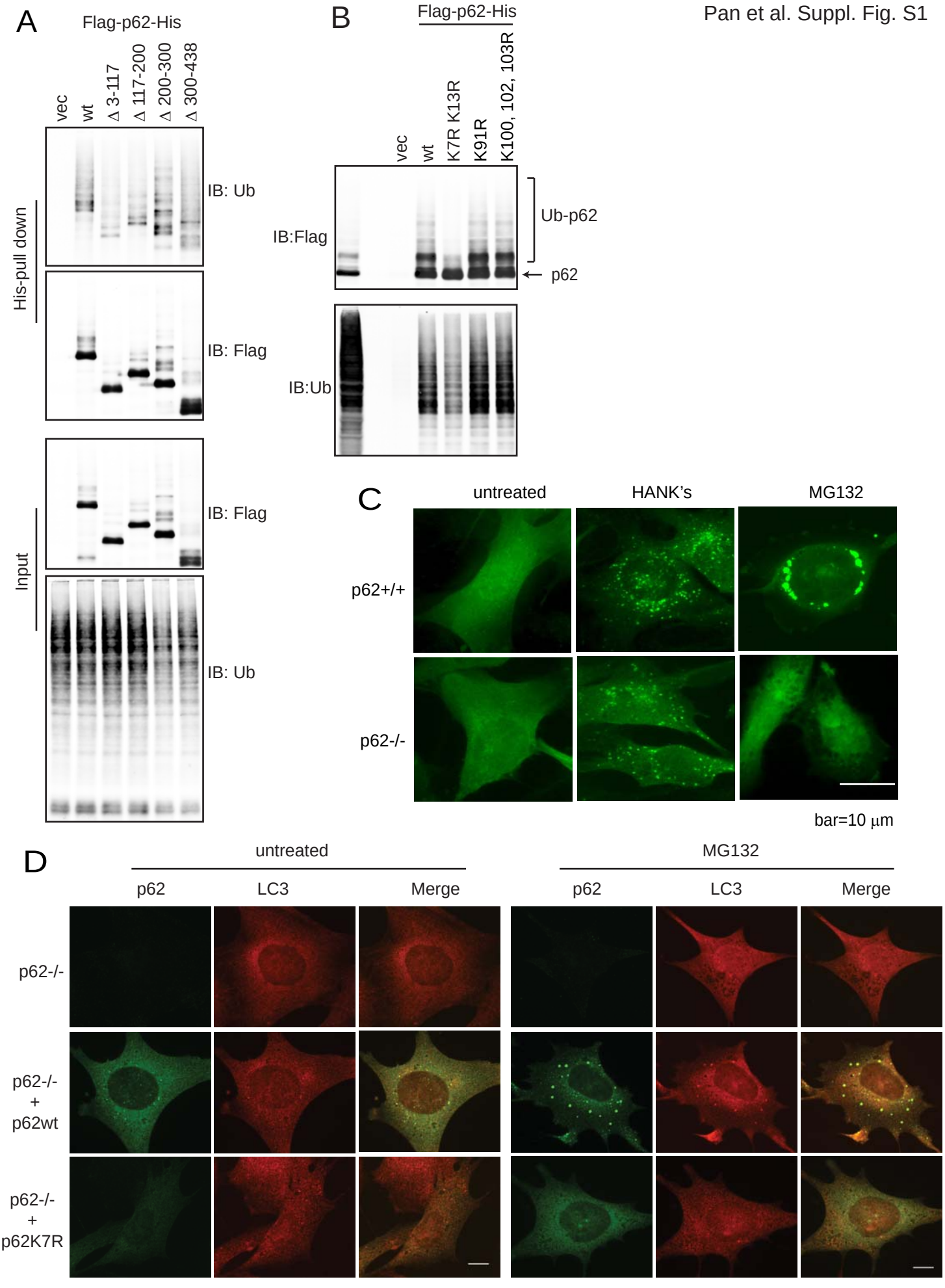
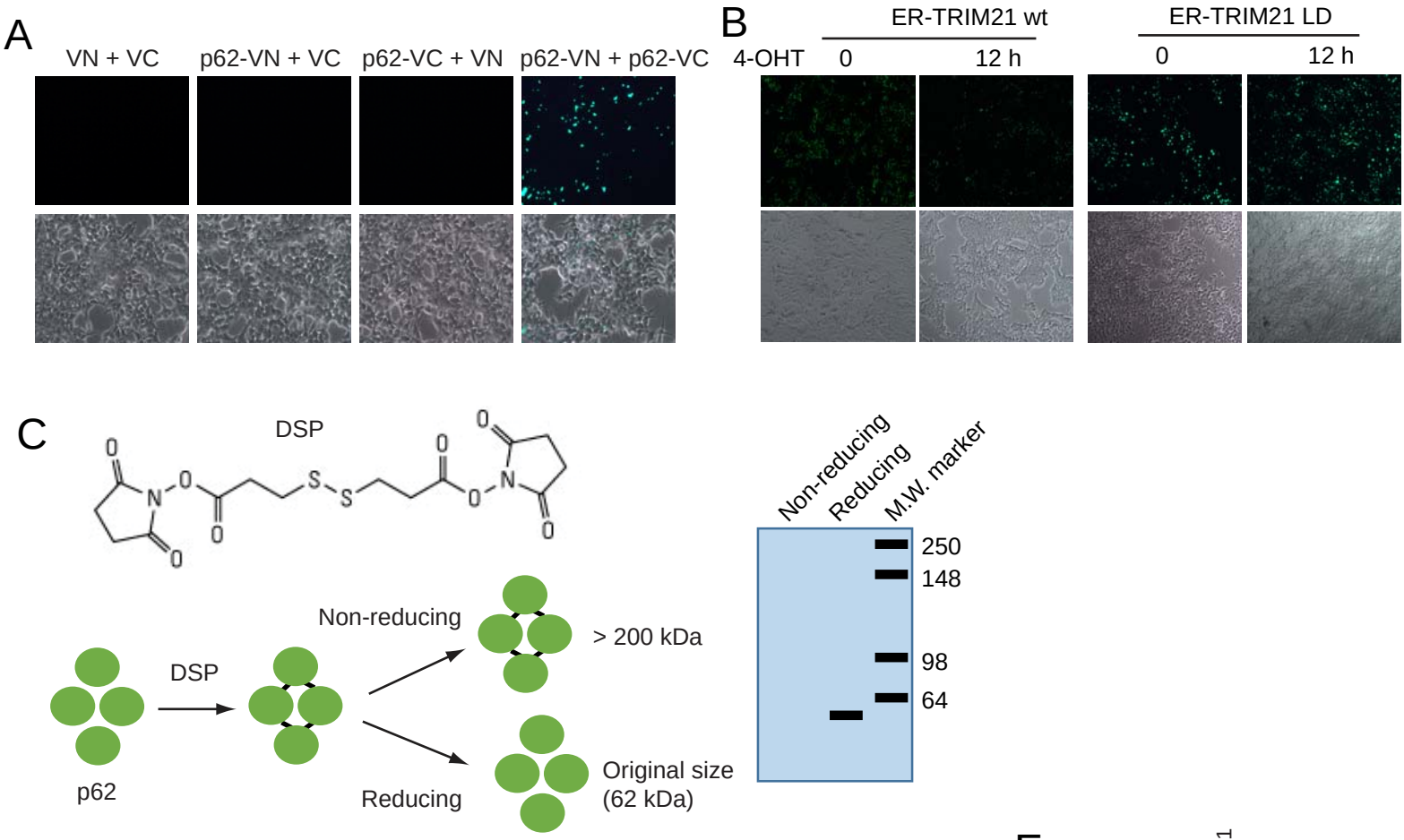
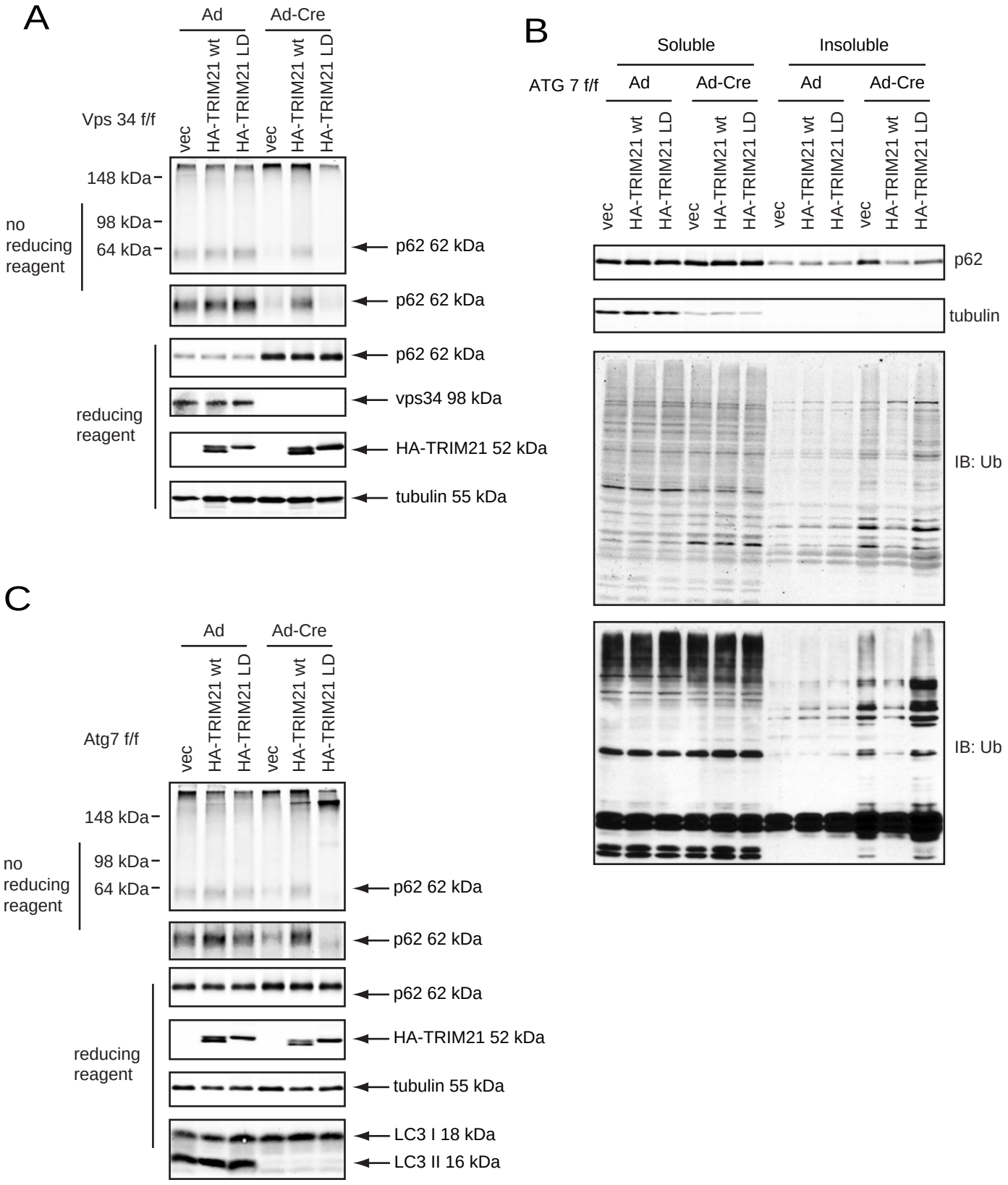
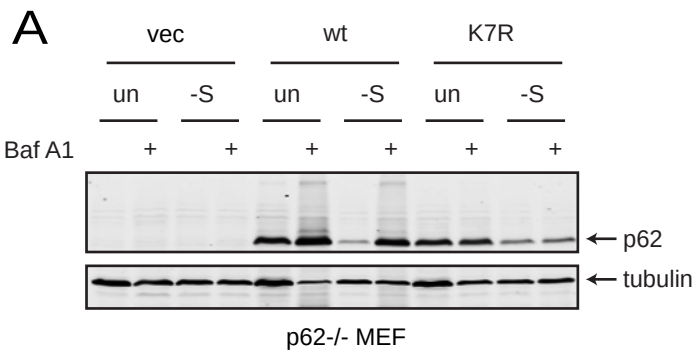
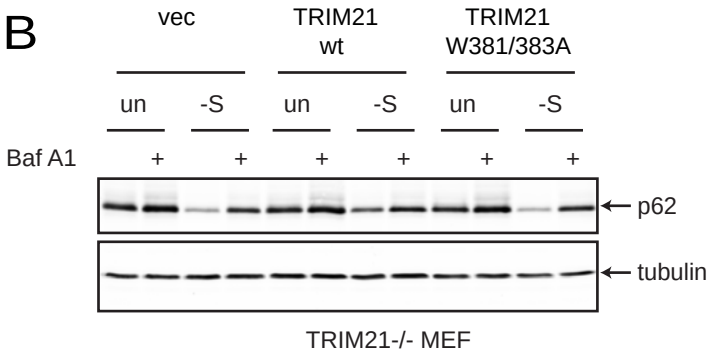
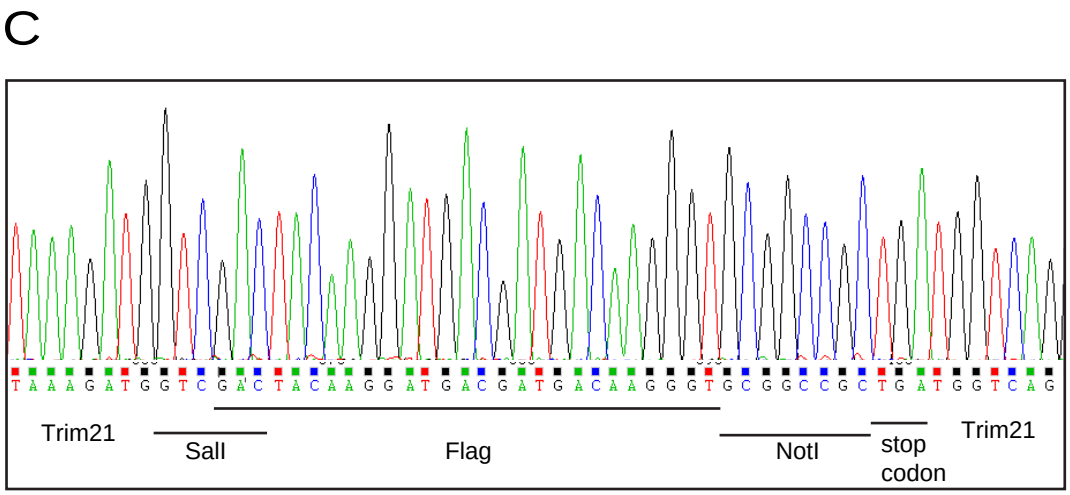

A
Flag-p62-His
vec
wt
Δ 3-117
Δ 117-200
Δ 200-300
Δ 300-438
His-pull down
IB: Ub
IB: Flag
Input
IB: Flag
IB: Ub
B
Flag-p62-His
vec
wt
K7R K13R
K91R
K100, 102, 103R
IB:Flag
Ub-p62
p62
IB:Ub
C
untreated
HANK's
MG132
p62+/+
p62-/-
bar=10 μm
D
untreated
MG132
p62
LC3
Merge
p62
LC3
Merge
p62-/-
p62-/- + p62wt
p62-/- + p62K7R

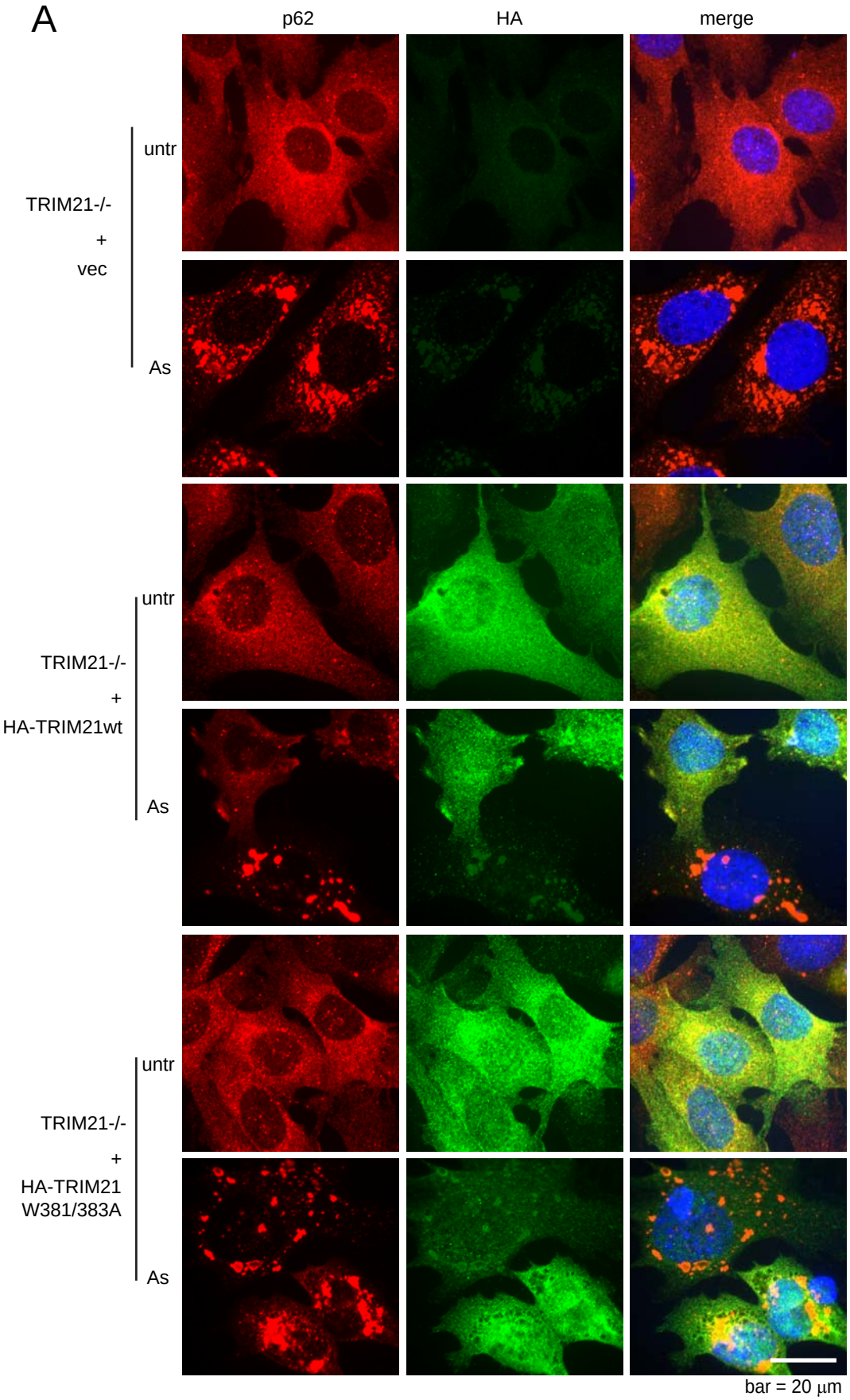

A
VN + VC
p62-VN + VC
p62-VC + VN
p62-VN + p62-VC
B
4-OHT
ER-TRIM21 wt
0
12 h
ER-TRIM21 LD
0
12 h
C
DSP
Non-reducing
> 200 kDa
DSP
p62
Reducing
Original size
(62 kDa)
Non-reducing
Reducing
M.W. marker
250
148
98
64
D
HeLa with shTRIM21
shNTC
shTRIM21
vector
wt
LD
un
MG
S
I
p62
p62 insol/sol
1.0
5.0
2.4
12.4
1.0
6.7
0.4
2.2
0.7
5.0
tubulin
E
parental
shNTC
shTRIM21
IP: Flag
IB: HA-Ub
148 kDa
98 kDa
64 kDa
Flag-p62
Input
Flag-p62
TRIM21
F
LPC
HA-TRIM21 wt
HA-TRIM21LD
HA
Ub
merge
untr
MG132
As(III)

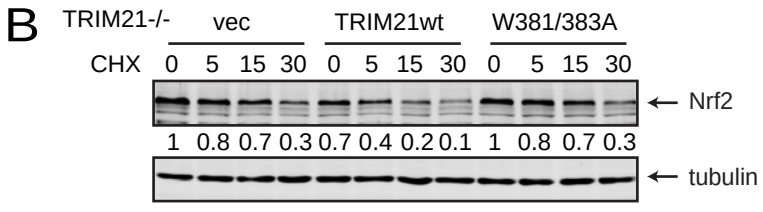

A
Ad
Ad-Cre
vec
HA-TRIM21 wt
HA-TRIM21 LD
Vps 34 f/f
148 kDa
98 kDa
64 kDa
no reducing reagent
p62 62 kDa
p62 62 kDa
p62 62 kDa
vps34 98 kDa
HA-TRIM21 52 kDa
tubulin 55 kDa
reducing reagent
B
Soluble
Insoluble
ATG 7 f/f
Ad
Ad-Cre
vec
HA-TRIM21 wt
HA-TRIM21 LD
p62
tubulin
IB: Ub
IB: Ub
C
Ad
Ad-Cre
vec
HA-TRIM21 wt
HA-TRIM21 LD
Atg7 f/f
148 kDa
98 kDa
64 kDa
no reducing reagent
p62 62 kDa
p62 62 kDa
p62 62 kDa
HA-TRIM21 52 kDa
tubulin 55 kDa
LC3 I 18 kDa
LC3 II 16 kDa
reducing reagent

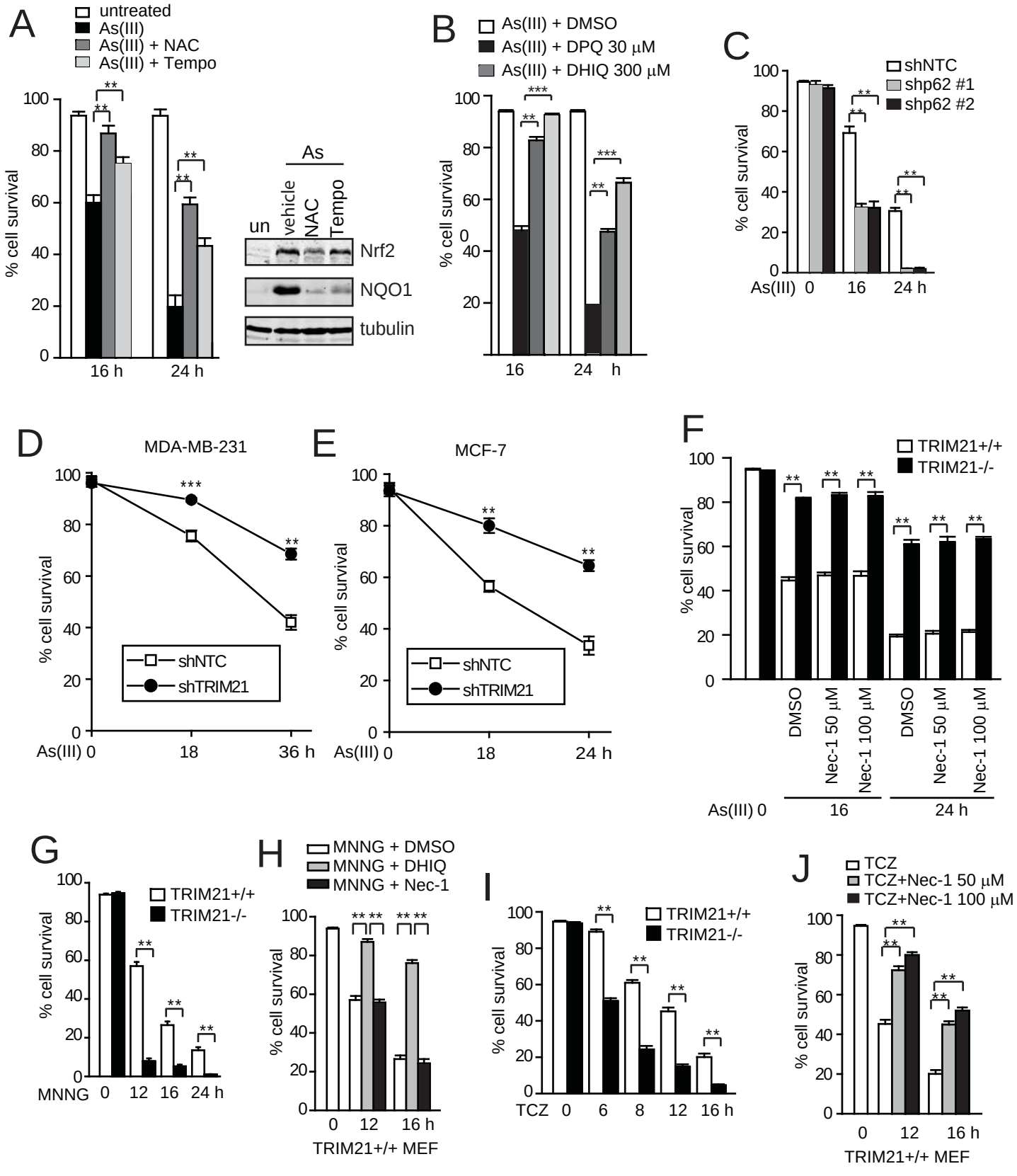

A
vec
wt
K7R
un
-S
un
-S
un
-S
Baf A1
+
+
+
+
+
+
p62
tubulin
p62-/- MEF

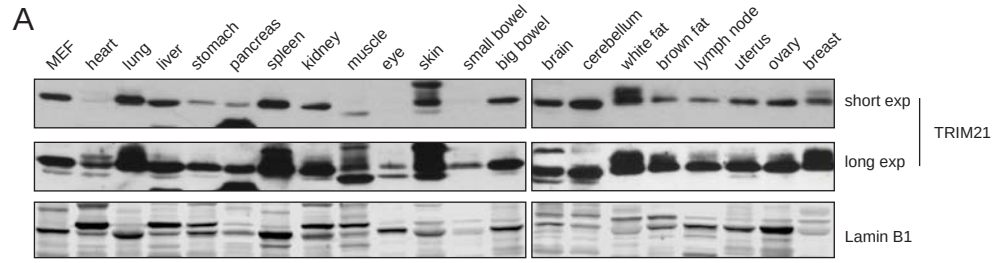

B
vec
TRIM21
wt
TRIM21
W381/383A
un
-S
un
-S
un
-S
Baf A1
+
+
+
+
+
+
p62
tubulin
TRIM21-/- MEF

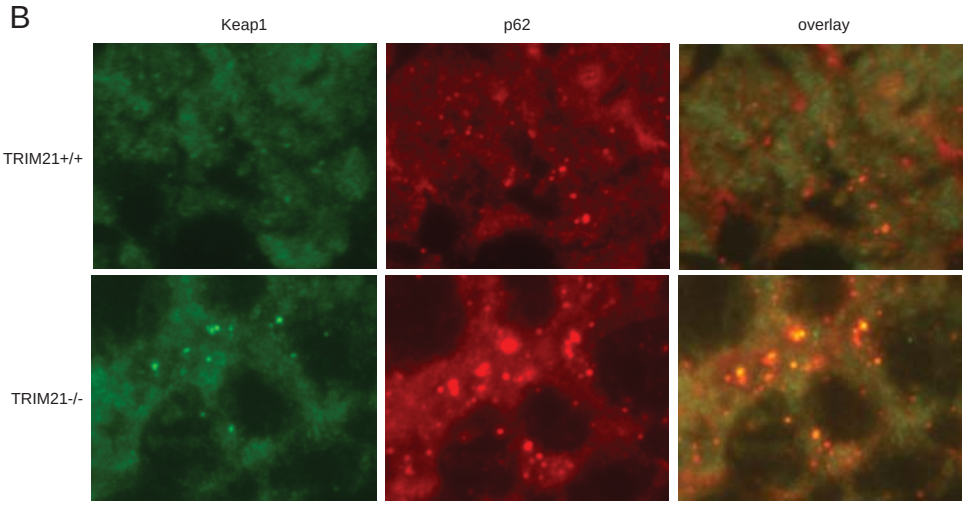

C
T A A A G A T G G T C G A C T A C A A G G A T G A C G A T G A C A A G G G T G C G G C C G C T G A T G G T C A G
Trim21
SalI
Flag
NotI
stop
codon
Trim21

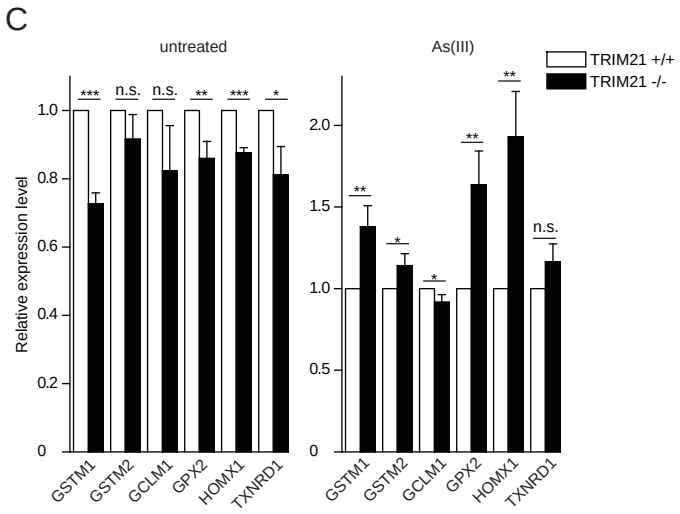

A
p62
HA
merge
TRIM21-/-
+
vec
untr
As
TRIM21-/-
+
HA-TRIM21wt
untr
As
TRIM21-/-
+
HA-TRIM21
W381/383A
untr
As
bar = 20 μm


B
TRIM21-/-
vec
TRIM21wt
W381/383A
CHX 0 5 15 30 0 5 15 30 0 5 15 30
Nrf2
1 0.8 0.7 0.3 0.7 0.4 0.2 0.1 1 0.8 0.7 0.3
tubulin


A
untreated
As(III)
As(III) + NAC
As(III) + Tempo
% cell survival
16 h
24 h
As
un
vehicle
NAC
Tempo
Nrf2
NQO1
tubulin
B
As(III) + DMSO
As(III) + DPQ 30 μM
As(III) + DHIQ 300 μM
% cell survival
16
24 h
C
shNTC
shp62 #1
shp62 #2
% cell survival
As(III) 0
16
24 h
D
MDA-MB-231
% cell survival
shNTC
shTRIM21
As(III) 0
18
36 h
E
MCF-7
% cell survival
shNTC
shTRIM21
As(III) 0
18
24 h
F
TRIM21+/+
TRIM21-/-
% cell survival
DMSO
Nec-1 50 μM
Nec-1 100 μM
DMSO
Nec-1 50 μM
Nec-1 100 μM
As(III) 0
16
24 h
G
TRIM21+/+
TRIM21-/-
% cell survival
MNNG 0
12
16
24 h
H
MNNG + DMSO
MNNG + DHIQ
MNNG + Nec-1
% cell survival
0
12
16 h
TRIM21+/+ MEF
I
TRIM21+/+
TRIM21-/-
% cell survival
TCZ 0
6
8
12
16 h
J
TCZ
TCZ+Nec-1 50 μM
TCZ+Nec-1 100 μM
% cell survival
0
12
16 h
TRIM21+/+ MEF


A
MEF
heart
lung
liver
stomach
pancreas
spleen
kidney
muscle
eye
skin
small bowel
big bowel
brain
cerebellum
white fat
brown fat
lymph node
uterus
ovary
breast
short exp
long exp
TRIM21
Lamin B1


B
Keap1
p62
overlay
TRIM21+/+
TRIM21-/-


C
untreated
As(III)
TRIM21 +/+
TRIM21 -/-
Relative expression level
GSTM1
GSTM2
GCLM1
GPX2
HOMX1
TXNRD1
n.s.

Supplemental Figure Legends

Figure S1. Related to Figure 1. (**A**) Flag- and His-doubly tagged wild-type or indicated truncation mutants of p62 were expressed in HEK293T cells and subjected to His pull-down followed by immunoblotting of indicated antibodies. (**B**) Wild-type or the lysine mutants of p62 were expressed in HEK293T cells and subjected to His pull-down followed by immunoblotting with indicated antibodies. (**C**) $p62^{+/+}$ and $p62^{-/-}$ MEFs stably expressing GFP-LC3 were left untreated or treated with the indicated conditions. Cells were observed under deconvolution microscope. (D) $p62^{-/-}$ MEFs were reconstituted with either p62wt or p62K7R. Cells were treated with MG132 (0.3 μM) for 12 h. Cells were stained for p62 and endogenous LC3 by IF, and observed under deconvolution microscope. Note that MG132 induced large size puncta positive for both p62 and LC3 in p62wt but not K7R cells.

Figure S2. Related to Figure 3. (**A**) Venus N-terminus (VN), Venus C-terminus (VC), p62-VN, and p62-VC were expressed in the indicated combinations in HEK293T cells, and were observed under fluorescence microscope. Representative micrographs are shown. Note that only the p62-VN and p62-VC combination resulted in fluorescent cells. (**B**) p62-VN and p62-VC were co-expressed in HEK293T cells with estrogen receptor (ER)-fused TRIM21wt or LD. 16 h post transfection, cells were treated with 4-OHT for 12 h to induce TRIM21 expression, then observed under fluorescence microscope. Note that TRIM21wt led to loss of Venus fluorescence while the LD mutant did not. (**C**) The schematic illustration of the strategy for DSP crosslinking and immunoblotting under reducing or non-reducing conditions. DSP crosslinking leads to the formation of bisulfite bond among proteins, which leads to large size protein complexes that are

undetectable in non-reducing SDS-PAGE. Treatment of reducing agent such as β-mercaptoethanol leads to disruption of the bisulfite bond hence detection of the protein monomers as illustrated in the diagram gel. **(D)** HeLa cells infected with lentiviral shNTC or shTRIM21 were left untreated or treated with MG132 (0.5 μM) for 12 h. Cells were lysed in IP lysis buffer. Each insoluble pellet was dissolved in RIPA buffer with 1% SDS with equal volume as the respective soluble fraction. 20 μg soluble fraction and the corresponding same volume of insoluble fraction of each sample were examined by IB. The same experiment is performed using HeLa cells with shTRIM21 reconstituted with vector, TRIM21wt, or TRIM21LD. (**E**) HeLa cells stably expressing Flag-p62 were infected with lentiviral shNTC or shTRIM21. HA-Ub was expressed in HEK293T cells for 24 h. Both HEK293T and HeLa cells were lysed in IP lysis buffer, and the same amount of lysates from HeLa cells were mixed with that from HEK293T cells. The mixed cell lysates were used for Flag IP followed by immunoblotting for indicated proteins. (**F**) TRIM21$^{-/-}$ cells reconstituted with vector, TRIM21wt, or TRIM21-LD were left untreated or treated with MG132 (0.25 μM) for 12 h and As(III) (10 μM) for 4 h. Immunofluorescence analysis using HA and Ub antibodies was performed, and observed under deconvolution microscope. Representative micrographs are shown. The cells are observed under deconvolution microscope. Note that expression of TRIM21wt (cells marked with an asterisk) but not LD mutant (cells marked with an asterisk) leads to disappearance of Ub-positive aggregates.

Figure S3. Related to Figure 3. (**A**) Vps34$^{f/f}$ MEFs stably expressing TRIM21wt or LD were infected with control or Cre-encoding adenovirus to knockout Vps34. 5 days post infection, cells were treated with DSP and analyzed by reducing and non-reducing SDS-PAGE to detect p62

monomer. Note that in Vps34$^{-/-}$ cells (Ad-Cre), TRIM21wt but not LD leads to increased p62 monomer under non-reducing condition. (**B** and **C**) Atg7$^{f/f}$ MEFs stably expressing TRIM21wt or LD were infected with control or Cre-encoding adenovirus to knockout Atg7. 5 days post infection, cells were fractioned into Triton X-100 soluble or insoluble fractions, the p62 monomers were detected by the reducing and non-reducing SDS-PAGE (**B**), and the distribution of p62 and polyubiquitylated proteins in the fractions was determined by immunoblotting (**C**).

Figure S4. Related to Figure 4. (**A**) p62$^{-/-}$ MEFs were reconstituted with vector, p62wt, and p62K7R. Cells were cultured in complete or serum-free medium were, in the absence or presence of bafilomycin A1 (Baf A1, 25 nM) for 6 h. Cells were lysed in RIPA buffer containing 1% SDS, and probed for p62 and tubulin by immunoblotting. Note that serum starvation induced p62wt autophagic degradation that was inhibitable by BafA1, whereas the degradation of p62K7R was not inhibited by BafA1 which is likely through proteasomal degradation of the p62K7R monomers. (**B**) TRIM21$^{-/-}$ MEFs were reconstituted with vector, TRIM21wt, and TRIM21W381/383A mutants. Cells were cultured in complete or serum-free medium were, in the absence or presence of bafilomycin A1 (Baf A1, 25 nM) for 6 h. Cells were lysed in RIPA buffer containing 1% SDS, and probed for p62 and tubulin by immunoblotting. (**C**) Sequencing result of the construct with the Flag tag flanked by murine TRIM21 genome sequences, which was introduced into MEFs via CRISPR. This led to the insertion of the Flag tag into the TRIM21 gene locus to generate the Flag-tagged TRIM21 that is expressed at the endogenous level.

Figure S5. Related to Figure 5 (**A**) TRIM21$^{-/-}$ MEFs reconstituted with vector, HA-TRIM21wt, or HA-TRIM21W381/383A mutants were left untreated or treated with As(III) (10 μM) for 6 h. Cells were subjected to IF with p62 and HA antibodies and observed under deconvolution microscope. Representative micrographs are shown. Note that TRIM21wt but not the W381/383A mutant inhibited As-induced p62 aggregation. (**B**) TRIM21$^{-/-}$ MEFs reconstituted with vector, HA-TRIM21wt, or HA-TRIM21W381/383A mutant were left untreated or treated with As(III) (10 μM) for 4 h. Cells were treated with cycloheximide (CHX) for indicated times, then lysed in RIPA buffer containing 1% SDS and probed for indicated proteins by immunoblotting. Note that Nrf2 degradation was enhanced by TRIM21wt but not the W381/383A mutant.

Figure S6. Related to Figure 6. (**A** and **B**) MEFs were treated with As(III) (10 μM) alone or together with ROS scavengers NAC (5 mM) or Tempo (5 mM) (**A**), or with PARP inhibitors DPQ or DHIQ (**B**), for indicated times. Cell death was determined by Trypan Blue staining (left panel). Data shown are the mean plus S.D. of three countings. **p<0.01, ***p<0.001. In (**A**), cell lysates were collected 6 h after the treatment and subjected to immunoblotting with indicated antibodies as a control showing that the ROS scavengers worked to suppress oxidative stress (right panel). (**C**) MEFs were infected with shNTC or two independent p62 shRNA clones. Cells were treated with As(III) for indicated times and cell viability determined by Trypan Blue staining. Data shown are the mean plus S.D. of three countings. **p<0.01. (**E** and **E**) MDA-MB-231 (**D**) and MCF7 (**E**) cells were infected with control shRNA (shNTC) or shTRIM21. Cells were treated with As(III) (10 μM) for indicated times. Cell viability was determined by Trypan Blue staining. Data shown are the mean plus S.D. of three countings. **p<0.01,

***p<0.001. (**F-J**) Cells were treated with various conditions. (**F**) WT or TRIM21$^{-/-}$ MEFs were treated with As(III) with DMSO or indicated amount of Nec-1 for 16 and 24 h. (**G**) WT or TRIM21$^{-/-}$ MEFs were treated with MNNG (0.5 mM). (**H**) WT MEFs were treated with MNNG (0.5 mM) in the presence of DMSO, DHIQ (300 μM), or Nec-1 (50 μM) for indicated times. (**I**) WT or TRIM21$^{-/-}$ MEFs were treated with TNFα (10 ng/ml)/cycloheximide (10 μg/ml)/z-VAD (10 μM) (TCZ) for indicated times. (**J**) WT MEFs were treated with TCZ in the presence of Nec-1 for indicated times. For all conditions, cell viability was measured by Trypan Blue staining. Shown are the mean plus S.D. of at least three countings. **p<0.01.

Figure S7. Related to Figures 6 and 7. (A) 40 μg of total protein isolated from each tissue was subjected to immunoblotting. Both short and long exposures of TRIM21 are shown. (B) Liver tissues were collected from mice treated with 30 mg/kg As_2O_3 in Fig. 6F-I. Cryosections from TRIM21$^{+/+}$ and TRIM21$^{-/-}$ animals were co-stained with anti-Keap1 and anti-p62 antibodies. Immunohistofluorescence was performed and the sections were observed and images captured by using an All-in-One microscope. Note increased protein aggregates containing p62 and Keap1 in As2O3-treated TRIM21$^{-/-}$ liver. (C) TRIM21$^{+/+}$ and TRIM21$^{-/-}$ MEFs were left untreated or treated with sodium arsenite (10 μM) for 5 h without inducing cell death. Total RNA was isolated and subjected to qRT-PCR. The relative abundance of specific mRNAs was normalized to mouse actin mRNA as the invariant control. n.s. non-significant; *p<0.05; **p<0.01; ***p<0.001.

Supplemental Experimental Procedures

Cell cultures, transfection and adenovirus infection

HEK293T cells and mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Lipofectamine 2000 (Invitrogen) was used for transfection. Vps34 $^{f/f}$ and Atg7$^{f/f}$ MEFs were infected with adenoviral GFP to generate Vps34$^{+/+}$ and Atg7$^{+/+}$ cells, or with adenoviral Cre-GFP for 24 h to delete Vps34 or Atg7, respectively. One day post infection, the virus-containing medium was replaced with fresh medium. Cells were cultured for at least 5 days to reach sufficient knockout that was verified by immunoblotting with Vps34 or Atg7 antibodies.

Plasmids

The human TRIM 21 cDNA was amplified using primers 5'-CCCAAGCTTACCATG TACCCATACGATGTTCCAGATTACGCG ATGGCTTCAG CAGCACGC-3' and 5'-CG GAATTCTCAATAGTCAGTGGATCCTTG-3', and were cloned into the retroviral LPC vector. The various TRIM21 mutants were generated following standard site-directed mutagenesis procedure. The primers used for TRIM 21 mutagenesis were: for C16A, 5'-GGAGGTCACAGCCCCTATCTGCCTGGACCCCTTCG-3' and 5'-GCAGATAGGGGCTGTGACCTCCTCCCACATC-3'; for C31A/H33A, 5'-CATCGAGGCTGGCGCCAGCTTCTGCCAGGAATGC-3' and 5'-GAAGCTGGCGCCAGCCTCGATGCTCACAGGCTC -3'; for shRNA resistant TRIM21, 5'-GCAGGAATTAGCGGAAAAATTAGAGGTGGAAATTGCAATAAAGAGAGCAG-3' and

5’-CCACCTCTAATTTTTCCGCTAATTCCTGCTTTCTTCTCAGTTCCCC-3’; for deletion of amino acid 8-251, 5’-CAGCACGCTTGGAAAGGAGTGAGTCCTGGAACC-3’ and 5’-CACTCCTTTCCAAGCGTGCTGCTGAAGCCAT-3’; for deletion of amino acid 252-476, 5’-GATAATTGTCCTGGGATCACAAGGATCCACTGAC-3’and 5’-CCTTGTGATCCCAGGACAATTATCACCTCCTGCA-3’; for deletion of amino acid 287-334, 5’-GGACATGTGCACAGCACTTTCACTCTGGAAAAC-3’ and 5’-GTGAAAGTGCTGTGCACATGTCCTCAGCATCTTC-3’; for W381/383A, 5’-CTGGACAATTGCGTTGGCGAACAAACAAAAATATGAGGCTG-3’ and 5’-TTTTGTTTGTTCGCCAACGCAATTGTCCAGAAGCCACTCTTG-3’. ER-TRIM21 is cloned into the ER-Ras construct by replacing Ras with TRIM21 cDNA between EcoRI and ClaI.

For p62 mutants, D69A and deletion of 321-342 were previously described (Pan et al., 2011). The other mutants were generated using the following primers: deletion of 3-117, 5’-CATGTTGCGGGGCGCCATGGTAAGCTTGTCATCGTCATC-3’ and 5’-GCTTACCATGGCGCCCCGCAACATGGTGCACCCCAATGTGATC-3’; deletion of 117-200, 5’-TCCTGGTGGACCCGCCTCCTGAGCACACGGTGGGCGG-3’ and 5’-CTCAGGAGGC GGGTCCACCAGGAAACTGGAGCCC-3’; deletion of 200-300, 5’-CGTGGCGCCCTCCATTTCCCATCCTGGCCACCCG-3’ and 5’-GG ATGGGAAATG GAGGGCGCCA CGCAGTCTCT GGCGG-3’; deletion of 300-438, 5’-GCAGAATTCTCAAACATTCCCACCCGGCTTGCTGG-3’ and 5’-GG TGGGAATGTTTGAGAATTCTGCAGATATCCATCACAC-3’; deletion of 1-61, 5’-GCTTACCATGTTCCAGGCGCACTACCGCGATGAG-3’ and 5’-GCGCCTGGAACATGGTAAGCTTCGCGTAATCTGG-3’; deletion of 62-117, 5’-

GCCTGGCGGCCCCCGCAACATGGTGCACC-3' and 5'-CATGTTGCGGGGGCCGCCAGGCCGCAGCGCGGG-3'; deletion of 117-163, 5'-CTCAGGAGGCGACCAAGCTCGCATTCCCCAGC 3' and 5'-GCGAGCTTGGTCGCCTCCTGAGCACACGGTGG 3'; deletion of 163-200, 5'-CCGGGGGCACGGTCCACCAGGAAACTGGAG-3' and 5'-CTGGTGGACCGTGCCCCCGGTGCAAGCCCTTTC-3'; K7/13R, 5'-TCCCTGCCCAGAAGGTAGGCCCTCACGGTGAGCGACGCCATGG-3' and 5'-TGAGGGCCTACCTTCTGGGCAGGGAGGACGCGGCGCGCGAG-3'; K7R, 5'-AGGTAGGCCCTCACGGTGAGCGACGCCATGG-3' and 5'-TCACCGTGAGGGCCTACCTTCTGGGCAAGG-3'; K13R, 5'-GCGTCCTCCCTGCCCAGAAGGTAGGCCTTCACG-3' and 5'-CTTCTGGGCAGGGAGGACGCGGCGCGCGAGATTC 3'; K91R, 5' AAGATGTCATCCCTCACGTAGGACATGGCCATTG-3' and 5'-ATGTCCTACGTGAGGGATGACATCTTCCGAATCTAC-3'; K100/102/103R, 5'-ACTCTCTTCTCTCTCTAATGTAGATTCGGAAGATGTCATC-3' and 5'-TACATTAGAGAGAGAAGAGAGTGCCGGCGGGACCACCGC-3'; K141R, 5'-AGACGCTGCACCTGTAGCGGGTTCCTACCACAGGC-3' and 5'-ACCCGCTACAGGTGCAGCGTCTGCCCAGACTACGAC-3'; K165R, 5'-TGGGGAATGCGAGCCTGGTGTGCCCCCGGTGCAAGCCC-3' and 5'-GGGGCACACCAGGCTCGCATTCCCCAGCCCCTTCGGGC-3'; K238R, 5'-TCCCCAACGTTCCTCAGGAAATTCACACTCGGATCCTC-3' and 5'-TGAATTTCCTGAGGAACGTTGGGGAGAGTGTGGCAGC-3'. The RNF138 is amplified from human cDNA with primers 5'-

CCCGGATCCACCATGTACCCATACGATGTTCCAGATTACGCGATGCGATCCCCCTCC CCC-3' and 5'-CGGAATTCTCAGATGTTTACTTGAAAAGATTCTTC-3'. The Bimolecular fluorescence complementation (BiFC) system for p62 were constructed following the strategy as previously described (Pan et al., 2011). VN (Venus a.a.1-173) and VC (Venus a.a.155-239) were fused to the C-terminals of p62wt and K7R.

Antibodies

The following antibodies were used: caspase 3 (Cell Signaling #9661 1:1,000 for WB), caspase 8 (R&D Systems AF1650, 1:1,000 for WB), caspase 9 (Cell signaling #9504 1:1,000 for WB), Flag (Sigma-Aldrich F7425, 1:1,000 for WB and F3165, 1:1,000 for WB, 1:500 for IF), anti-Flag M2 Affinity Gel (Sigma-Aldrich A2220), GFP (Santa Cruz Biotechnology sc-9996, 1:1,000 for WB, 1 μg/500 μl for IP), rabbit GFP antibody (a kind gift from Dr. Zhenyu Yue, The Mount Sinai Medical Center, 1:2,000 for WB, 0.3 μg/500 μl for IP), phospho H2A.X (Cell Signaling #2577, 1:1,000 for WB), HA (Rockland 21345, 1:5,000 for WB, 1:1,000 for IF and 0.5 μg/500 μl for IP), LC3 (MBL PM036, 1:1,000 for WB), NQO1 (Abcam ab2346, 1:2,000 for WB), Nrf2 (Santa Cruz sc-13032, 1:500 for WB), tubulin (Sigma-Aldrich T4026, 1:10,000 for WB), p62 (Novus Biologicals C11, H00008878-MOL, 1:5,000 for WB and 1:1,000 for IF), PAR (BD Pharmingen 551813, 1:1,000 for WB), PARP (Cell Signaling #9542 1:1,000 for WB), TRIM21 (Santa Cruz sc-25351, 1:2,000 for WB, 1:500 for IF).

His-Ub assay for mammalian cells

1×10^6 HEK293T cells were plated into 6-cm petri dish. After overnight recovery, cells were transfected with 0.5 μg His-Ub, His-Ub-K63R, His-Ub-K48R or 0.5 μg p62-His constructs by

Lipofectamine 2000. Two days after transfection, cells were harvested. One tenth of cells were lysed in RIPA buffer and the remaining cells were dissolved in Buffer A (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole, pH 8.0). After 10 s sonication, each sample was added with 50 µl Ni-NTA-agarose (Qiagen) equilibrated with Buffer A and incubated for 3 h at room temperature with agitation. The agarose beads were precipitated by centrifugation and washed once with Buffer A, then twice with Buffer B (10 mM Tris-Cl, pH 8.0, 8 M urea, 0.1 M NaH_2PO_4) and three times with 1:4 diluted Buffer B supplied with 25 mM imidazole. The precipitates were resuspended in 100 µl 2X Laemmli buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl pH 6.8) with 200 mM imidazole, boiled at 95℃ for 10 min and subjected to western blotting.

In vitro ubiquitination assay

Flag-p62 was expressed in HEK293T cells. After Flag-agarose pull-down, Flag-p62 was eluted with elution buffer supplemented with Flag peptide. HA-TRIM21 was expressed in HEK293T cells and isolated by HA-agarose pull-down. Flag-p62 and HA-TRIM21 were incubated together at 30°C in a 25 µl reaction mixture containing 20 mM HEPES buffer (pH 7.4), 10 mM MgCl2, 1m M DTT, 1 mM ATP, 5 mM creatine phosphate, 1 U creatine kinase, 2 µM ubiquitin, 50 nM (UBE1), and 500 nM UBE2N or UBE2D2 for 3 h. The reaction was stopped by adding 2X loading buffer, and boiled for 10 min and subjected to immunoblotting.

Co-immunoprecipitation

Cells were scrapped off the plates and precipitated at 800 x g for 5 min. After being washed twice with cold PBS, cells were lysed in IP lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl,

10% glycerol, 1% Triton X-100, 0.5 mM EDTA, 10 mM NaF, 100 µM orthovanadate, 200 µM PMSF) supplemented with protease inhibitor cocktail (Biosciences) on ice for 30 min. The cell lysates were cleared by centrifugation at 4°C, and pre-cleared with protein A/G agarose. The lysates were incubated with the primary antibody or anti-FLAG M2 Affinity Gel overnight at 4°C with agitation. The complexes were precipitated, washed twice with IP lysis buffer supplemented with 500 mM NaCl, then four times with IP lysis buffer, if p62 is involved in IP assay, or 6 times with IP lysis buffer, and boiled in 2X SDS sample buffer at 95°C for 5 min.

DSP crosslinking

Cells with less than 80% confluence were washed twice with ice cold washing puffer (Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, NaCl 137 mM, KCl 2.7 mM, $CaCl_2$ 0.1 mM, and $MgCl_2$ 1 mM), and incubated in washing buffer with 0.4 mg/ml DSP at 4°C for 2 h. Cells were lysed in IP lysis buffer with 1% SDS and sonicated for 10 seconds. For IP, the lysate is diluted with IP lysis buffer at ratio 1:10 and subjected to IP assay as described before.

CRISPR insertion of Flag tag into the TRIM21 gene

For Crisper-TRIM21, the oligos 5'-CACCGTCTGTCCACTAAAGATGTGA-3' and 5'-AAACTCACATCTTTAGTGGACAGAC-3' were annealed to form dsDNA and inserted into Lenticrispr vector (Addgene) followed the recommended protocol. For the Flag integration template, fusion PCR strategy is employed. Two PCR fragments with the Flag coding sequence were amplified from mouse genomic DNA with two pairs of primer, 5'-CCTGTGGACATTGAATGCATGTG-3' /5'-GCGGCCGCACCCTTGTCATCGTCATCCTTGTAGTCGACCATCTTTAGTGGACAGAGC

T-3' and 5'-GTTATTGGCTATGGGGGAAAGC-3' / 5'-CGATGACAAGGGTGCGGCCGCTGATGGTCAGGAGCCAGTGC-3'. Then the two PCR products were used as the template for PCR with primers 5'-TCTAGATCTGAGCCGTCCATGAAGGAGAGC-3' and 5'-GAGCTCGAGTCCCAATTCCATTCAGTGGAG-3' with the PCR product spanning the region from +1,000 bps of stop codon to -1,000 bps in TRIM21 gene. This PCR fragment was used as the template to obtain the 90-nt integration oligos. The 90-nt oligos were amplified by PCR with primers 5'-TTTGCTGGACCTCTGCGACCTTTC-3'/5'-GGTCCCCAGGAGTCACTGGATC-3' and purified by MinElute PCR Purification Kit (Qiagen). The purified DNAs were boiled for 10 min and immediately transferred into ice cold water for 10 min. 3×10^5 MEFs were resuspended in 25 µl MEF nucleofactor solution 1 (Amaxa). CRISPR-TRIM21 (0.5 µg) and the 90-nt oligos (0.6 µg) were used for electroporation following the protocol as described(Ran et al., 2013). After electroporation, cells were resuspended in 300 µl pre-warmed medium, transferred to 6-cm petri dish and recovered for at least 24 h before being used for other assays.

Cell death assay

Cell viability was quantified by either propidium iodide (PI) exclusion or Trypan Blue staining. For PI exclusion, cells were collected and resuspended in culture medium with PI at 1 µg/ml. Cell viability was determined by flow cytometry using a FACs caliber. For Trypan Blue staining, 0.5 ml of cells (1×10^5 cells per ml) were mixed with 0.1 ml of 0.4% Trypan Blue and incubated at room temperature for 5 min. Cells were counted under a phase-contrast light microscope.

ROS detection

$5x10^5$ cells were plated into 6-cm petri dish. After overnight recovery, cells were untreated or treated with 10 μM sodium arsenate. 4 h after treatment, cells were incubated with CMH_2DCFDA (5 μM final concentration) for 30 min in dark and then harvested. Cells were resuspended in PBS containing 10% FBS and analyzed with a flow cytometer using the CellQuest program.

Immunofluorescence

Immunofluorescence was performed as previously described (Pan et al., 2011). The slides were observed and imaged using a Zeiss LSM 510 META NLO two-photon laser scanning confocal microscope or a Zeiss inverted Axiovert 200M deconvolution microscope.

RNA extraction, cDNA synthesis, PCR

After treatment, total RNA of the cells was isolated with RNeasy kit (Qiagen). Reverse transcription was carried out with 2 μg of total RNA using the SuperscriptIII First Strand Synthesis system(Invitrogen). The synthesized cDNA was used for real-time quantitative PCR (qPCR) with the PerfeCTa SYBR Green Super mix (Quanta Bioscience 95055) on theStepOnePlus (Applied Biosystems). The following primers were used for qRT-PCR: GSTM1 5'-TTTGTCCTGCCCACGTTTCT-3' and 5'-TCAAAGTCGGGAGCGTCAC-3'; GSTM2 5'-TGGACTTTCCCAATCTGCCC-3' and 5'-ATCTTCTCAGGGAGACCCTCT-3'; GCLM1 5'-TGGGCACAGGTAAAACCCAAT-3' and 5'-TTCCCTGCTCTTCACGATG-3'; GPX2 5'-TCGGACATCAGGAGAACTGTC-3' and 5'-CGCACGGGACTCCATATGAT-3'; HMOX1 5'-ACAGCCCCACCAAGTTCAAA-3' and 5'-TCTGCAGGGGCAGTATCTTG-3'; TXNRD1 5'-GCGAGAAGAGCTGGTGGTTT-3' and 5'-TTTTTGTTCGGCTTCAGGGC-3'.

Histology, immunohistochemistry, and immunohistofluorescence analyses

Liver tissues were fixed and processed for H&E staining and IHC staining for p62, ubiquitin and 8-oxodG staining. In brief, paraffin-embedded liver sections were deparaffinized, rehydrated, and microwave heated for 15 min in 0.01 mol/L citrate buffer (pH 6.0) for antigen retrieval. 3% hydrogen peroxide was applied to block endogenous peroxidase activity. After 30 min of blocking with goat serum, the primary antibody, anti-p62 (American Research Products, Inc. Belmont, MA), anti-ubiquitin (Dako, Glostrup, Denmark), anti-8-oxodG (Abcam, Cambridge, MA) or control IgG were applied and incubated overnight at 4°C. After wash, slides were incubated with biotinylated secondary antibody and the streptavidin-biotin complex (Vector Laboratories, Inc. Burlingame, CA) were applied, each for 30 min at room temperature with an interval washing. After rinsing with PBS, the slides were immersed for 5 min in the coloring substrate 3,3′-diaminobenzidine (DAB, Sigma-Aldrich Co. LLC. St. Louis, MO) 0.4 mg/mL with 0.003% hydrogen peroxide, then rinsed with distilled water, counterstained with hematoxylin, dehydrated, and coverslipped.

For immunohistofluorescence analysis, liver OCT cryosections were fixed in cold acetone for 15 min and immunostained with anti-Keap1 (1:100, Proteintech, IL, USA) and anti-p62 (1:100, Progen, Heidelberg, Germany) for overnight at 4 ^{o}C. After wash with PBS for 3 times, cryosections were incubated with goat anti-rabbit IgG-Alexa488 (1:500, Invitrogen, Grand Island, NY) and donkey anti-guinea pig IgG-Alexa651 (1:500, Millipore, Temecula, USA) for 1 h at room temperature. Images were visualized and captured using the All-in-One Fluorescence Microscope, BZ-X700 (Keyence, Elmwood Park, NJ).

Arsenic trioxide treatment in vivo

For arsenic trioxide treatment, age (6-12 week) and gender-matched pairs of TRIM21 wild-type and knockout mice were gavaged with one dose of water or 30 mg/kg arsenic trioxide (As_2O_3, Sigma-Aldrich). After 24 h of treatment, mice were weighted and liver tissues harvested for histology and immunohistochemistry analyses.

Transverse aortic constriction (TAC)

Mice were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg) given intraperitoneally. The skin was cleaned with an iodine solution, and the operation was performed under sterile conditions. The surgical procedure was performed as previously described (Hu et al., 2003) with modifications. Briefly, mice were placed in a supine position and a horizontal skin incision ~1.0 cm in length was made at the level of the suprasternal notch. The thyroid was retracted, and a 3-5 mm longitudinal cut was made in the proximal portion of the sternum. This allowed visualization of the aortic arch under low-power magnification. A wire with a snare on the end was passed under the aorta between the origin of the right innominate and left common carotid arteries. A 6-0 silk suture was snared with the wire and pulled back around the aorta. A bent 27-gauge needle was then placed next to the aortic arch, and the suture was snugly tied around the needle and the aorta. After ligation, the needle was quickly removed and the skin was closed. The sham procedure was identical except that the aorta was not ligated. Animals were imaged by echocardiography 4 days after aortic banding. Echocardiography were performed as previously described (Lu et al., 2009).

Mouse experiments

All mouse experiments were done in compliance with the Institutional Animal Care and Use Committee guidelines at Stony Brook University and Rutgers University. For tissue harvesting, different tissues were removed from the wide type C57BL/6 mice and washed in the cold PBS. 5-10 mg tissues were lysed in 500 µl RIPA buffer with 1% SDS and homogenized/sonicated, except that one whole eye was lysed in 800 µl RIPA buffer with 1% SDS and followed by homogenization and sonication. Samples were spun down at 12,000 x g for 15 min.

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