antibody information: Cell Signaling, 4970S, 1:1000

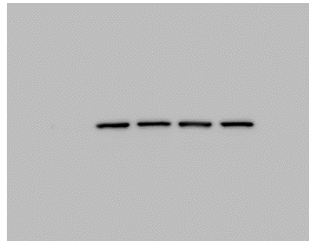


Figure S5E-GPX4  
Antibody information: Abcam, ab41787, 1:1000

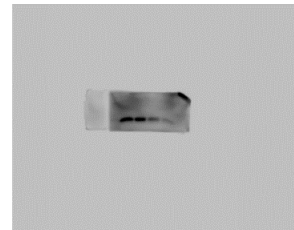


Figure S5E-OTUD5  
Antibody information: Cell signaling, 20087S, 1:1000

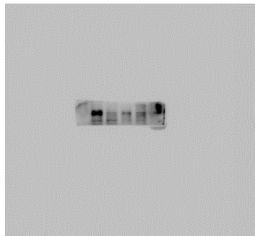


Figure S5C-HSC70  
Antibody information: Santa Cruz Biotechnology, Sc-7298, 1:500



Figure S5F-UBR5

Antibody information: Cell signaling, 65344S, 1:500

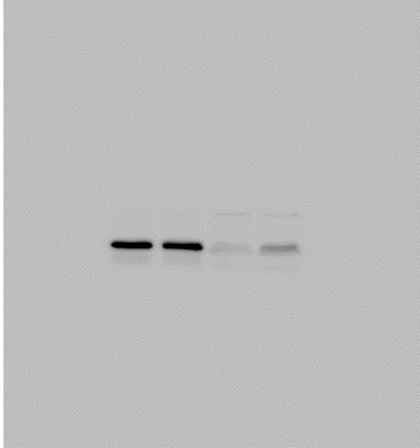


Figure S5F-GPX4

Antibody information: Abcam, ab41787, 1:1000

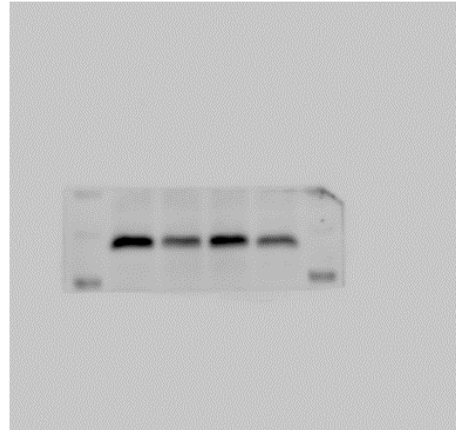


Figure S5F-OTUD5

Antibody information: Cell signaling, 20087S, 1:1000

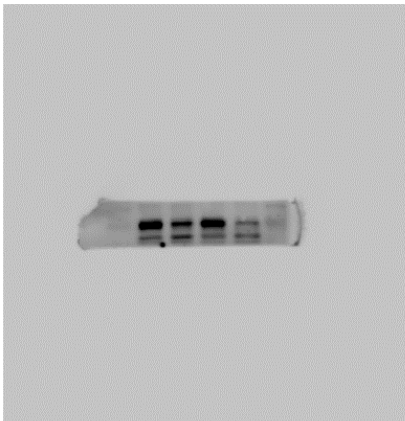
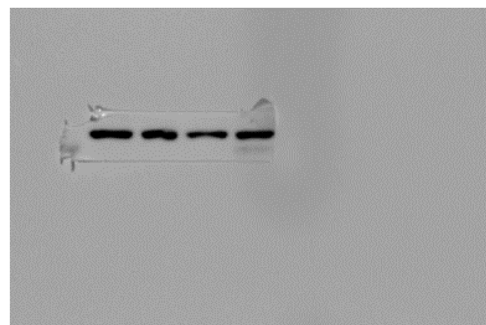


Figure S5F-Actin

Antibody information: Cell Signaling, 4970S, 1:1000



## Uncropped immunoblot gels for Supplementary Figure 6

Figure S6A-mTOR

Antibody information: Cell signaling, 2983S, 1:1000

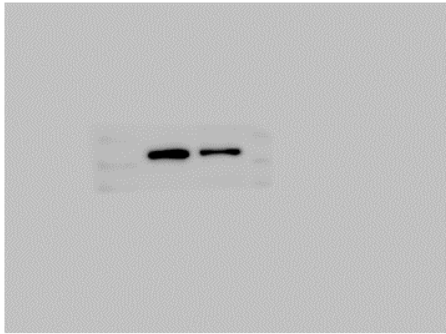


Figure S6A-mTOR-Ser2448

Antibody information: Cell signaling, 5536S, 1:500

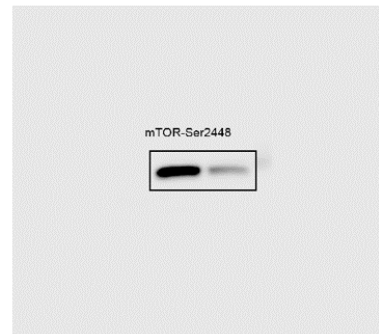


Figure S6A-mTOR-Ser2481

Antibody information: Cell signaling, 2974S, 1:500

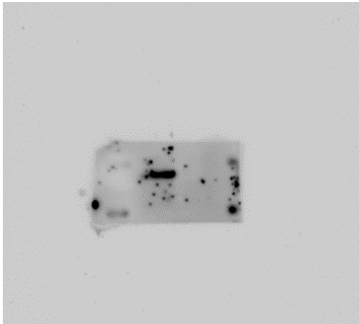


Figure S6A-S6K

Antibody information: Cell signaling, 9202S, 1:1000



Figure S6A-S6K- Ser371

Antibody information: Cell signaling, 9208S, 1:500

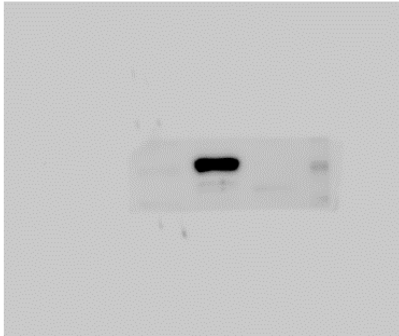


Figure S6A- Actin

Antibody information: Cell Signaling, 4970S, 1:1000



## Uncropped immunoblot gels for Supplementary Figure 7

Figure S7A-Flag

Antibody information: Cell Signaling, 14793S, 1:1000

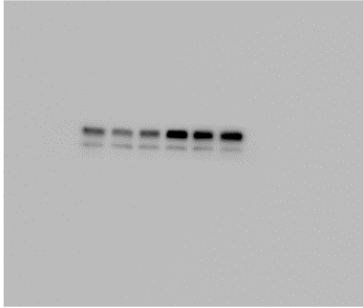


Figure S7A-GPX4

Antibody information: Abcam, ab125066, 1:1000

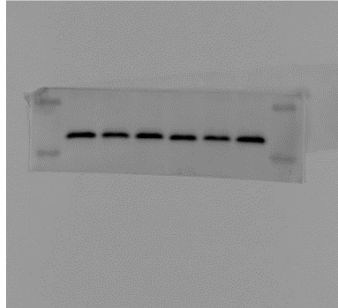


Figure S7A-Actin

Antibody information: Cell Signaling, 4970S, 1:1000



Figure S7c-Flag

Antibody information: Cell Signaling, 14793S, 1:1000



Figure S7c -GPX4

Antibody information: Abcam, ab125066, 1:1000

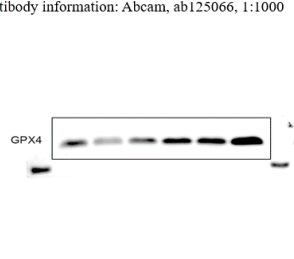


Figure S7c-S6K

Antibody information: Cell signaling, 9202S, 1:1000

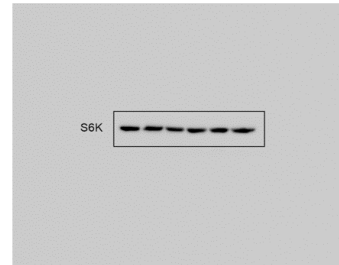


Figure S7c-p-S6K

Antibody information: Cell signaling, 9208S, 1:500

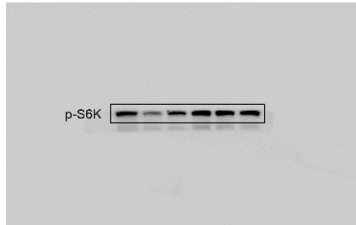


Figure S7c-4-HNE

Antibody information: Abcam, ab48506, 1:500

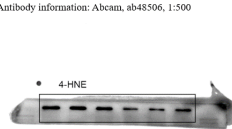


Figure S7c-LC3

Antibody information: Cell Signaling, 4108S, 1:1000

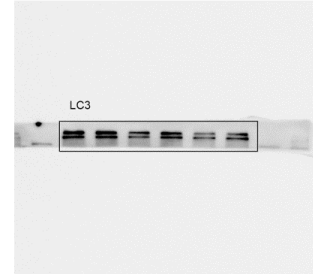


Figure S7c-ACTIN

Antibody information: Cell Signaling, 4970S, 1:1000

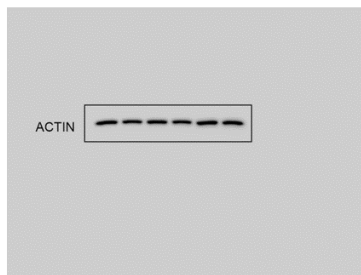


Figure S7c-IP-GPX4

Antibody information: Santa Cruz Biotechnology,  
Sc-166437, 2 µg per 500 µg protein]



Figure S7c-IP-Ubi

Antibody information: Cell Signaling, 20326S, 1:1000

