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Supplementary Information for
The N-terminal Cysteine is a Dual Sensor of Oxygen and Oxidative Stress

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This PDF file includes:

Supplementary Information Text

Extended Methods.

Plasmids and other reagents

The Myc tagged RGS4 plasmid was constructed using pcDNA 3.1/myc-His A vector (Invitrogen) at EcoRI/XhoI sites. Human RGS16 gene were cloned from cDNA of 293T cell. Then cloned RGS16 gene were inserted into pCMV14/3xFLAG vector using HindIII/BamHI sites. C-RGS4 genes are sub-cloned into previously established pcDNA3 plasmid URT construct (1) and subjected to site-directed-mutagenesis to generate RC/V-RGS4 mutant.

Plasmid mentioned above are transfected to indicated cell lines using Lipofectamine 2000 reagent (HEK 293T and PC-12), Lipofectamine LTX reagent (MEFs), and Lipofectamine 3000 reagent (SH-SY5Y) from Thermo Fisher Scientific. All the chemicals other than mentioned are purchased from Sigma Aldrich.

RNA interference analysis

Cells were cultured in 6-well plate (1.2 x 106 per well) and transfected with either negative control siRNA (Bioneer, 4390843), or sip62 (Bioneer), siATE1(Invitrogen, s21887), siUBR4(Invitrogen, s23628), siUBR3 (Bioneer), siUBR6 (Bioneer), siUBR7(Invitrogen, s30283), siADO (Bioneer), siTOM70 (Bioneer), siKCMF1 (Bioneer) using Lipofectamine RNAiMAX reagent (Invitrogen, 13778150). Final concentrations of siRNAs except for siUBR4 were 40nM, and that of siUBR4 was 20nM. 48h after transfection, cells were treated for indicated chemicals or harvested for immunoblotting and immunocytochemistry. The sequences of pre-designed siRNAs are as follows: sip62 (sense, 5'-GUGAACUCCAGUCCCUACA-3'; antisense, 5'-UGUAGGGACUGGAGUUCAC-3'), siATE1 (sense, 5′-ACCCACCAUCUUUGUUUCCACCAAA-3'; antisense, 5'-UUUGGUGGAAACAAAGAUGGUGGGU-3′), siUBR4 (sense, 5′-GCCUGUUCGAAAGCGCAAA-3′; antisense, 5′-UUUGCGCUUUCGAACAGGC-3′) siUBR3 (sense, 5'-CCGUCUUUGAAAGAUUUAA-3'; antisense,5'-UUAAAUCUUUCAAAGACGG-3'), siUBR6 (sense, 5'-GCAGACUGGAGGAAUAUAU-3'; antisense,5'-

AUAUAUUCCUCCAGUCUGC-3'), siUBR7 (sense, 5'-GCAAGAGACCUUAUCCUGA-3'; antisense,5'-UCAGGAUAAGGUCUCUUGC-3').

Antibodies

Following list of antibodies were used for this study: rabbit polyclonal anti-RGS4 (Merck, ABT-17, 1:2000), rabbit polyclonal anti-RGS5 (Bioworld, BS5897, and Sigma Aldrich, HPA001821, both in 1:1000), rabbit polyclonal anti-RGS16 (Bioworld, BS70592, 1:1000), rabbit polyclonal anti-UBR4 (Abcam, ab86738, 1:3000), rabbit polyclonal anti-KCMF1 (Thermo Scientific, PA5-56453, 1:2000), polyclonal anti-LC3 (Sigma Aldrich, L7543, 1:20000), mouse monoclonal anti-p62(Abcam, ab56416, 1: 10000), mouse monoclonal anti-b-actin (Sigma Aldrich, A1978, 1:10000), mouse monoclonal b-tubulin (Santa Cruz, SC-55529, 1:1000), rabbit polyclonal anti-GAPDH (Bioworld, AP0063, 1:20000), mouse monoclonal anti-FK2 (Enzo, BML-PW8810, 1:3000), rabbit polyclonal anti-K48 linkage specific ubiquitin (Abcam, ab140601, 1:2000), rabbit polyclonal anti-K63 linkage specific ubiquitin (Abcam, ab179434,1:3000), anti-K27 linkage specific ubiquitin (Abcam, ab181537, 1:3000), rabbit polyclonal anti-Myc (Cell signaling, 2278S, 1:1000), mouse monoclonal anti-FLAG M2 (Sigma, F1804, 1:1000), rabbit polyclonal anti-FLAG (Sigma, F7425, 1:10000), rabbit polyclonal anti-PARP (Cell signaling, 9542, 1:3000), mouse monoclonal anti-CHOP (Cell signaling, 2895, 1:2000), rabbit polyclonal anti-cleaved caspase3 (Cell signaling, 9661,1:1000), rabbit anti-p38 MAPK (Cell signaling, 9212, 1:1000), rabbit anti-phospho p38 MAPK (Cell signaling, 9211, 1:1000), mouse anti-MTCO2 (Abcam, ab110258, 1:2000), rabbit anti-TOMM22 (Sigma Aldrich, , 1:2000), anti-FLAG M2 affinity gel agarose beads (Sigma), anti-Myc affinity gel agarose beads (Thermo Scientific).

Following list of secondary antibodies were used: alexa fluor 488 goat anti-rabbit IgG (Invitrogen, A11034, 1:400), alexa fluor 488 goat anti-mouse IgG (Invitrogen, A11029, 1:400), alexa fluor 555 goat anti-rabbit IgG (Invitrogen, A32732, 1:400), alexa fluor 555 goat anti-mouse IgG (Invitrogen, A32727, 1:400), alexa fluor 633 goat anti-rabbit IgG (Invitrogen, A21071, 1:400), alexa fluor 633 goat anti-mouse IgG (Invitrogen, A21052, 1:400), anti-rabbit IgG-HRP (Jackson

ImmunoResearch, 111-035-003, 1:10000), and anti-mouse IgG-HRP (Jackson ImmunoResearch, 115-035-003, 1:10000).

Cell culture and immunoblotting

HeLa and HEK293T cells were purchased from ATCC (American Type Culture Collection). SH-SY5Y and PC-12 cells were obtained from Korean Cell Line Bank. Wild type and 62-/- MEF cells were obtained from Keiji Tanaka's laboratory (Tokyo Metropolitan Research Institute, Tokyo, Japan) with the permission of T. Ishii. The constructions of MEFs were previously mentioned; *ATE1*$^{-/-}$ (2), *UBR1*$^{-/-}$*UBR2*$^{-/-}$ (1), *UBR4*$^{-/-}$ (3). Cells were resuspended at PBS and lysed using 2× Laemmli sample buffer (1610747; Bio-Rad, [277.8 mM Tris-HCl, pH 6.8, 4.4% LDS, 44.4% (v/v) glycerol]) with 10% beta-mercaptoethanol. Then whole cell lysates were separated by SDS/PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in PBST (PBS and 0.1% Tween 20) for 30 min at room temperature. Subsequently, the membranes were incubated with primary antibodies and washed 3 times with PBST for 10 min. Then, membranes were incubated with host-specific secondary antibodies conjugated with HRP for 1 h and washed for 3 times as mentioned above. For detection, a mixture of SuperSignal West Pico Chemiluminescent Substrate (34080; ThermoFisher) or SuperSignal West Femto Maximum Sensitivity Substrate (34095; ThermoFisher) were used to visualize immunoreactive proteins on to X-ray films.

Immunocytochemistry

Cells were cultured on coverslips coated with poly-L-Lysine in 24-well plate followed by fixation with 4% paraformaldehyde in PBS (pH7.4) for 15 min and washed with PBS for 5 min three times. Subsequently, the cells were permeabilized with 0.5% Triton X-100 in PBS for 15 min followed by washing three times with PBS for 5 min. After blocked with 2% BSA in PBS for 1 hr, the cells were incubated with primary antibodies overnight at 4 °C. The cells were washed for 10 min three times and incubated with Alexa Fluor conjugated secondary antibodies for 30 min at room temperature. Then, washing three times, the coverslips were mounted on slide glasses using

VECTASHIELD hard set mounting medium with DAPI (H-1500). Confocal images were taken using laser scanning confocal microscope 510 Meta (Zeiss) and analyzed by Zeiss LSM Image Browser (Blue edition, ver. 4.2.0.121). Subsequently, for puncta counting analysis, cells were counted as showing significant co-localization if more than ten clear puncta structures of the proteins in question showed association and/or co-localization. Quantification results are shown as mean +/- S.D. of at least three independent experiments.

Co-immunoprecipitation

Co-immunoprecipitation assay was performed to demonstrate physical interaction of the proteins of interest. Cells were harvested by scraping and centrifuged using ice-cold PBS. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail) and incubated on rotator for 30 min at 4°C. After incubation cell debris were removed by centrifugation at 13000 rpm for 10 min. Supernatant were subjected to pre-clearing using normal mouse IgG and A/G agarose bead (Santa Cruz) overnight on rotator at 4°C. After incubation, samples were centrifuged at 13000 rpm for 10 min to remove non-specific protein bound IgG and bead. Pre-cleared supernatant was incubated with anti-FLAG M2 affinity agarose beads (Sigma) or anti-c-Myc affinity agarose beads (Thermo) for 3 h on rotator at 4°C. Next, incubated anti-FLAG M2 beads were precipitated and washed four times with identical lysis buffer. Then bound proteins were eluted at 2X Laemmli sample buffer by heating 10 min at 95 °C, separated by SDS PAGE, and immunoblotted using antibodies of interests.

Denaturation-ubiquitylation assay

Ubiquitylation status of substrate proteins were investigated under denaturation immunoprecipitation. Either WT or selective Lys (K48 or K63) only remained ubiquitin coding plasmid were transfected to cells of interests. Cells were harvested using ice-cold PBS by scraping. Harvested pellets were lysed using 100 μl of N-ethylmaleimide (NEM)-based buffer (1% SDS, 1 mM NEM in PBS) and heated at 100°C for 10 min. Then lysates were 1/10 diluted using

lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail) and following steps are identical to the method used in co-IP.

***In vitro* peptide pull-down assay**

A set of 12-mer peptides corresponding N-terminal 11mer sequences reflecting MetAP cleavage and post translational modification of RGS4 and Lys at the C-terminal for biotinylation was synthesized. A set of X-RGS4 peptides (X-KGLAGLPASCLK-biotin) was composed of Arg-Cys^2_{ox}(Cys^2_{ox} reflects tri-oxidized second Cys residue), Cys^2(native), Val^2(Cys^2-to-Val^2 mutant). 400 μg of synthesized peptides were mixed to 1 ml of high -capacity streptavidin agarose resin (20361; Thermo) and conjugated at 4 °C overnight. Streptavidin resin were washed 5 5imes with PBS. Harvested cells were resuspended in hypotonic buffer [10 mM KCl, 1.5 mM MgCl2, and 10 mM HEPES (pH 7.9)] with a protease inhibitor mix (P8340; Sigma) and lysed by repetitive freezing and thawing process. Protein lysates were centrifuged at 13000 rpm for 10 min. for removal of cellular debris. Supernatant were quantified by BCA protein assay kit (23227; Thermo). 600 μg of protein lysates were mixed with 50 ul of peptide conjugated resin and binding buffer [0.05% Tween-20, 10% glycerol, 0.2 M KCl, and 20 mM HEPES (pH 7.9)] was added up to 300ul. The mixture of lysates and resin were incubated at 4 °C for 4 hr on a rotator. Protein-bound bead were washed for 5 times with binding buffer and resuspended in 35 μl of 2X Laemmli sample buffer. Samples were heated for 10 min at 95 °C, separated by SDS PAGE, and immunoblotted.

Digitonin based mitochondria fractionation

Cells were harvested via centrifugation at 1500xg for 5min. The plasma membranes of the harvested cells were resuspended using digitonin based lysis buffer (0.01% digitonin, 110 mM potassium acetate, 25 mM HEPES in pH7.2, 2.5 mM sodium acetate and 1mM EGTA) and incubated on ice for 15 min. Mitochondria were pelleted by centrifugation at 1000xg for 5 min. Resulting cytosolic supernatant was transferred to new tube and re-centrifuged at 15000xg for 15min for complete removal of contaminating organelle portion. Mitochondria containing pellet

was washed for three times using identical lysis buffer following centrifugation at 15000xg for 5 min. Laemmli sample buffer was added to cytosolic portion and 0.01% digitonin-insoluble pellets which were resuspended by PBS. Samples were heated for 10 min at 100°C, separated by SDS PAGE, and immunoblotted.

Cycloheximide based protein degradation assay

Either WT or C2V mutant RGS4 coding plasmids were transfected to selected cell lines using indicated lipofectamine reagent mentioned above. After 24 h or transfection, oxidative stress was induced by indicated chemicals for 6 h. Then, media was exchanged into the mixture of media with either 10 μM cycloheximide (CHX) alone or in combination with oxidative stress inducing chemicals. From the point of CHX treatment, cells were harvested at indicated time point and subjected to analyzation using SDS-PAGE and immunoblotting.

Quantitative real-time PCR analysis

Upon treatment of oxidative stress inducing chemicals for indicated time, mRNA of treated cells was extracted using Trizol reagent (Invitrogen). 1 μg of extracted RNA were used to synthesize cDNA via using PrimeScript 1st strand cDNA synthesis kit (Takara, 6110). Synthesized cDNAs are 1/2 diluted using 20 μl D.W. and 2 μl of the cDNA were used to run quantitative RT PCR. 2X Fast Q-PCR master mix with SYBR (SMOBIO, TQ1210) were used to measure the expression of the gene of interest. Following sequences are the primer sequences for measuring indicated gene expression: GAPDH (5'-GCGCCCAATACGACCAA-3', 5'-CTCTCTGCTCCTCCTGTTC-3'), RGS4 (5'-TGGTGCAAGAATCCAGGTTC-3', 5'-CCACAACAAGAAGGACAAAGTG-3') iNOS (5'-CACCATCCTCTTTGCGACA-3', 5'-CGAGCTCAGCCTGTACT-3'), SOD1 (5'-CCTCGGAACCAGGACCT-3', 5'-TTAATGCTTCCCCACACCTT-3'), TGF-β (5'-CCGACTACTACGCCAAGGA-3', 5'-GTTCAGGTACCGCTTCTCG-3'), COX2 (5'-GCACTACATACTTACCCACTTCA -3', 5'-GCCATAGTCAGCATTGTAAGTTG-3').

Statistical analysis

For immunocytochemistry, data values shown represent the mean ± S.D. of at least three independent experiments. For each experiment, sample size (n) was determined as stated in the figure legends. For quantitative real time PCR analysis, each set of experiments was triplicated and performed three times. P-values were determined using ANOVA with Prism 6 software (GraphPad) or two-tailed student's t-test (degree of freedom = n-1). Statistical significance was determined as values of $p < 0.05$ (***$p < 0.001$; **$p < 0.01$; *$p< 0.05$).

Mass spectrometry analysis

Peptide sample preparation

$RGS4^{MYC}$ proteins are transiently expressed in HEK293T cells (4X 100mm dishes per sample) and treated with either MG132 (10 μM) or combination treatment of tBHP (250 μM) and BAF (20 nM) for 6hrs. Cells were harvested and lysed according to co-IP method and immunoprecipitated with MYC-agarose beads overnight (Invitrogen, 20169,100ul slurry per sample). Beads were washed with lysis buffer for 1hr at 4℃ and washes are repeated 5 times. Protein digestion of IP eluent was performed via the Filter-aided sample preparation (FASP) procedure as described with some modifications (PMID: 24753479, PMID:26227174). Briefly, 50ul of the elutes were mixed with 300ul of UA buffer (8 M Urea in 0.1 M Tris pH 8.5) and loaded onto a 30 K Amicon filter (Milipore). The buffer exchanges were performed with UA solution via centrifugation at 14,000xg for 15min. Following the exchange of buffer with 40 mM ammonium bicarbonate (ABC), protein digestion was performed at 37℃ overnight using a trypsin/LysC mixture (Promega) at a 100:1 protein-to-protease ratio. The digested peptides were collected by centrifugation. After the filter units were washed with 40 mM ABC, additional digestion was performed at 37℃ for 2 hours using trypsin (enzyme-to-substrate ratio [w/w] of 1:1000). All resulting peptides were acidified with 10% TFA and desalted using homemade SDB-C18-StageTips as described (PMID: 24753479, PMID:26227174). Desalted samples were completely dried with a vacuum dryer and stored at -80℃.

LC-MS/MS analysis

LC-MS/MS analysis was performed using hybrid quadrupole Orbitrap mass spectrometers, Q-exactive plus (Thermo Fisher Scientific, Waltham, MA) coupled to an Ultimate 3000 RSLC systems (Dionex) via a nano electrospray source, as described with some modifications (PMID:24753479, PMID:29950347). Peptide samples were separated on the 2-column setup with a trap column (300 μm I.D. x 5 mm, C18 3 μm, 100 Å) and an analytical column (50 μm I.D. x 50 cm, C18 1.9 μm, 100 Å). After the samples were loaded onto the nano LC, a 180-minute gradient from 8% to 26% solvent B (100% acetonitrile and 0.1% formic acid) was applied to all samples. The spray voltage was 2.0 kV in the positive ion mode, and the temperature of the heated capillary was set to 320°C. Mass spectra were acquired in data-dependent mode using a top 15 method. The Orbitrap analyzer scanned precursor ions with a mass range of 300–1800 m/z and a resolution of 70,000 at m/z 200. HCD scans were acquired at a resolution of 17,500 with the stepped normalized collision energy (NCE) of 27 ± 2%. The maximum ion injection time for the survey and MS/MS scans was 25 ms and 50 ms, respectively.

Data processing

MS raw files were processed by the Maxquant software version 1.6.1.0 (PMID:27809316). MS/MS spectra were searched against the sequence of Human RGS4 protein with C-terminal Myc-tag using the Andromeda search engine (PMID:21254760). Primary searches were performed using a 6-ppm precursor ion tolerance. The MS/MS ion tolerance was set to 20 ppm. Digestion mode was set as “no digestion”. To identify modification sites of cysteine, carbamido-methylation, di-oxidation, and tri-oxidation on cysteine, N-terminal acetylation, and oxidation on methionine were set as variable modifications. The minimum score for the modified peptides was set to 40. To assign modification sites, localization probability of 0.75 was set as a threshold.

Supplementary figure 1. Heo et al.

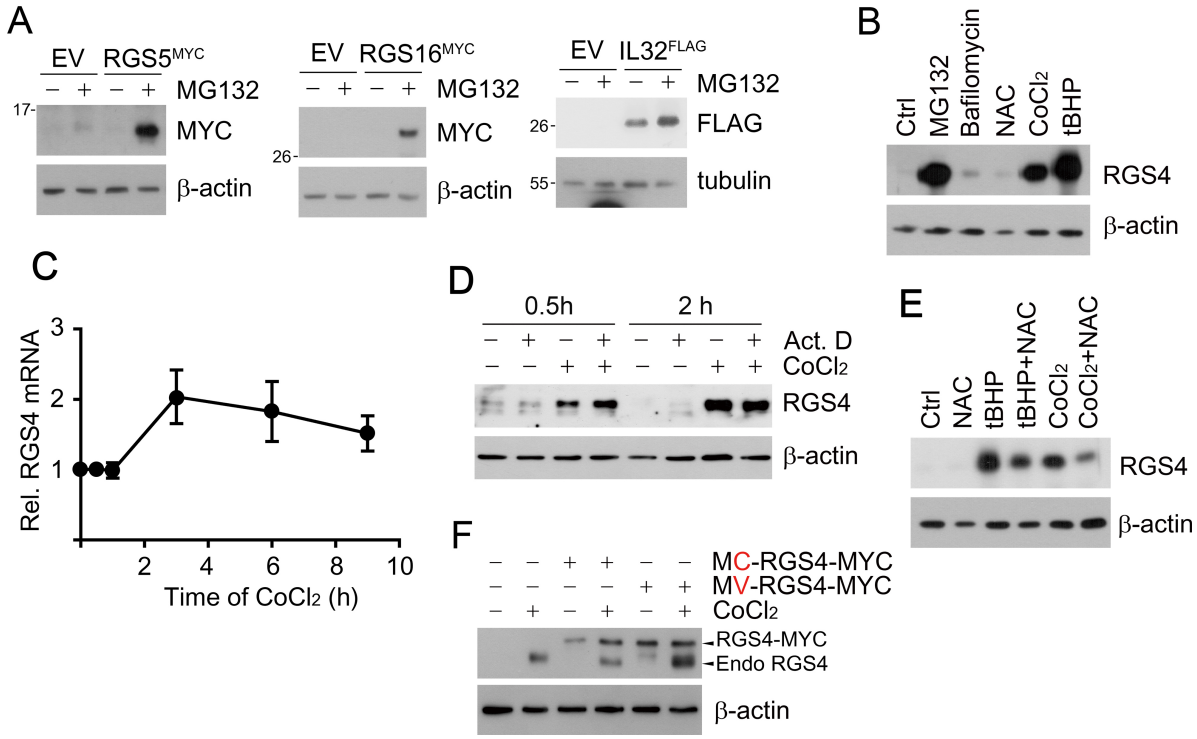

Figure S1. Cys/N-degron substrates are post-translationally stabilized under oxidative stress. (A) WB of RGS5^{MYC}, RGS16^{MYC} and IL32^{FLAG}-overexpressing HEK293T cells treated with MG132 (10 μM, 6 h). **(B)** WB of RGS4 in SH-SY5Y cells treated with a combination of MG132 (10 uM), BAF (200 nM), NAC (0.5 mM), $CoCl_2$ (250 μM) and tBHP (250 μM) for 6h. **(C)** Relative RGS4 mRNA levels in SH-SY5Y cells treated with $CoCl_2$ (250 μM) at the indicated time points. **(D)** WB of RGS4 in SH-SY5Y cells treated with actinomycin D (5 nM) and $CoCl_2$ (250 μM **(E)** Same as with **B** but with a combination of tBHP, $CoCl_2$ and NAC. **(J)** WB of SH-SY5Y cells treated with $CoCl_2$ (250 μM) at the indicated time points. **(F)** WB of SH-SY5Y cells expressing wild-type and C2V-mutant RGS4-MYC and treated with $CoCl_2$ (250 μM, 24 h). (I) Immunoblotting analysis of RGS4 and HIF1a in SH-SY5Y cells.

Supplementary figure 2. Heo et al.

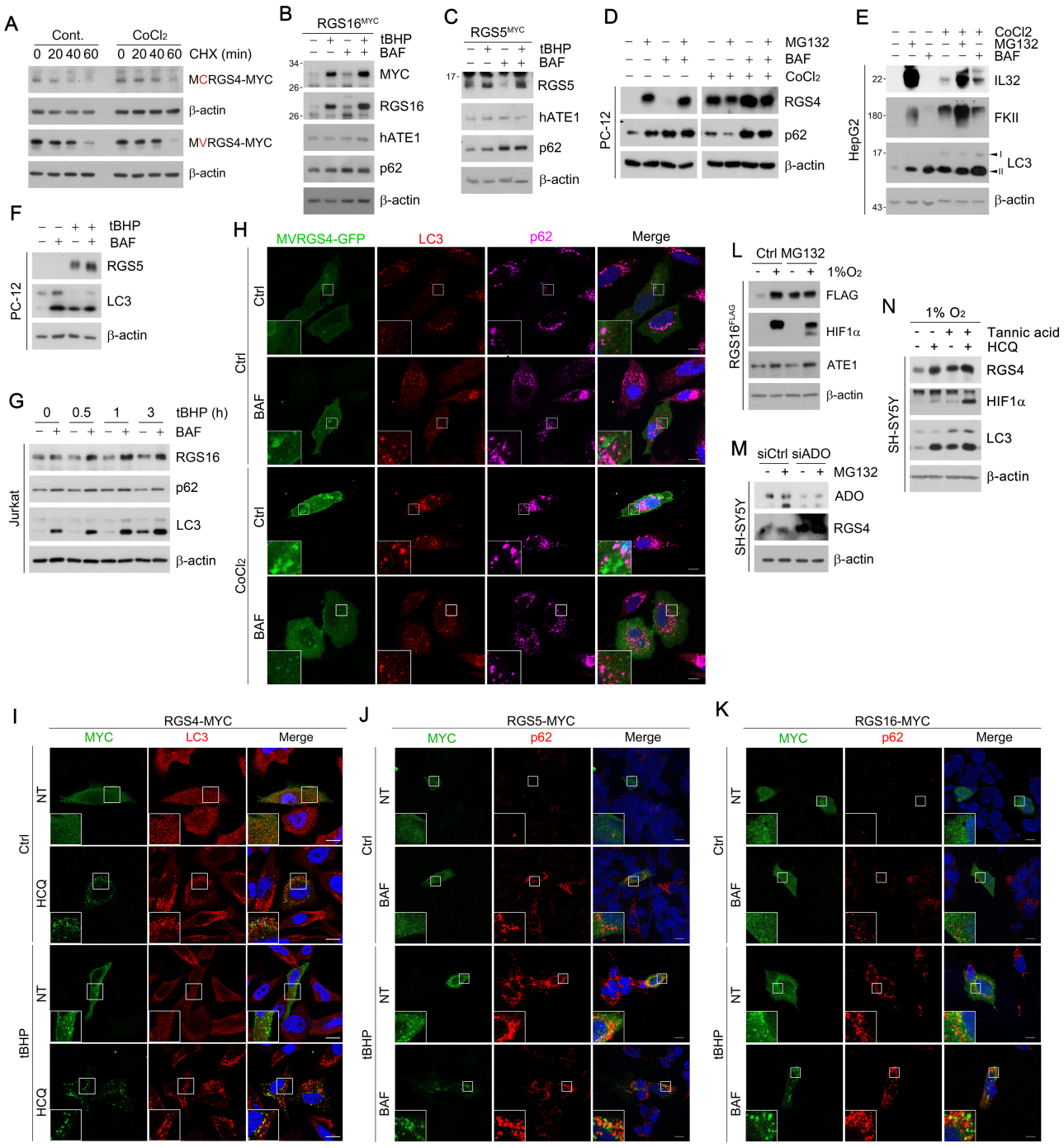

Figure S2. Cys/N-degron substrates are degraded via autophagy under oxidative stress. **(A)** CHX chase analysis of HEK293T cells expressing MC-RGS4^{MYC} or C2V-RGS4^{MYC} mutant and treated with $CoCl_2$ (250 μM, 24 h). **(B,C)** WB of RGS16^{MYC} (**B**) or RGS5^{MYC} (**C**) overexpressed 293T cells treated with tBHP (250 μM, 6 h), BAF (200 nM, 6 h) or both. **(D)** WB of endogeneous RGS4 in PC12 cells treated with a combination of $CoCl_2$ (250 μM, 6 h), BAF (200 nM, 6 h) and MG132 (10 μM, 6 h). (**E**) Same as **D** but HepG2 cells were used. (**F**) Same as **D** but treated with a combination of tBHP (250 μM, 6 h) and BAF (200 nM, 6 h). **(G)** WB of endogeneous RGS16 in Jurkat cells treated with tBHP (250 μM, 6 h) and BAF (200 nM, 6 h) for indicated time. (**H**) Co-localization assay of the GFP, p62 and LC3 puncta in C2V RGS4-GFP expressing HeLa cells treated with 6h BAF (200 nM, 6 h), $CoCl_2$, or both. Scale bar, 10 μm **(I)** Co-localization analysis of RGS4^{MYC} and LC3 in HeLa cells treated with tBHP (250 μM, 6 h) and HCQ (25 μM, 24 h). **(J)** ICC in RGS5^{MYC} overexpressing HeLa cells treated with tBHP (250 μM, 6 h) and BAF (200 nM, 6 h). **(K)** Same as **J** but with RGS16^{MYC} overexpression. (**L**) WB of 293T cells expressing RGS16^{FLAG} and exposed to acute hypoxia (1% O_2, 6 h) and MG132 (10 μM, 6 h).(**M**) WB of SH-SY5Y cells under *ADO* interference (20 nM, 48 h) treated with MG132 (10 μM, 6 h). (**N**) WB of SH-SY5Y cells exposed to chronic hypoxia (1% O_2, 24h)and treated with tannic acid (35 μM) and HCQ (25 nM, 24h).

Supplementary figure 3. Heo et al.

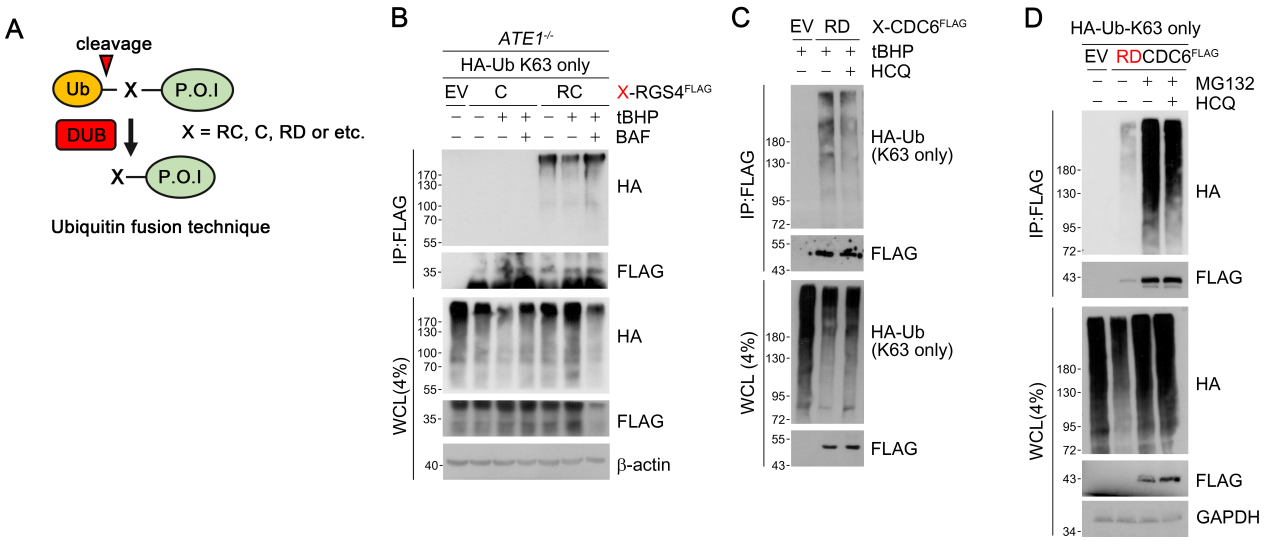

Figure S3. Nt-Cys substrate-specific Ub repriming induces autophagic degradation under oxidative stress (A) Graphical demonstration of the ubiquitin fusion technique (UFT). **(B)** Denaturation IP assay in *ATE1*$^{-/-}$ MEFs expressing HA-Ub-K63-only mutant and C- or RC-RGS4^{FLAG}, treated with tBHP (250 μM, 6 h) and BAF (200 nM, 6 h). **(C)** Denaturation IP assay in RD-CDC6^{FLAG} and HA-Ub-K63-only mutant-expressing HEK293T cells treated with tBHP (250 μM, 6 h) and HCQ (25 nM, 24 h). **(D)** Same as with **C** but treated with MG132 (3 μM, 24 h) and HCQ (25 nM, 24 h).

Supplementary figure 4. Heo et al.

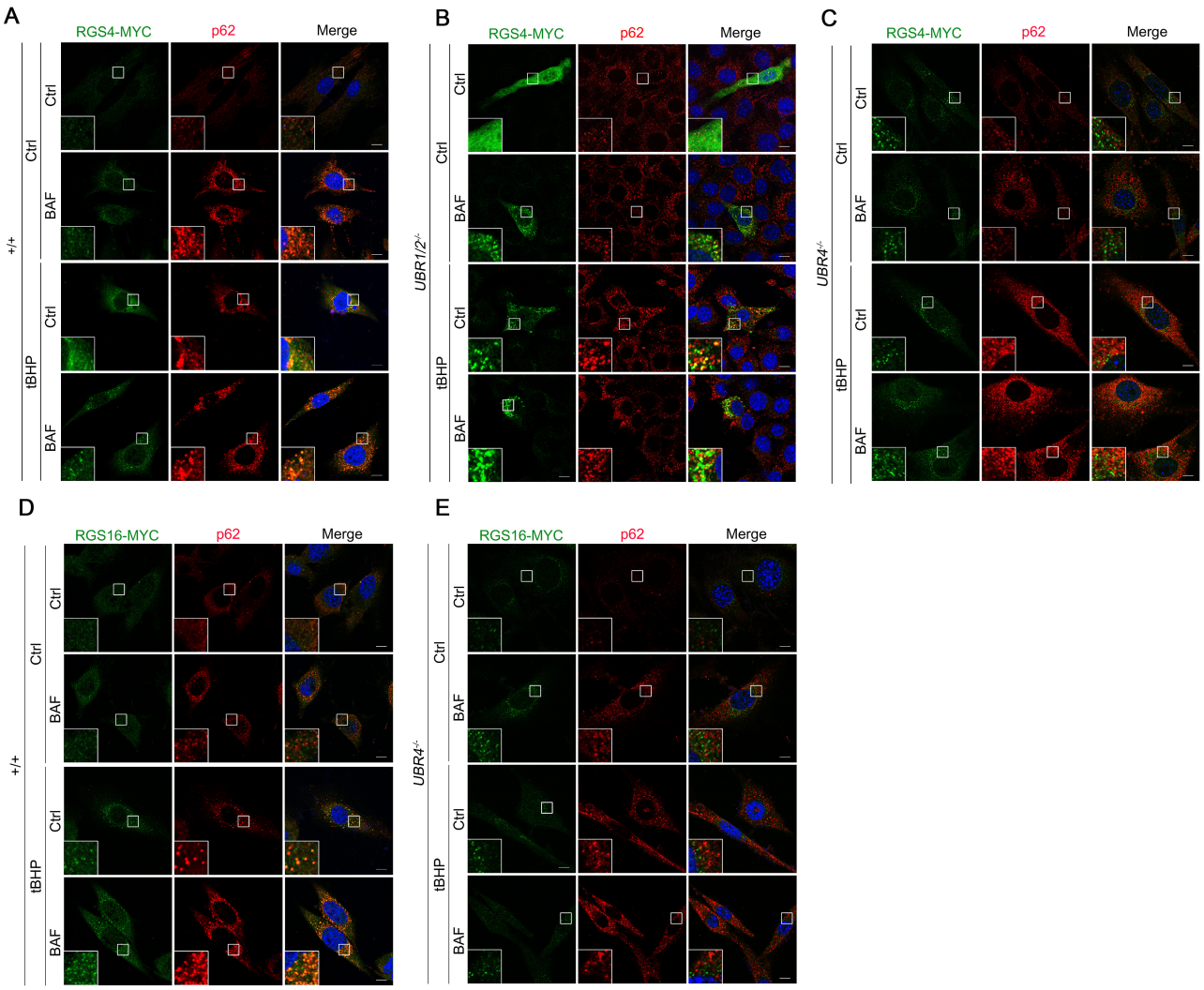

Figure S4. UBR4 is responsible for autophagic targeting of Cys/N-degron substrates. (A) Co-localization analysis of RGS4 and p62 in RGS4^{MYC}-overexpressed MEF cells treated with tBHP (250 μM, 6 h) and BAF (200 nM, 6 h). **(B,C)** Same as with **A** but in *UBR1*$^{-/-}$*UBR2*$^{-/-}$or *UBR4*$^{-/-}$ MEFs. **(D)** Same as **A** but with RGS16. **(E)** Same as **D** but in *UBR4*$^{-/-}$MEFs.

Supplementary figure 5. Heo et al.

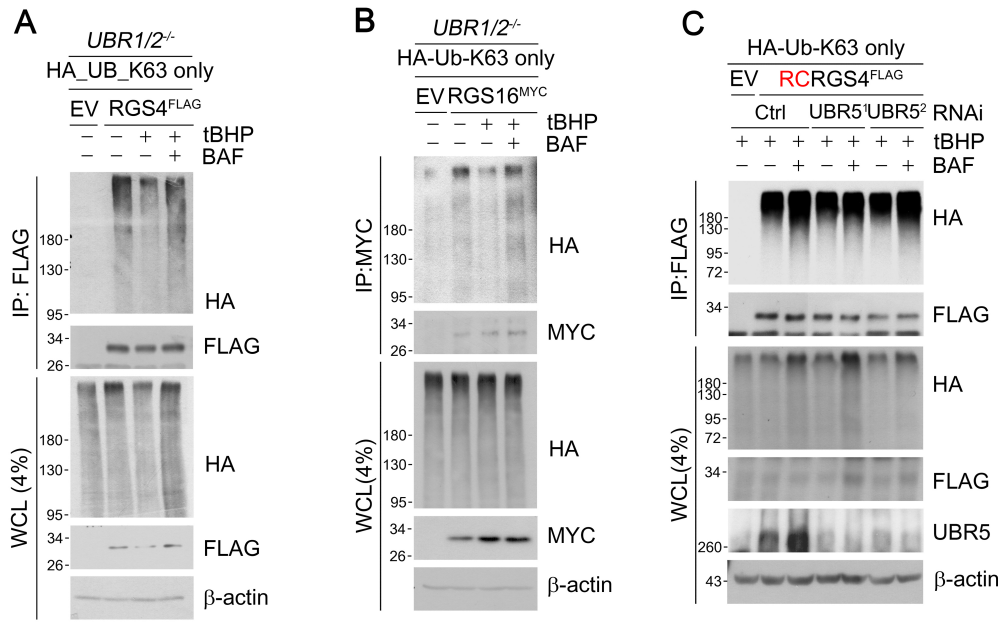

Figure S5. UBR4 is responsible for ubiquitylation of the Cys/N-degron substrates under oxidative stress. (A) Denaturation IP assay in *UBR1*$^{-/-}$*UBR2*$^{-/-}$ MEF cells transfected with HA-Ub-K63 only mutant and RGS4^{MYC} and treated with tBHP (250 μM, 6 h), BAF (20 nM, 6 h) or both. **(B)** Same as with **C** but with RGS16^{MYC}. **(C)** Denaturation IP assay in RCRGS4^{FLAG} and HA-Ub-K63-only mutant-transfected HEK293T cells following *UBR5* interference and treatment with tBHP (250 μM, 6 h) and BAF (200 nM, 6 h).

Supplementary figure 6. Heo et al.

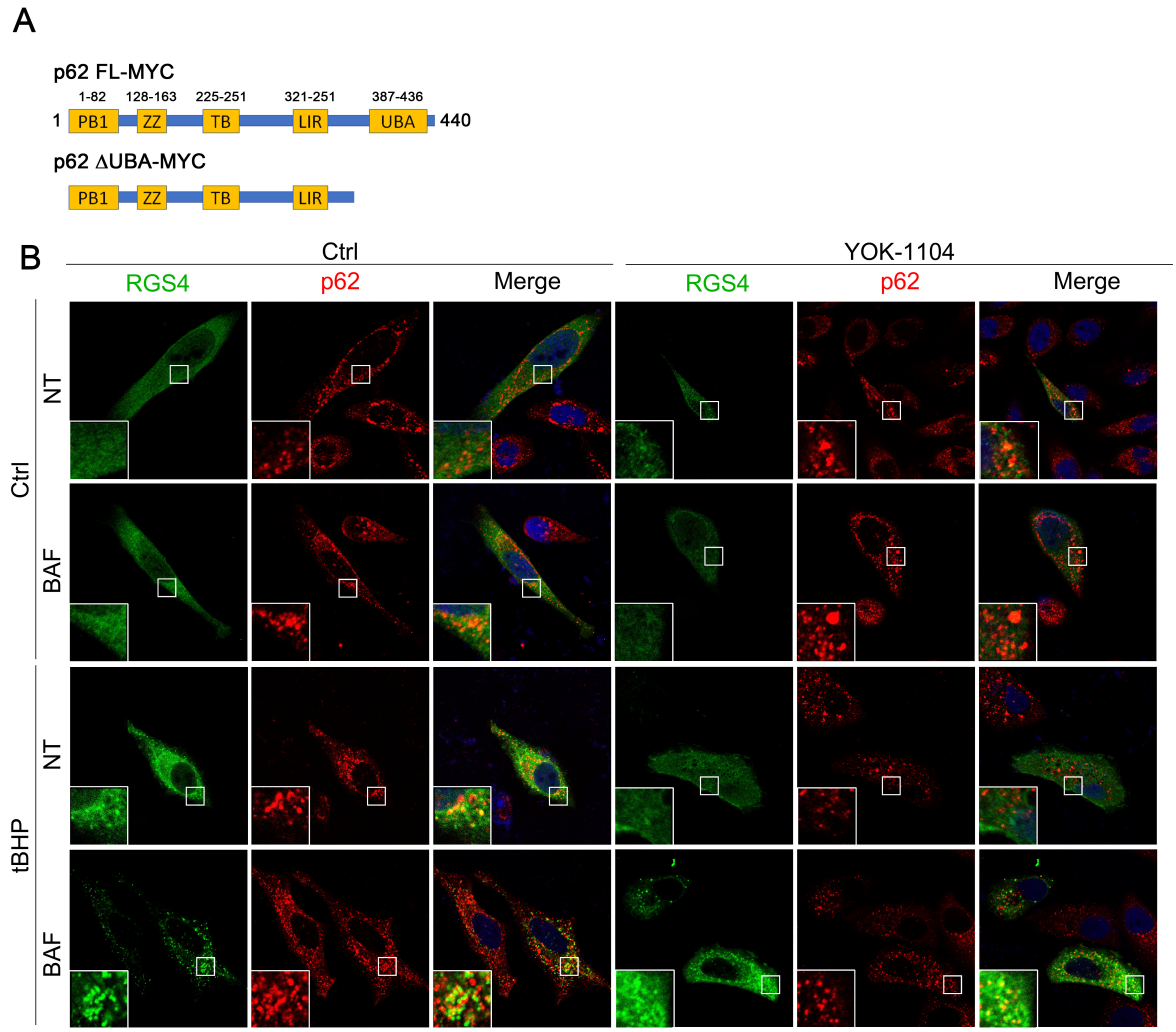

Figure S6. Stimulation of p62 activity enhances autophagic targeting of the N-degron substrates. (A) Graphical figure of p62 plasmid constructs. **(B)** ICC of SH-SY5Y cells treated with tBHP (250 μM, 6 h), BAF (200 nM, 6 h) and YOK-1104 (10 μM, 6 h).

Supplementary figure 7. Heo et al.

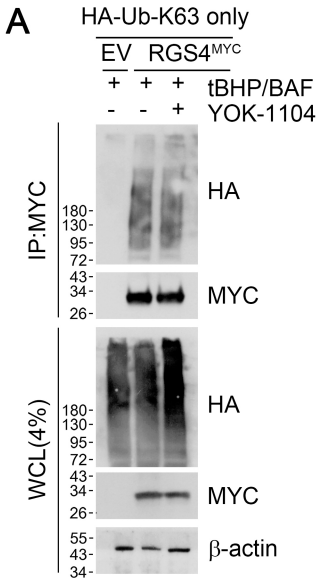

Figure S7. N-degron mimicking substrates do not alter KCMF1 activity. (A) denaturation immunoprecipitation assay of the HEK293T cells transfected with $RGS4^{MYC}$ and HA-Ub-K63 only construct with co-treatment of tBHP (250 μM, 6 h) and BAF (200 nM, 6 h) and YOK-1104 (10 μM, 6 h).

Supplementary figure 8. Heo et al.

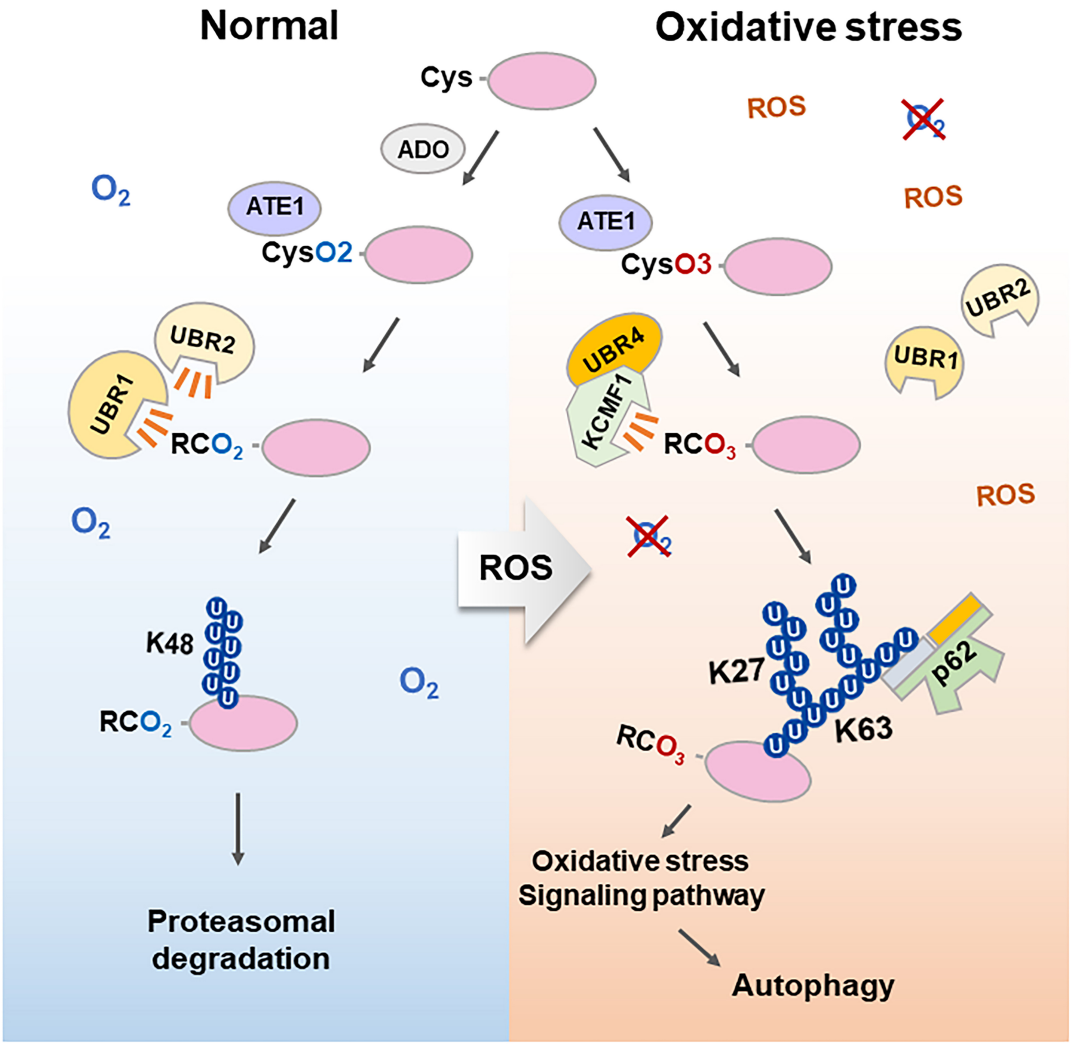

Figure S8. Graphical abstract of the Cys/N-degron pathway. (A) Graphical abstract demonstrating mammalian Cys/N-degron pathway under normoxia and oxidative stress.

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1. Y. T. Kwon, Z. Xia, I. V. Davydov, S. H. Lecker, A. Varshavsky, Construction and analysis of mouse strains lacking the ubiquitin ligase UBR1 (E3alpha) of the N-end rule pathway. *Mol Cell Biol* **21**, 8007-8021 (2001).
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3. S. T. Kim *et al.*, The N-recognin UBR4 of the N-end rule pathway is targeted to and required for the biogenesis of the early endosome. *J Cell Sci* **131** (2018).