This file contains Supplementary Methods for the “Assurance of mitochondrial integrity and mammalian longevity by the p62-Keap1-Nrf2-Nqo1 cascade” by Kwon et al. created in Acrobat files (.pdf).
Supplementary Methods

Mice The p62\(^{-/-}\) mice were generated by homologous recombination, following the protocols described previously (Hogan, 1994). A p62 genomic clone was isolated from a mouse 129/Sv genomic library. For targeting vector construction, a DNA fragment between the EcoRI and SspI restriction sites, containing the first and second exons of the p62 gene, was removed and replaced by neomycin resistant cassette in the vector, pPNT. The targeting construct was linearized by NotI, and introduced by electroporation into the HPRT-deficient mouse embryonic stem (ES) cell line, E14TG2a, which was derived originally from the 129/Ola mice. Gap-repair targeting was detected in 6 out of 256 colonies, surviving G418 and ganciclovir selection. ES cells that homologously integrated the mutated allele were injected into the blastocysts derived from the C57BL/6J mice. Chimeric mice able to transmit the mutated allele through germ line were obtained, and the heterozygous mice were crossed to obtain mice homozygous for the targeted p62 gene mutation. Genotyping was performed by PCR using three oligomeric primers (F1, R1, and R2) described in Supplementary Fig. 3. and confirmed by Southern blot analysis of DNA from the tail tissue. The mutation at the p62 gene locus was then transferred onto the inbred strain C57BL/6J by backcrossing more than 6
times and used for rest of experiments. All p62 mutant mice were bred by mating 10- to 20-week-old heterozygous male and female mice. Water and regular chow (LabDiet 5L79 containing 5.2 % fat) were available ad libitum, and all mice were handled in the AAALAC accredited Sungkyunkwan Medical School Animal Care Facility. Animal procedures complied with NIH guidelines and were approved by the Sungkyunkwan University Animal Care and Use Committee. Kaplan-Meier method was used to determine cumulative survival of wild-type and p62⁻/⁻ mice.

**Aging phenotype analyses** Lordokyphosis was evaluated by dorsal spine angles measured from X-ray radiographs (20 kV for 20 s) of anesthetized mice. Dorsal skins (n = 6 per group) were fixed, embedded in paraffin, sectioned in 4 μm, and stained with haematoxylin and eosin. Serum concentrations of glucose and insuline were determined by the Fuji automated blood chemistry analyzer and RIA kits (Linco), respectively. For this, serum was prepared from the blood withdrawn from mice after fasting for 16 hr.

**Indirect calorimetry** Oxygen consumption was determined by indirect calorimetry (Butler et al., 2001) using a four-chamber calorimetry system (Oxymax, Columbus Instruments) with one mouse per chamber. Mice were acclimatized to the chambers for
2 hr and oxygen consumption was recorded for 1 hr during the middle of light cycle. Measurements were recorded every 20 sec with the room air reference taken every 30 min and the air flow to chambers 1 L/min.

**GSH/GSSG in isolated tissues** GSH/GSSG was measured by a GSH reductase-mediated recycling assay, using a BIOXYTECH® GSH/GSSG-412™ kit (Oxis International). Briefly, tissues from the 8-week-old male mice fed *ad libitum* were homogenized in an ice-cold 5% metaphosphoric acid solution and centrifuged at 1000 x g for 10 min. Supernatants were immediately used to determine the total glutathione contents (GSH + GSSG). For instance, 5 µl supernatant was mixed with 355 µl assay buffer (100 mM NaHPO₄, 5 mM EDTA, pH 7.5), then with 1.262 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 15 units/ml GSH reductase. The reaction mixture was incubated at room temperature for 5 min, added 3.8 µmol NADPH, and the absorbance was recorded at 412 nm for 3 min, using a spectrophotometer (Molecular Devices, Spectra MAX 340). GSSG content was separately measured following the same protocol after GSH in homogenates was scavenged by 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate. Concentrations of total and oxidized glutathiones were
calculated using standard curves prepared with known amounts of purified GSH and GSSG.

**Measurement of oxidized macromolecules** Immunohistochemical staining of 4-HNE was performed for the brains of 95-week-old p62<sup>++</sup> and p62<sup>-/-</sup> mice. Anesthetized mice were perfused with 100 ml phosphate-buffered saline (PBS), and isolated brain was fixed with 4% paraformaldehyde, embedded in paraffin, and coronally sectioned. Sections (4 μm thick) were then immunostained with a monoclonal anti-4-HNE antibody (JaICA) and Vectastain ABC kit (Vector Laboratories, Burlingame, CA) (40 x magnification). Carbonylated protein levels in p62<sup>++</sup> and p62<sup>-/-</sup> MEFs were assessed using the Oxyblot kit (Chemicon Co.) and following protocols suggested by the manufacturer. Protein carbonyls were measured by densidometric analysis of the anti-protein carbonyl immunoblot. 8-oxoguanine (8-ohG) levels in the p62<sup>++</sup> and p62<sup>-/-</sup> MEFs were determined using the fluorogenic OxyDNA Assay Kit (Calbiochem), following protocols suggested by the manufacturer. Cells containing the resultant FITC-conjugate-bound 8-ohG were resuspended, and analyzed by flow cytometry using FACScan (Becton Dickinson).
**Analysis of tissue mitochondria** Mitochondria were isolated by differential centrifugation and iodixanol density gradient centrifugation (Sharer et al., 2002). Briefly, whole tissues were homogenized in the isolation buffer (10 mM Hepes, 250mM sucrose, 1 mM EDTA, pH 7.4, supplemented with protease inhibitor cocktail) and centrifuged twice at 2,000 x g for 5 min at 4°C. Supernatant was then centrifuged at 17,000 x g for 10 min at 4°C, and the resultant pellet suspended in the isolation buffer (P17; mitochondria-enriched fraction). The P17 fraction was further purified on an iodixanol gradient centrifugation using OptiPrep kit (Nycomed Pharm) and following protocols suggested by manufacturers (Op-M fraction). The rate of mitochondrial H$_2$O$_2$ generation in the presence of glutamate and malate (5 mM each) was determined by linear increase in fluorescence (excitation at 312 nm, emission at 420 nm) of oxidized homovanillic acid in the presence of horseradish peroxidase (Barja, 2002). Briefly, Op-M fraction (250 µg/ml protein) in 30 mM Hepes (pH 7.4), 145 mM KCl, 5 mM KH$_2$PO$_4$, 3 mM MgCl$_2$, 0.1 mM EGTA were mixed with 6 U/ml horseradish peroxidase and 0.1 mM homovanillic acid, and the fluorescence of oxidized homovanillic acid was detected every 30 seconds for 30 min at 30°C, using SPECTRAmax Plus microplate fluorometer equipped with SOFTmax Pro software (Molecular Devices). A standard curve prepared from known amounts of H$_2$O$_2$ was used to convert the measured fluorescence to the
amount H$_2$O$_2$ production in nmoles H$_2$O$_2$ per min per mg protein. The respiration rate was measured using a Clark-type oxygen electrode connected to the Biological Oxygen Monitor Micro System (YSI Model 5300, Yellow Springs Instrument). All measurements were conducted at 30 °C in a 0.6 ml chamber, containing P17 mitochondria-enriched fraction (0.3 mg protein) in 20 mM HEPES (pH 7.4), 125 mM KCl, 2 mM KH$_2$PO$_4$, 1 mM MgCl$_2$. The state 2 respiration rate was measured in the presence of glutamate and malate, and the state 3 respiration rate after additional supplementation of ADP (200 nM final concentration). Oxygen consumption was determined as the amount of oxygen disappearing from respiration chamber per unit time per 1 mg mitochondrial protein. The mtDNA deletion was detected by PCR amplification of an mtDNA segment flanking three direct repeats in the regions 8884–13357. For this, mtDNA was prepared from the liver P17 fraction using the mtDNA Extractor CT Kit (Wako), and oligonucleotide primers, 5’-CAAGTCCATGACCATTACTGG-3’ (forward) and 5’-GATTTTATGGGTGTAATG CG-3’ (reverse), were used for PCR reaction. As undeleted mtDNA controls in each sample, the mtDNA segment (471-670) encoding 12S rRNA, using 5’-GACAGCTAAGACCCAAACTG-3’ (forward) and 5’-TTAGCAAGAGATGGTGAGG T-3’ (reverse) primers, were also amplified.
Electron microscopy Mice were perfused through the left cardiac ventricle with 35–50 ml 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Isolated striata were diced and fixed overnight at 4 °C. After incubation for 2 h in 1% OsO₄ in 0.1 M cacodylate buffer, the specimens were dehydrated in an ethanol series, passed through propylene oxide, and embedded in epoxy resin (Embed 812, RT 14120, Electron Microscopy Sciences, USA). Ultrathin sections (70 nm) were collected on 200 mesh copper grids and stained for 20 min in 0.1% uranyl acetate and lead citrate. The specimens were observed with a JEOL 1200EX-II electron microscope at 80 kV.

Cell culture and manipulation of gene expression MEFs were isolated from day-13.5 embryos, and sex of each line was typed by genomic PCR of a locus on the Y chromosome containing Sry gene using oligonucleotide primers (Forward, 5’-AGAGATCAGCAAGCAGCTGG-3’ and Reverse, 5’-TCTTGCTGTATGTGATGGC-3’). Littermate male MEF pairs with passage numbers between 2 and 5 were used in most experiments. MEFs and HCT116 cells were cultured at 37°C in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 µg/ml of streptomycin. Expression of specific genes
was manipulated by transfection of pcDNA3.1-based vectors encoding p62, Keap1, Nrf2 and Nqo1 or by transfection of specific siRNA oligos (5'-GAUGCGGAAGAU GUCAUCU(dTdT)-3' for mouse p62, 5'-GAUUCGAAGAUGUCAUCC(dTdT)-3' for human p62, 5'-UCCAUGUCCUCUUAUGC(dTdT)-3' for mouse Nrf2, 5'-UUG GAACCAUGGUAGUCU(dTdT)-3' for human Nrf2, 5'-UCUCUGGAUAGUAACA UUC(dTdT)-3' for mouse Keap1, 5'-UUAGUCCCGGUUUUUGUAC(dTdT)-3' for human Keap1, 5'-AUUGAAACCAGGAUGUAUC(dTdT)-3' for mouse Nqo1, and 5'- UCAAGGUAUCUGUACUG(dTdT)-3' for human Nqo1) with a control scrambled siRNA.

**Flow cytometry and ARE reporter assay** Intracellular oxidant levels were assessed by measuring the fluorescence of dichlorofluorescin (DCF), an oxidation product of 2',7'-dichlorofluorescin diacetate (DCF-DA) (Royall and Ischiropoulos, 1993). Cells (1 X 10^6/ml) incubated with 10 μM DCF-DA at 37°C for 30 min were washed three times with PBS, and the DCF fluorescence was analyzed in a FACScan equipped with CellQuest software (Becton Dickinson). In each analysis, 10,000 events were recorded. Mitochondrial membrane potential (ΔΨ) was assessed by measuring both red and green fluorescence of 5,5',6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine
iodide (JC1), in that higher $\Delta \Psi$ emits more red fluorescence and less green fluorescence (Bass et al., 1983). Cells were treated as similarly as the DCF fluorescence measurement except using 1 $\mu$M JC1 (Molecular Probes) and analyzed in the FACScan equipped with FCS express V3 software (De Novo). JC1 fluorescence of the cells treated with 250 nM Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Sigma) or 2 $\mu$M Oligomycin (Sigma) was also measured as controls for the mitochondria with low and high $\Delta \Psi$, respectively. In order to assess anti-oxidant response in the cell, the relative luciferase reporter activity (human Nqo1-ARE-luciferase vs. pRL-TK renilla luciferase) was measured at 24 hrs post-transfection of plasmids.

**Antibodies** Antibodies used for this study include monoclonal anti-p62 antibody (Abnova), rabbit polyclonal anti-Keap1 antibody (Protein Tech Group), monoclonal anti-$\beta$-actin (Sigma), and rabbit polyclonal anti-Nqo1 antibody (Protein Tech Group).

**RT-PCR.** Total RNA was isolated using RNeasy mini kit (Qiagen), and complementary DNA was prepared using PrimeScript RT reagent kit (Takara). Semiquantitative RT-PCR was performed on an GeneAmp PCR system 9700 (Perkin Elmer) according to the
manufacturer’s instructions. Primer sets used were; p62/sqstm1 F: 5’- CATGGTGCA CCCCAATGTGATC-3’ and R: 5’- AGGAGGACGTGGGCTCCAGTT-3’, 36B4 F: 5’- CAGCTCTGGAGAAACTGCTG-3’ and R: 5’- GTGTACTCATGTCTCCACAGA-3’, Gclc F: 5’- CCGCTGTCCAAGGTTGACGA-3’ and R: 5’- GTTGCCGCTTTCGAGAT -3’, Gpx F: 5’- CCTCAAGTACGTCGGACCTG-3’ and R: 5’- CAATGTCGTT GCGGCACACC-3’, Gstp1 F: 5’- ATGCCACCATACACCCATTGTC-3’ and R: 5’- GG GAGCTGCCCATAGACAC -3’, Hmox1 F: 5’- CCTGGTGCAAGATATGCCC -3’ and R: 5’- GAAGACTGAGAGTGAGGCCA GTCGCTTGCAACACAC -3’, Mtr2 F: 5’- CTGTGCCCTCCGATGG ATCCT -3’ and R: 5’- CTTGTCGAAGTGAGGACCCA-3’, Prdx1 F: 5’- CAATGTCGTT GTGGCACCAGTTTGTTGTC-3’ and R: 5’- AATGGGTCGCTTTGGGATCTG TCGGCCCTTTTGCTG-3’ and R: 5’- TGCTCTCCTGAGAGTGA GAT-3’, Sod1 F: 5’- GTGAACCAGTTTGTTGTC-3’ and R: 5’- TGCTCTCCTGAGAGTGA GAT-3’, Txnrd1 F: 5’- TACTGCACTCAGCTGATGATC-3’ and R: 5’- CCATGTCTCTCCAT GTGTTCA -3’, Ugt1a6a F: 5’- GTTTGCTTTTCTAGTGCTTTGGG -3’ and R: 5’- CCTCGTTCACTGAGATGTTCTAC-3’, Nqo1 F: 5’- CATTGTGAAGGCTGGTTT GA-3’ and R: 5’- TTTCTTCCCATGCTCAGGAATGTA-3’, Keap1 F: 5’- GCCAGGACCAG TTGAAACATGCTGCTTCCTCAGGAATGTA-3’, Atg5 F: 5’- AGCCAG GTGATGATGCAGCCG -3’ and R: 5’- GGCTGGGGGACAAATGCTAA -3’, Atg7 F: 5’- GTTTGCCCCCTTTAATAGTGC-3’ and R: 5’- TGAACCTCAACGTCAAGGCGG -3’,
Lonp1 F: 5’- CGGATGTGTTTCCTCACCTG -3’ and R: 5’- ACGCCAACATAGGGCT GTG -3’.

**Statistical analyses** All data are expressed as the mean ± s.d. of at least triplicate samples. Data were analyzed by two-tailed, unpaired Student t-tests. For all experiments, $P$ values < 0.05 were considered significant.
Supplementary Fig. S1. Generation of p62−/− mice. A, Schematic representation of the p62 gene allele, targeting vector, and targeted allele. B, PCR analysis of genomic DNA from the tails of p62+/+, p62+− and p62−/− mice, using three oligomeric primers (F1, R1, and R2). Amplification by two sets of primers (F1-R1 and F1-R2) yielded 540bp and 200bp long wild-type and targeted alleles, respectively. C, Immunoblot analysis of p62 in the brain, liver, and pancreas of wild-type and p62−/− mice.
Supplementary Fig. S2. Aging phenotypes developed in p62^-/- mice. A, Representative X-ray radiographs of 95-week-old p62^+/+ and p62^-/- mice (n=5 per group). Average spinal angles of p62^+/+ and p62^-/- male mice were 106° and 71°, respectively (p < 0.00005). B, Rough fur coat of the 95-week-old p62^-/- mice C, Haematoxylin and eosin staining of the representative 90-week-old p62^+/+ and p62^-/- dorsal skin sections. (40 x magnification). D, Immunohistochemical staining of 4-HNE in the brains of 95-week-old p62^+/+ and p62^-/- mice. E,F, Intracellular levels of carbonylated protein (* p < 0.05) (h) and 8-oxoguanine (8-ohG) (i) in p62^+/+ and p62^-/- MEFs.
Supplementary Fig. S3. Role of p62 for Lon protease expression and mitochondrial morphology. A. message (upper panel) and protein (bottom panel) levels of Lon protease in skeletal muscles of p62\(^{+/+}\) and p62\(^{-/-}\) mice. B. Representative mitochondrial morphology in control and p62 knockdown HeLa cells at G1 phase. C. % HeLa cell population containing tubular or fragmented mitochondrial morphology.
Supplementary Fig. S4. Electrophile/oxidant-independent nature of p62-induced Nrf2 activation. A, p62 dose-dependent activation of anti-oxidant response (filled bars) was completely disappeared by Nrf2 knockdown (open bars) (* p < 0.01 and ** p < 0.001). B, Anti-oxidant response upon exposure for 6 hr to an electrophile, tertiary butylhydroquinone (tBHQ) in p62+/+ and p62-/- MEFs. C, Effect of an anti-oxidant, NAC, on p62- and tBHQ-induced anti-oxidant response (* p < 0.01).
Supplementary Fig. S5. ΔΨm and oxidant levels modulated by p62 and Keap1. A-E, ΔΨm and oxidant level in p62+/+ (A,E), p62-/ MEFS (B,E), and p62--/-- MEFS after ectopic expression of p62 (C,E) or p62Δ335-357 (D,E). F-H, ΔΨm (F,G) and oxidant levels (H) in HCT116 cells after Keap1 overexpression.