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Molecular evolution of Keap1 was essential for adaptation of vertebrates to terrestrial life

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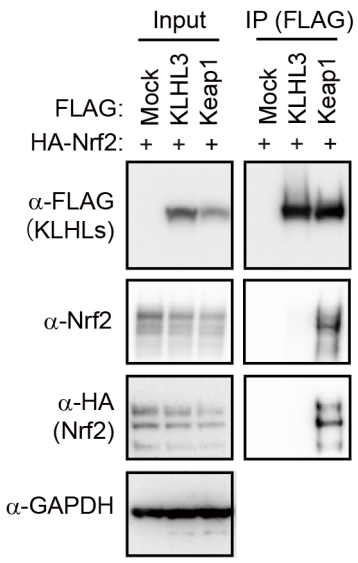

Fig. S1. Overexpressed Keap1 interacts with Nrf2. Lysates of HEK293T cells expressing FLAG-tagged human KLHL3 or Keap1 together with HA-tagged human Nrf2 were subjected to immunoprecipitation with antibodies to FLAG, and the resulting precipitates as well as the original lysates were subjected to immunoblot analysis with the indicated antibodies.

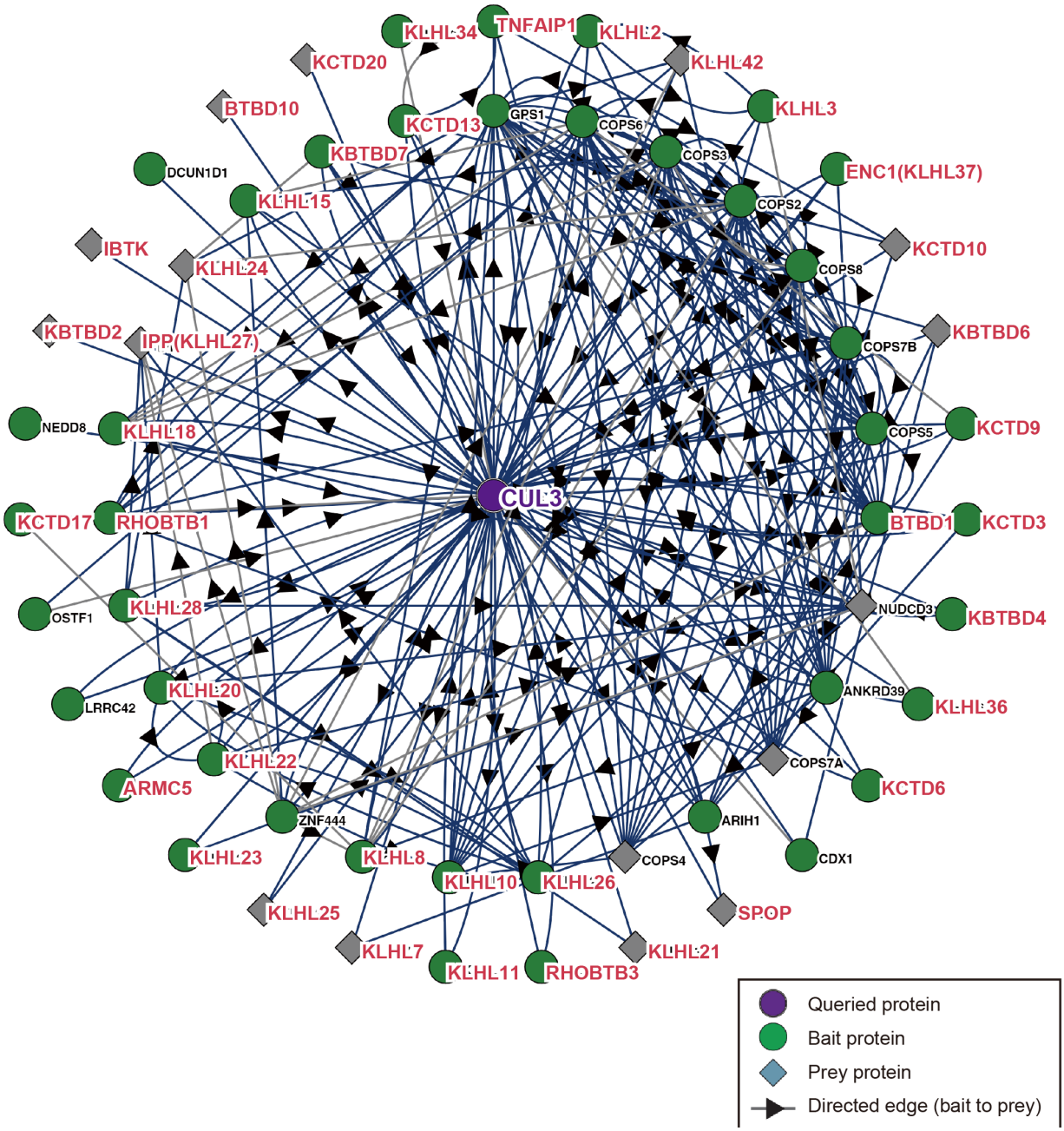

Fig. S2. Cul3 binding proteins listed in the BioPlex 3.0 interactome database. Binding proteins of Cul3 in HEK293T or HCT116 cells were identified from the BioPlex 3.0 database (https://bioplex.hms.harvard.edu). The names of proteins with BTB domains are shown in red letters, whereas binding detected in HEK293T or HCT116 cells is indicated by blue and gray lines, respectively.

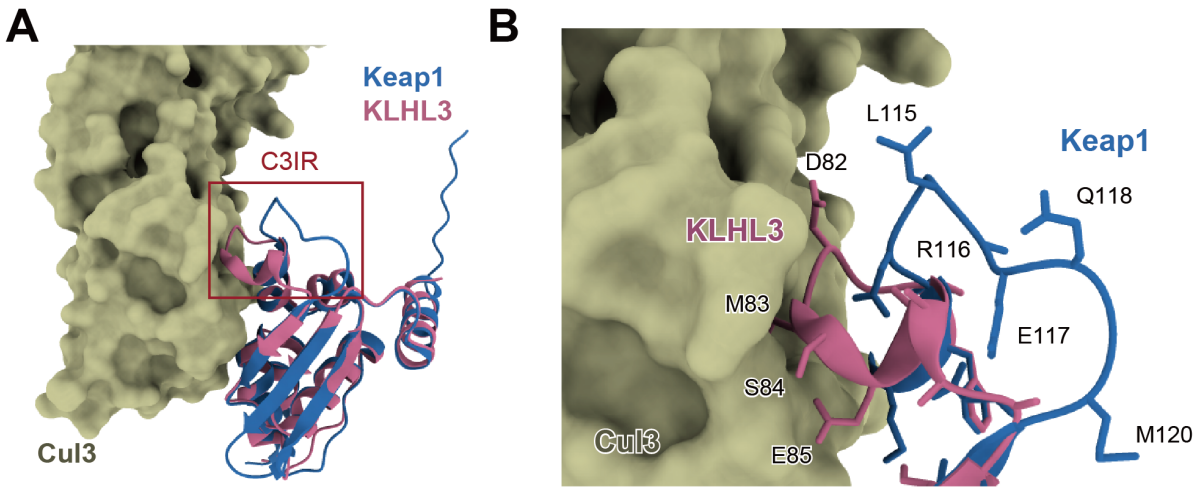

Fig. S3 Structural alignment of the human Cul3-KLHL3 complex (PDB: 4HXI) with the BTB domain of human Keap1 (4CXI). The boxed region containing C3IR of Keap1 in (**A**) is shown enlarged in (**B**).

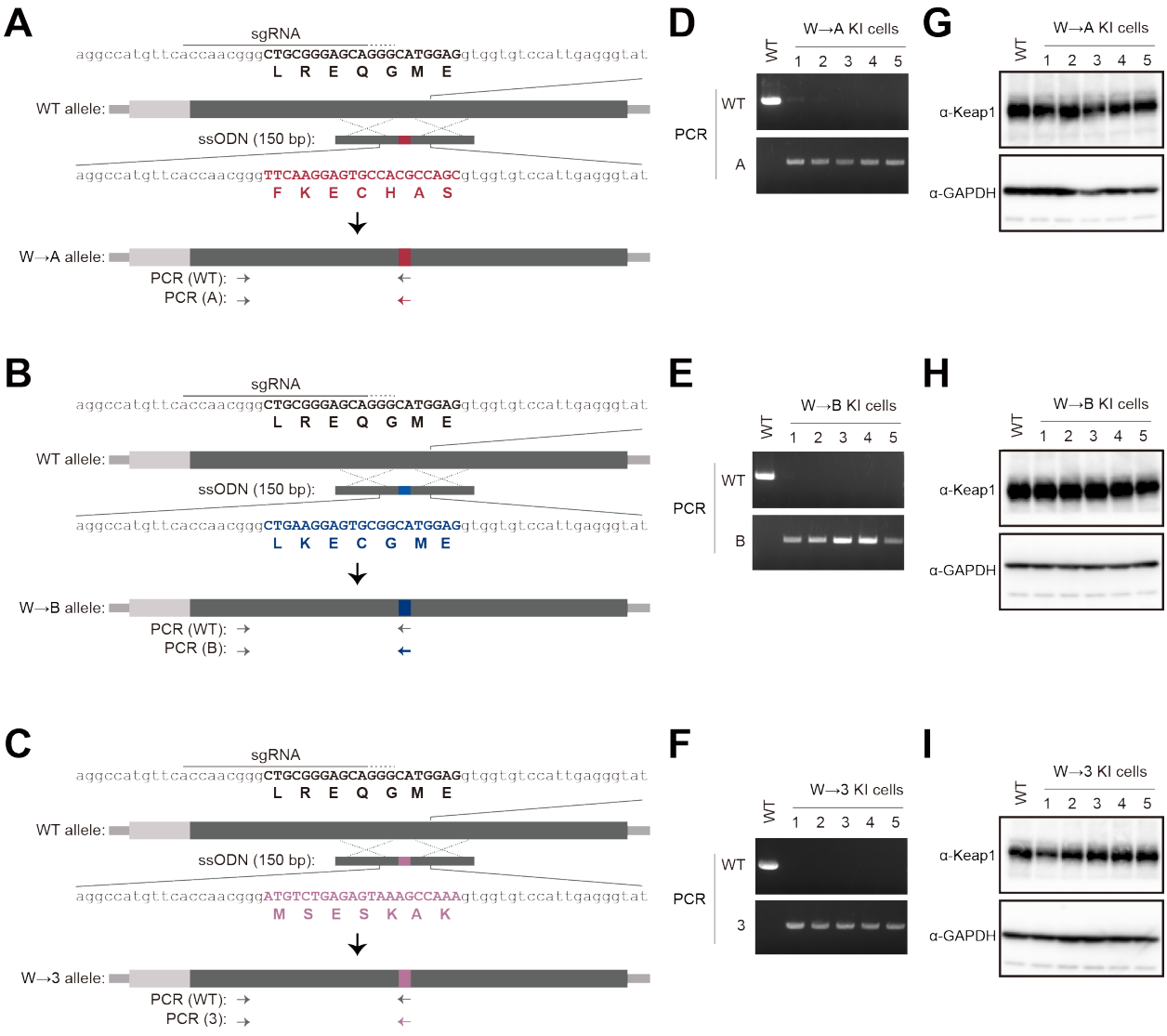

Fig. S4. Construction of C3IR knock-in HEK293T cells. (**A** to **C**) Schematic representation of exon 2 of the WT human Keap1 allele, the single-stranded oligodeoxynucleotide (ssODN) for knock-in of the W→A (A) W→B (B), or W→3 (C) mutation, and the mutant allele after homologous recombination. The single guide RNA (sgRNA) for the CRISPR-Cas9 system and its protospacer adjacent motif (PAM) are indicated by continuous and dotted overlines, respectively. The 5′ untranslated region and open reading frame of the Keap1 gene are represented by the light and dark gray boxes, respectively. Each mutation is shown in red, blue, or pink. (**D** to **F**) PCR analysis of genomic DNA for WT (D to F) or five clones of W→A (D), W→B (E), or W→3 (F) knock-in (KI) HEK293T cells. PCR primers are indicated in (A) to (C), respectively. (**G** to **I**) Protein extracts of WT (G to I) or W→A (G), W→B (H), or W→3 (I) knock-in HEK293T cells were subjected to immunoblot analysis with the indicated antibodies.

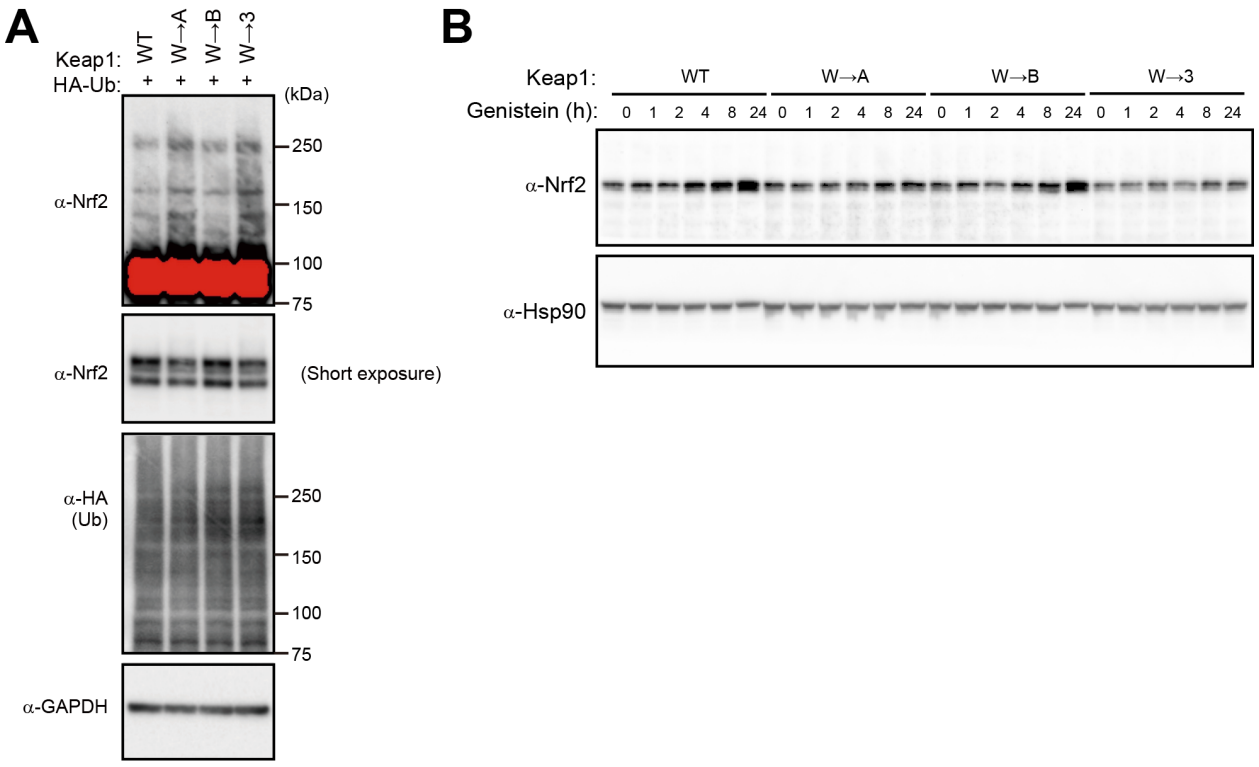

Fig. S5. Nrf2 ubiquitylation is increased and Nrf2 accumulation in response to oxidative stress is attenuated in Keap1(W→A) or Keap1(W→3) knock-in cells. (**A**) WT or Keap1 C3IR mutation knock-in HEK293T cells expressing HA-tagged ubiquitin (Ub) were treated with 1 μM MLN4924 for 24 h, lysed, and subjected to immunoblot analysis with the indicated antibodies. (**B**) WT or Keap1 C3IR mutation knock-in HEK293T cells were exposed to 100 μM genistein (B) for the indicated times, lysed, and subjected to immunoblot analysis with antibodies to Nrf2.

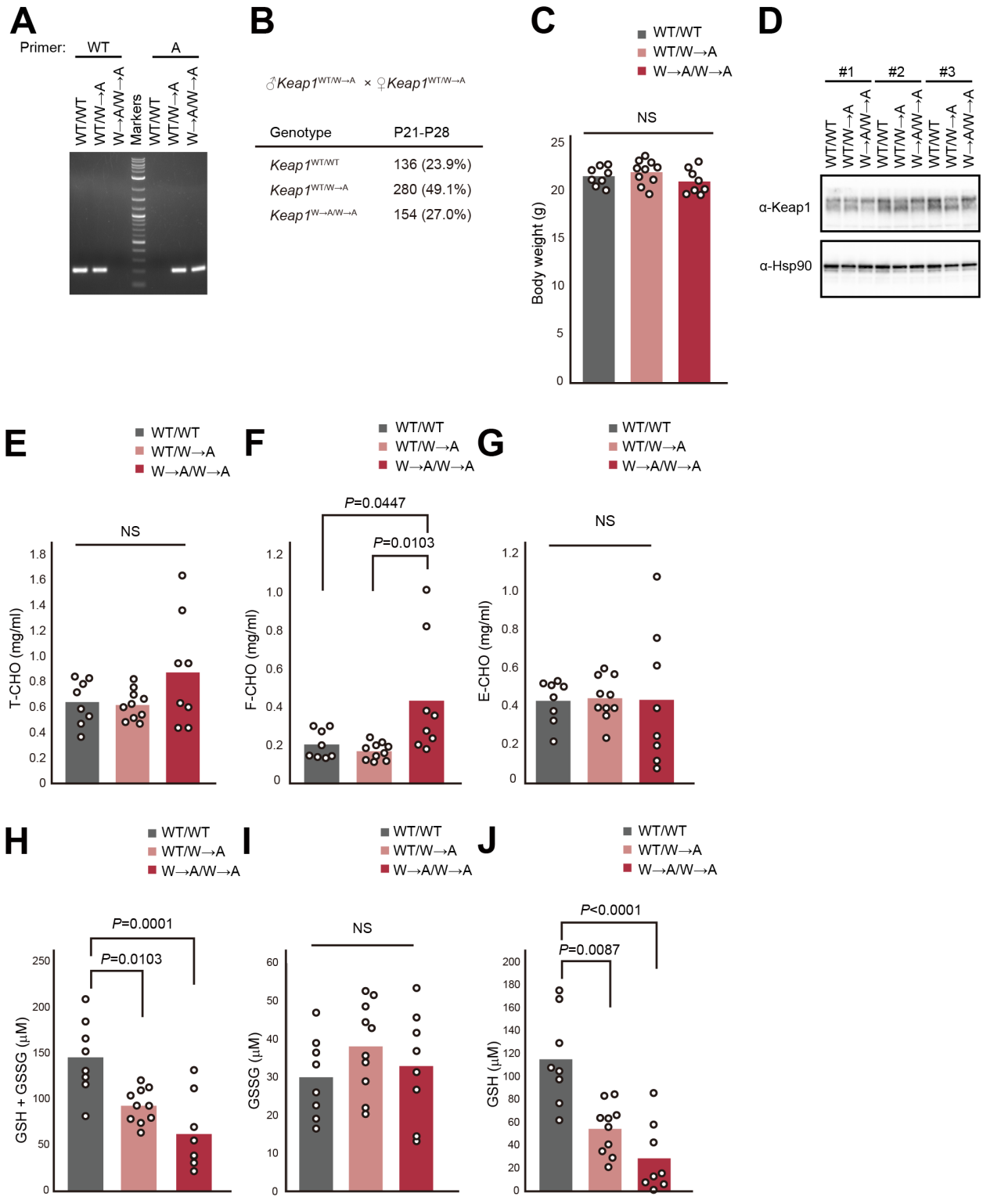

Fig. S6. *Keap1*$^{W→A/W→A}$ knock-in mice show a severe phenotype for APAP-induced liver injury. (**A**) PCR analysis of genomic DNA from the tail of mice of the indicated genotypes. PCR primers are indicated in Figure 5A. (**B**) Genotype frequencies for live offspring at postnatal day (P) 21 to P28 produced from *Keap1*$^{WT/W→A}$ mouse intercrosses. (**C**) Body weight of male WT, *Keap1*$^{WT/W→A}$, or *Keap1*$^{W→A/W→A}$ mice at 8 weeks of age. Data are means for 8 to 10 mice of

each genotype. NS, not significant (one-way ANOVA followed by the Tukey-Kramer post hoc test). (**D**) Protein extracts of the liver of WT, *Keap1*$^{WT/W\rightarrow A}$, or *Keap1*$^{W\rightarrow A/W\rightarrow A}$ mice at 8 weeks of age were subjected to immunoblot analysis with the indicated antibodies. Three animals were examined for each genotype. (**E** to **G**) Serum concentrations of total cholesterol (T-CHO) (E), free cholesterol (F-CHO) (F), and esterified cholesterol (E-CHO) (G) for WT, *Keap1*$^{WT/W\rightarrow A}$, or *Keap1*$^{W\rightarrow A/W\rightarrow A}$ mice at 8 weeks of age. Data are means for 8 to 10 mice of each genotype. Statistical analysis was performed by one-way ANOVA followed by the Tukey-Kramer post hoc test. (**H** to **J**) Concentrations of total glutathione (GSH + GSSG) (H), oxidized glutathione (GSSG) (I), and reduced glutathione (GSH) (J) in liver extracts of WT, *Keap1*$^{WT/W\rightarrow A}$, or *Keap1*$^{W\rightarrow A/W\rightarrow A}$ mice at 8 weeks of age. Data are means for 8 to 10 mice of each genotype. Statistical analysis was performed by one-way ANOVA followed by the Tukey-Kramer post hoc test.

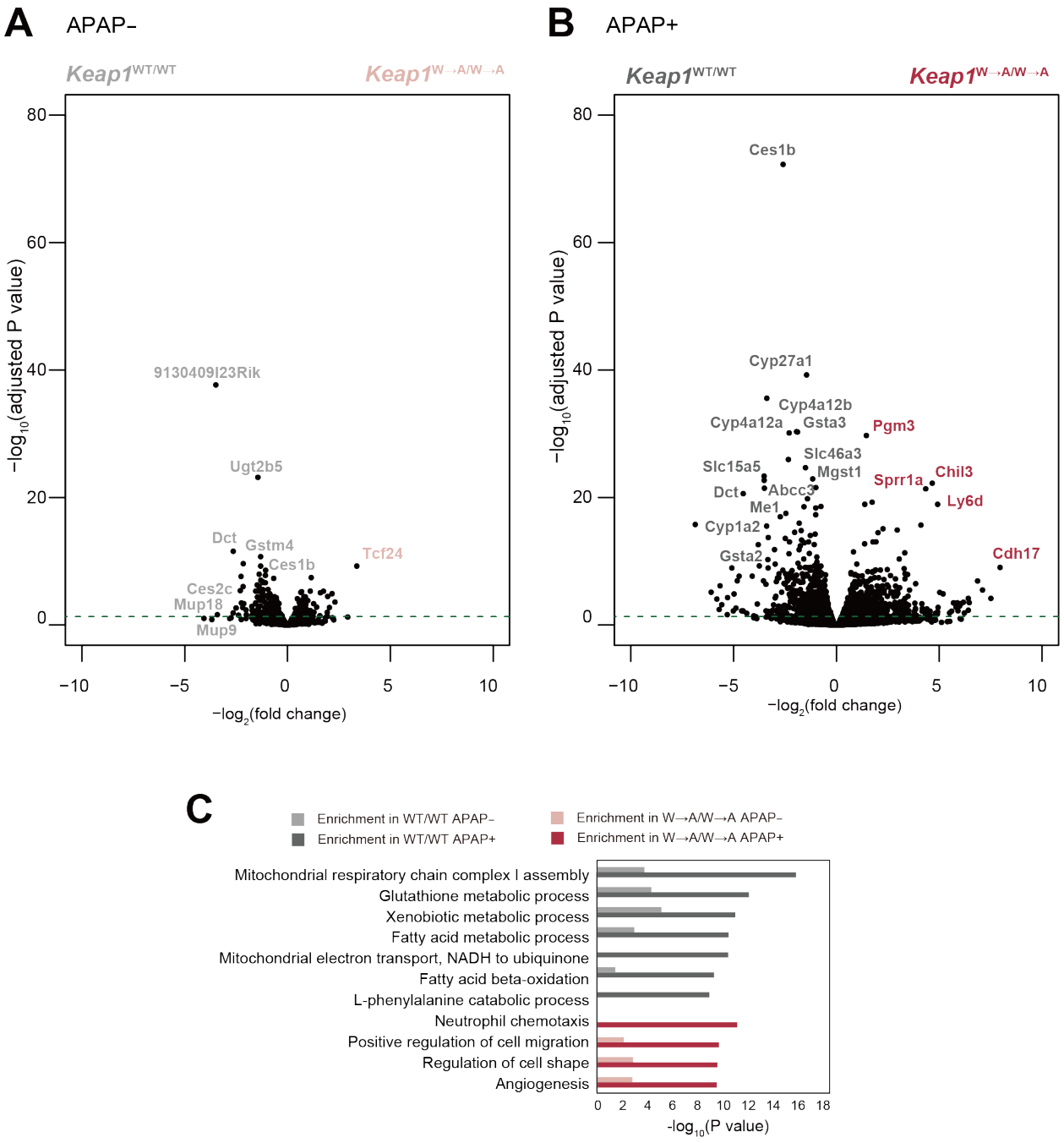

Fig. S7. RNA-seq analysis for the liver of *Keap1*$^{W→A/W→A}$ and WT mice with or without administration of an overdose of APAP. (**A** and **B**) Volcano plots for RNA-seq data obtained from the liver of WT or *Keap1*$^{W→A/W→A}$ mice that were either left untreated (A) or treated with APAP (B) as in Figure 5D. (**C**) GO analysis of differentially expressed genes identified from RNA-seq analysis as in (A) and (B).

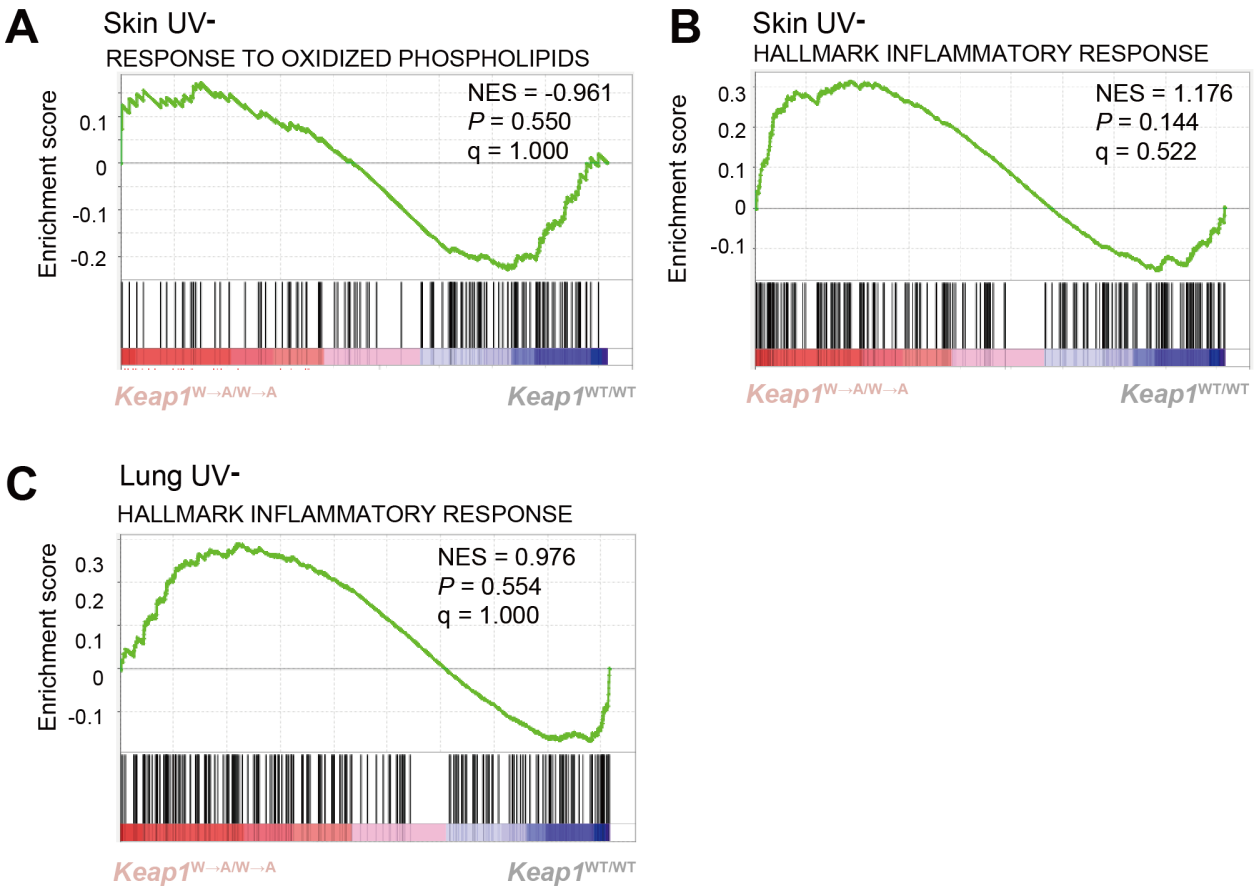

Fig. S8. GSEA plots constructed from RNA-seq data for nonirradiated *Keap1*$^{W→A/W→A}$ and WT mice at P6.5. (**A** and **B**) GSEA plots for gene sets associated with the response to oxidized phospholipids (A) or the inflammatory response (B) were constructed from RNA-seq data for skin. (**C**) A GSEA plot for the gene set associated with the inflammatory response was constructed from RNA-seq data for lung.

Table S1. List of antibodies used in this study

ANTIBODIES	SOURCE	IDENTIFIER
FLAG-M2	Sigma	F1804 or F7425
MYC-9E10	Sigma	M5546
HA-HA11	Covance	MMS-101R
Cul3	BD Transduction Laboratories™	611848
Nrf2-H300	Santa Cruz Biotechnology	sc-13032
Keap1-D1G10	Cell Signaling Technology	7705
GAPDH-1D4	Enzo Life Sciences	A-335E
α Tubulin-TU1	Invitrogen™	13-8000
Hsp90	BD Transduction Laboratories™	610419
4-HNE	JaICA	MHN-100P
Anti-Mouse IgG (H+L), HRP Conjugate	Promega	W4021
Anti-Rabbit IgG (H+L), HRP Conjugate	Promega	W4011
Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™)	BD Pharmingen™	553141
FITC anti-mouse CD45.2 Antibody	BioLegend	109806
APC/Cyanine7 anti-mouse/human CD11b Antibody	BioLegend	101226
PE anti-mouse F4/80 Antibody	BioLegend	157304
Brilliant Violet 421 anti-mouse CD11c Antibody	BioLegend	123110
BV510 Rat Anti-Mouse Siglec-F	BD OptiBuild™	740158
APC anti-mouse CD80 Antibody	BioLegend	104714
PE/Cyanine7 anti-mouse CD206 (MMR) Antibody	BioLegend	141720

Table 2 | List of oligonucleotides used in this study

Oligonucleotides	
Human NQO1 Forward Primer	5’-CCGCAGACCTTGTGATATTCC-3’
Human NQO1 Reverse Primer	5’-TCTCCTATGAACACTCGCTCAAAC-3’
Human HO1 Forward Primer	5’-GCCAGCAACAAAGTGCAAGAT-3’
Human HO1 Reverse Primer	5’-AGTGTAAGGACCCATCGGAGAA-3’
Human RPS18 Forward Primer	5’-GATCCCTGAAAAGTTCCAGCA-3’
Human RPS18 Reverse Primer	5’-CATGAGCATATCTTCGGCCC-3’
ssODN for W→A_mutation Knockin in HEK293T	5’-CTGGCCTCATCCAGCCCTGTCTTCAAGGCCATGTTCACCAACGGGTTCAAGGAGTGCCACGCCAGCGTGGTGTCCATTGAGGGTATCCACCCCAAGGTCATGGAGCGCCTCATTGAATTCGCCTACACGGCCTCCATCTCCATGGGCGAG-3’
ssODN for W→B_mutation Knockin in HEK293T	5’-CTGGCCTCATCCAGCCCTGTCTTCAAGGCCATGTTCACCAACGGGCTGAAGGAGTGCGGCATGGAGGTGGTGTCCATTGAGGGTATCCACCCCAAGGTCATGGAGCGCCTCATTGAATTCGCCTACACGGCCTCCATCTCCATGGGCGAG-3’
ssODN for W→3_mutation Knockin in HEK293T	5’-CTGGCCTCATCCAGCCCTGTCTTCAAGGCCATGTTCACCAACGGGATGTCTGAGAGTAAAGCCAAAGTGGTGTCCATTGAGGGTATCCACCCCAAGGTCATGGAGCGCCTCATTGAATTCGCCTACACGGCCTCCATCTCCATGGGCGAG-3’
oligonucleotides of the human Keap1 sgRNA target sequences_Forward	5’-CACCACCAACGGGCTGCGGGAGCA-3’
oligonucleotides of the human Keap1 sgRNA target sequences_Reverse	5’-AAACTGCTCCCGCAGCCCGTTGGT-3’
human Keap1(Common)_Forward	5’-AGGTTGATCAGGTCGGGGAAG-3’
human Keap1(WT)_Reverse	5’-CTCCATGCCCTGCTCCCGCAG-3’
human Keap1(W→A_mutant)_Reverse	5’-CTGGCGTGGCACTCCTTGAAC-3’
human Keap1(W→B_mutant)_Reverse	5’-GACACCACCTCCATGCCGCAC-3’
human Keap1(W→3_mutant)_Reverse	5’-GGCTTTACTCTCAGACATC-3’

ssODN for W®A_mutation Knockin in mice	5’-ATGAGGACATCCCAGCTGCCCAATTCATGGCTCACAAAGTGGTGCTGGCCTCCTCCAGCCCAGTCTTTAAAGCCATGTTCACCAACGGGTTCAAGGAGTGCCACGCCAGCGTGGTGTCCATCGAAGGCATCCACCCTAAGGTCATGGAAAGGCTTATTGAGTTCGCCTACACGGCCTCCATCTCCGTGGGCGAGAAGTGT-3’
oligonucleotides of the mouse Keap1 sgRNA target sequences_Forward	5’-CACCACCAACGGGCTTCGGGAGCA-3’
oligonucleotides of the human Keap1 sgRNA target sequences_Reverse	5’-AAACTGCTCCCGAAGCCCGTTGGT-3’
mouse Keap1(Common)_Forward	5’-CAGCTACACACTAGAGGATC-3’
mouse Keap1(WT)_Reverse	5’-CCATGCCCTGCTCCCGAAGC-3’
mouse Keap1(W→A_mutant)_Reverse	5’-CACGCTGGCGTGGCACTCCTTG-3’

Table S3 List of NCBI Gene accession used in this study

	NCBI Gene accession
H. sapiens Keap1 sequence	9817
M. musculus Keap1 sequence	50868
M. gallopavo Keap1 sequence	104917088
C. picta bellii Keap1 sequence	101953638
X. tropicalis Keap1B sequence	493386
O. latipes Keap1B sequence	101160787
D. rerio Keap1B sequence	100003679
C. carpio Keap1B sequence	109053857
X. tropicalis Keap1A sequence	100489128
O. latipes Keap1A sequence	101159396
D. rerio Keap1A sequence	321837
C. carpio Keap1A sequence	109054162
C. intestinalis Keap1 sequence	100177101
S. purpuratus Keap1 sequence	100889922
O. bimaculoides Keap1 sequence	106877646
A. californica Keap1 sequence	101863786