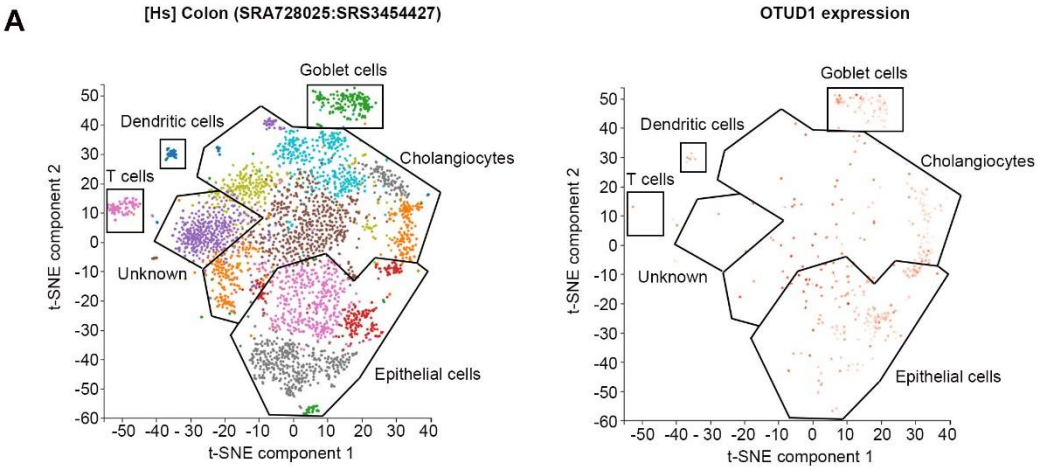
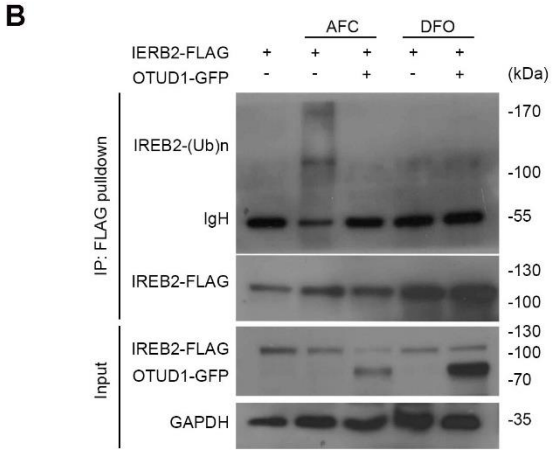
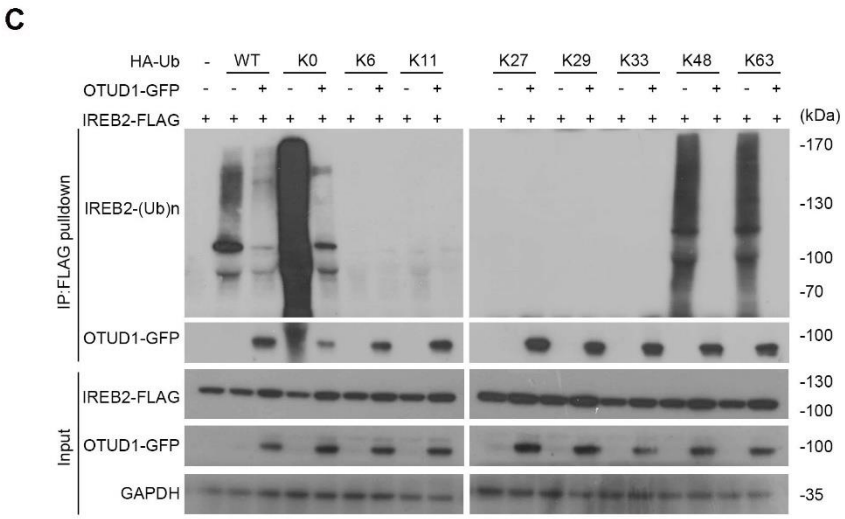
Apppendix


A
[Hs] Colon (SRA728025:SRS3454427)
OTUD1 expression
Goblet cells
Dendritic cells
Cholangiocytes
T cells
Unknown
Epithelial cells
t-SNE component 1
t-SNE component 2

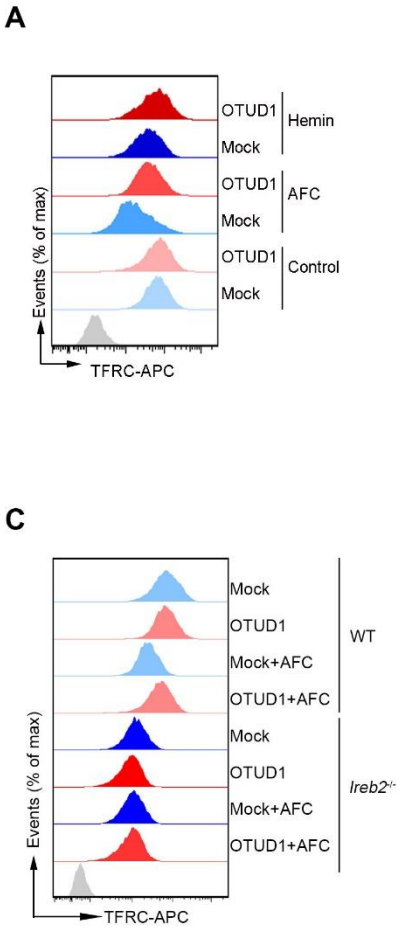

B
AFC
DFO
IERB2-FLAG
OTUD1-GFP
(kDa)
IP: FLAG pulldown
IREB2-(Ub)n
IgH
IREB2-FLAG
Input
IREB2-FLAG
OTUD1-GFP
GAPDH
-170
-100
-55
-130
-100
-130
-100
-70
-35


C
HA-Ub
WT
K0
K6
K11
K27
K29
K33
K48
K63
OTUD1-GFP
IREB2-FLAG
(kDa)
IP:FLAG pulldown
IREB2-(Ub)n
OTUD1-GFP
Input
IREB2-FLAG
OTUD1-GFP
GAPDH
-170
-130
-100
-70
-100
-130
-100
-100
-35

Appendix Figure S1. OTUD1 deubiquitinases IREB2.

(**A**) t-Distributed stochastic neighbor embedding (t-SNE) plot of RNA-sequenced (scRNA-seq) single cells from human colon tissues (Left) and the expression of OTUD1 in different cell population (Right).

(**B** and **C**) *In vivo* ubiquitination assay of IREB2. HEK293T cells were co-transfected with indicated plasmids, treated with indicated reagents and subjected to immunoprecipitation with anti-FLAG antibody followed by western blot analysis.

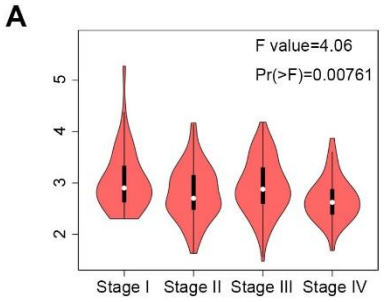


Appendix Figure S2. OTUD1 promotes TFRC expression.

(**A**) Flow cytometric analysis of TFRC expression in mock and OTUD1 expressing CT26 cells with Hemin (100 μM) and AFC (50 μM) treatment. Gray shaded curve indicates isotype-matched control antibody.

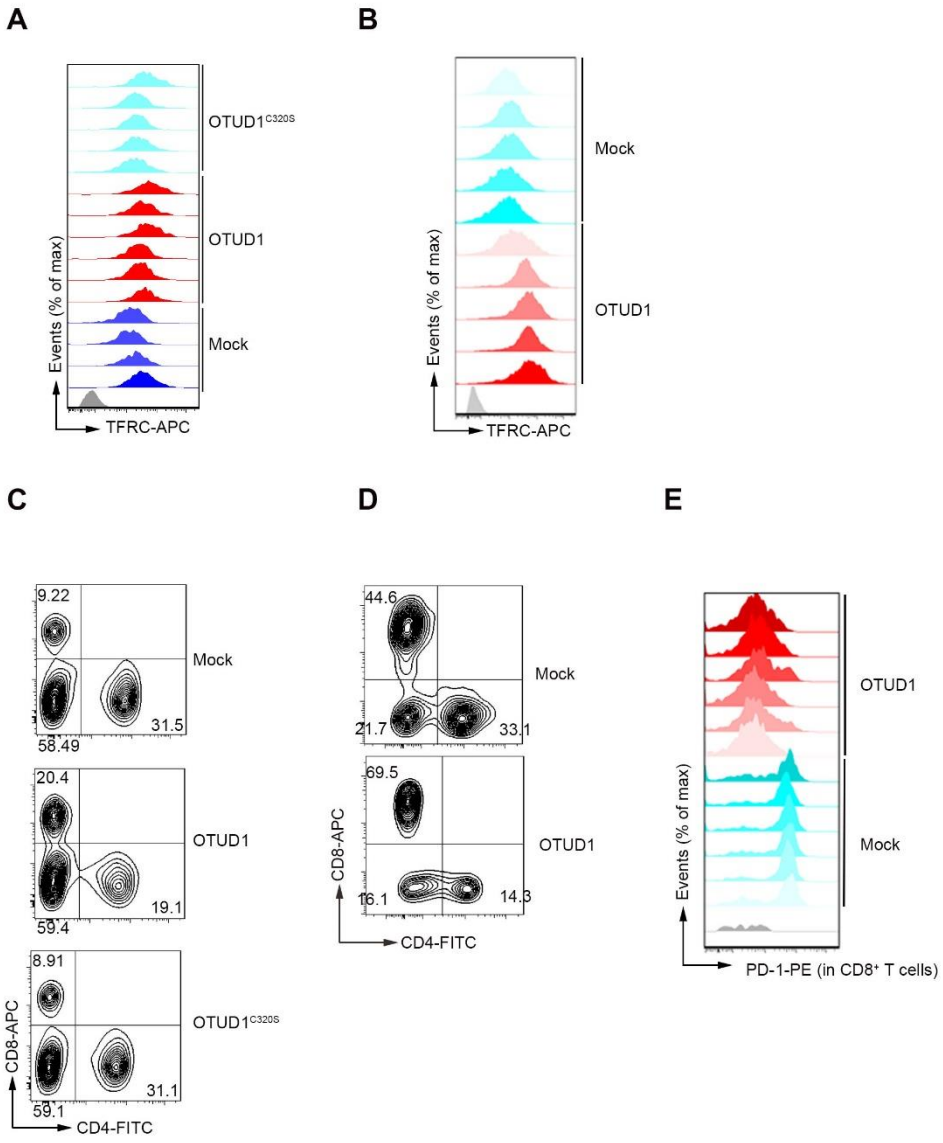
(**B**) Flow cytometric analysis of TFRC expression in wild-type (WT) and *OTUD1*$^{-/-}$ NCM460 cells with DFO (100μM) treatment. Gray shaded curve indicates isotype-matched control antibody.

(**C**) Flow cytometric analysis of TFRC expression in wild-type (WT) or *Ireb2*$^{-/-}$ CT26 cells with or without OTUD1 overexpression treated with AFC (50 μM). Gray shaded curve indicates isotype-matched control antibody.



Appendix Figure S3. Downregulation of OTUD1 is correlated with poor outcome of colorectal cancer.

(**A**) Violin plot of *OTUD1* mRNA levels in colorectal cancers at different clinical stages.



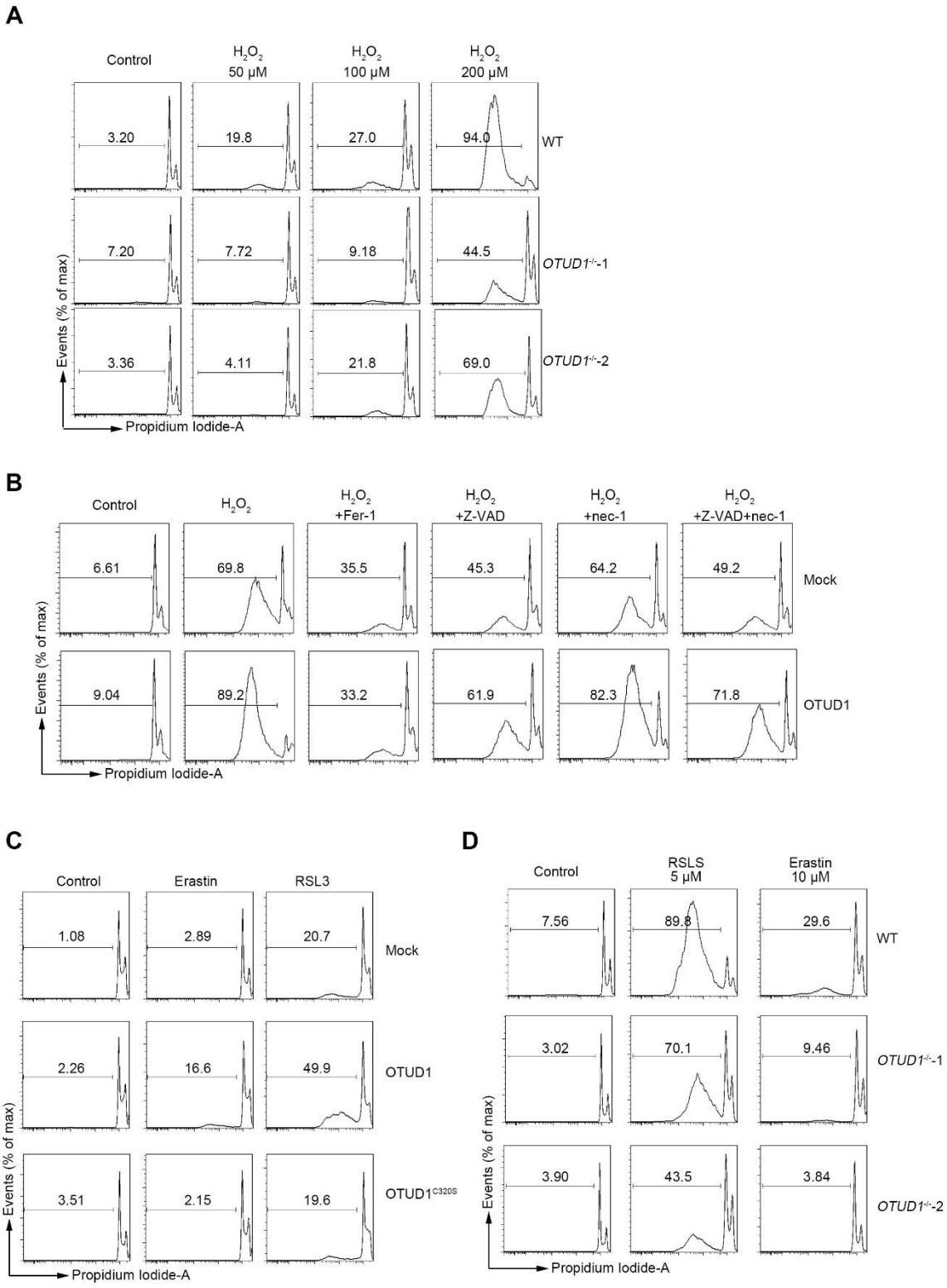
Appendix Figure S4. OTUD1 augments host anti-tumor immunity.

(**A**) Flow cytometric analysis of TFRC expression in mock (n = 4 tumors), OTUD1 (n = 6 tumors) and OTUD1^{C320S} (n = 5 tumors) expressing CT26 tumors. Gray shaded curve indicates isotype-matched control antibody.

(**B**) Flow cytometric analysis of TFRC expression in mock or OTUD1 expressing LLC tumors. Gray shaded curve indicates isotype-matched control antibody.

(**C**) Flow cytometric analysis of $CD8^+$ T cells, $CD4^+$ T cells isolated from mock, OTUD1 and $OTUD1^{C320S}$ expressing CT26 tumors in BALB/c mice.

(**D** and **E**) Flow cytometric analysis of $CD8^+$ T cells, $CD4^+$ T cells (**D**) and PD-1 expression in T cells (n = 6 mice) (**E**) isolated from mock and OTUD1 overexpressing LLC tumors. Gray shaded curve indicates isotype-matched control antibody.


A
Control
H2O2 50 μM
H2O2 100 μM
H2O2 200 μM
3.20
19.8
27.0
94.0
WT
7.20
7.72
9.18
44.5
OTUD1-/--1
3.36
4.11
21.8
69.0
OTUD1-/--2
Events (% of max)
Propidium Iodide-A
B
Control
H2O2
H2O2 +Fer-1
H2O2 +Z-VAD
H2O2 +nec-1
H2O2 +Z-VAD+nec-1
6.61
69.8
35.5
45.3
64.2
49.2
Mock
9.04
89.2
33.2
61.9
82.3
71.8
OTUD1
Events (% of max)
Propidium Iodide-A
C
Control
Erastin
RSL3
1.08
2.89
20.7
Mock
2.26
16.6
49.9
OTUD1
3.51
2.15
19.6
OTUD1C320S
Events (% of max)
Propidium Iodide-A
D
Control
RSLS 5 μM
Erastin 10 μM
7.56
89.8
29.6
WT
3.02
70.1
9.46
OTUD1-/--1
3.90
43.5
3.84
OTUD1-/--2
Events (% of max)
Propidium Iodide-A

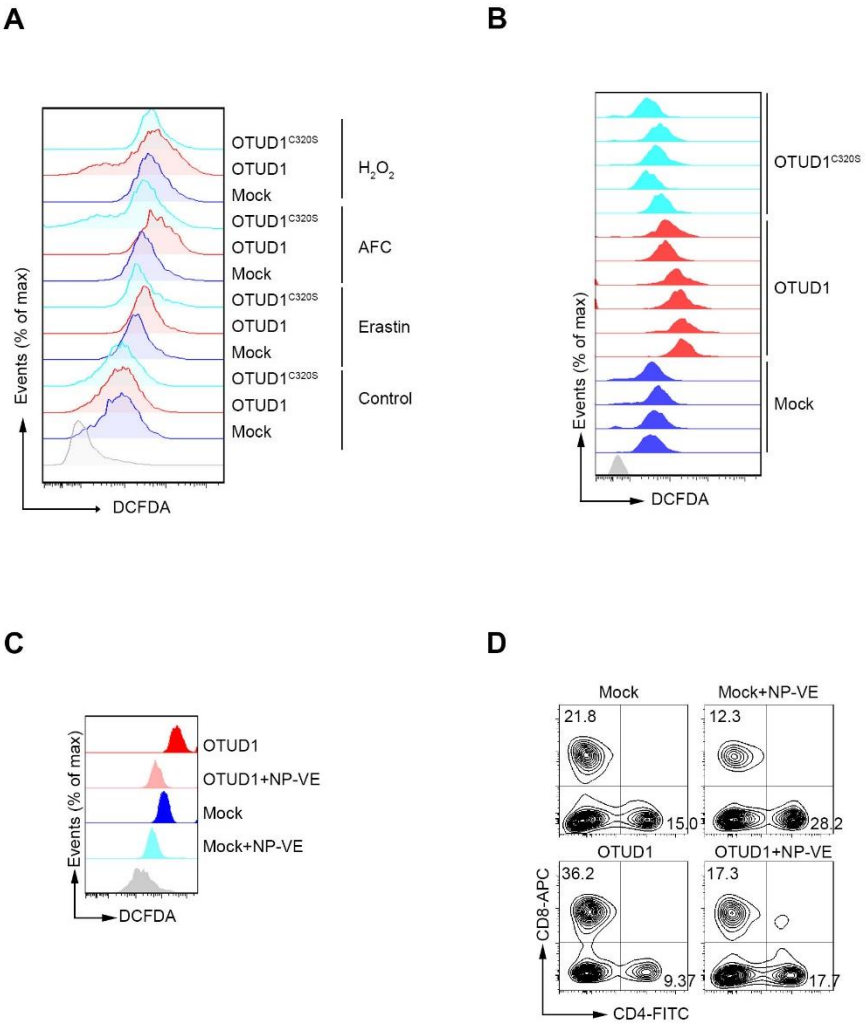
Appendix Figure S5. OTUD1 promotes ferroptosis.

(**A**) Histogram of propidium iodide (PI) in wild-type (WT) and *OTUD1*$^{-/-}$ NCM460 cells treated by H_2O_2.

(**B**) Histogram of propidium iodide (PI) in mock and OTUD1 expressing CT26 cells treated by H_2O_2 and cell death inhibitors.

(**C**) Histogram of propidium iodide (PI) in mock, OTUD1 or OTUD1^{C320S} expressing CT26 cells treated by ferroptosis activators.

(**D**) Histogram of PI in wild-type (WT) and *OTUD1*$^{-/-}$ NCM460 cells treated by ferroptosis activators.



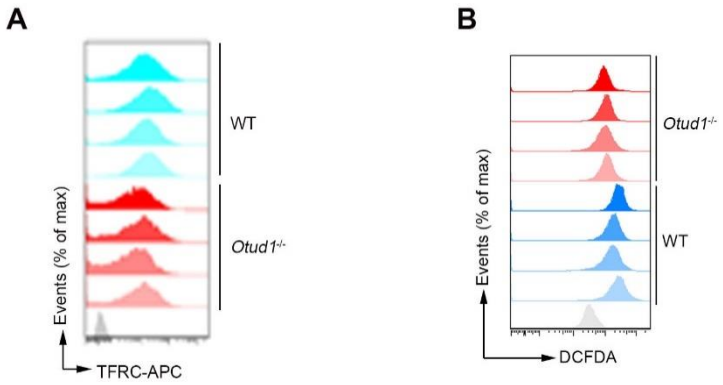
Appendix Figure S6. OTUD1 promotes ROS accumulation.

(A) Intracellular ROS levels in mock, OTUD1 and OTUD1^{C320S} overexpressing CT26 cells treated with Erastin (10 μM), AFC (50 μM) or H_2O_2 (100 μM) were detected by DCFDA staining. Gray shaded curve indicates isotype-matched control antibody.

(B) Intracellular ROS levels in mock (n = 4 tumors), OTUD1 (n = 6 tumors) or OTUD1^{C320S} (n = 5 tumors) expressing CT26 tumors were detected by DCFDA staining. Gray shaded curve indicates isotype-matched control antibody.

(**C**) Flow cytometric analysis of DCFDA in mock or OTUD1 expressing CT26 tumors with or without intratumoral injection of NP-VE. Gray shaded curve indicates isotype-matched control antibody.

(**D**)Tumor infiltrating $CD4^+$ T cells and $CD8^+$ T cells of mock or OTUD1 expressing CT26 tumors with or without intratumoral injection of NP-VE.



Appendix Figure S7. Loss of OTUD1 impedes host anti-tumor immunity.

(**A**) Flow cytometric analysis of TFRC expression in intestinal epithelial cells (IECs) from wild-type (WT) and *Otud1*$^{-/-}$ mice treated with AOM/DSS. Gray shaded curve indicates isotype-matched control antibody. (n = 4 mice).

(B) Intracellular ROS levels in colon tissues from wild-type (WT) or *Otud1*$^{-/-}$ mice treated with AOM/DSS (n = 4 mice) were detected by DCFDA staining. Gray shaded curve indicates isotype-matched control antibody. (n = 4 mice).

Appendix Table S1. The primers used for quantitative real-time PCR.

Primer name	Sequence (5'->3') -Forward	Sequence (5'->3') -Reverse
OTUD1	GATTCCCTGAGGCCTAGTATTT	CAGTTGTCGTACTCTGGGTTAG
TFRC	GGTGACCCTTACACACCTGGATT	TGATGACCGAGATGGTGGAA
IREB2	CTGCCGAGGATCTTGTGATTC	GGGTGTATTCTCAATCTGCGAA
GAPDH	ACCCACTCCTCCACCTTTGA	CTGTTGCTGTAGCCAAATTCGT