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Supplementary Materials for

Deubiquitination of proteasome subunits by OTULIN regulates type I IFN production

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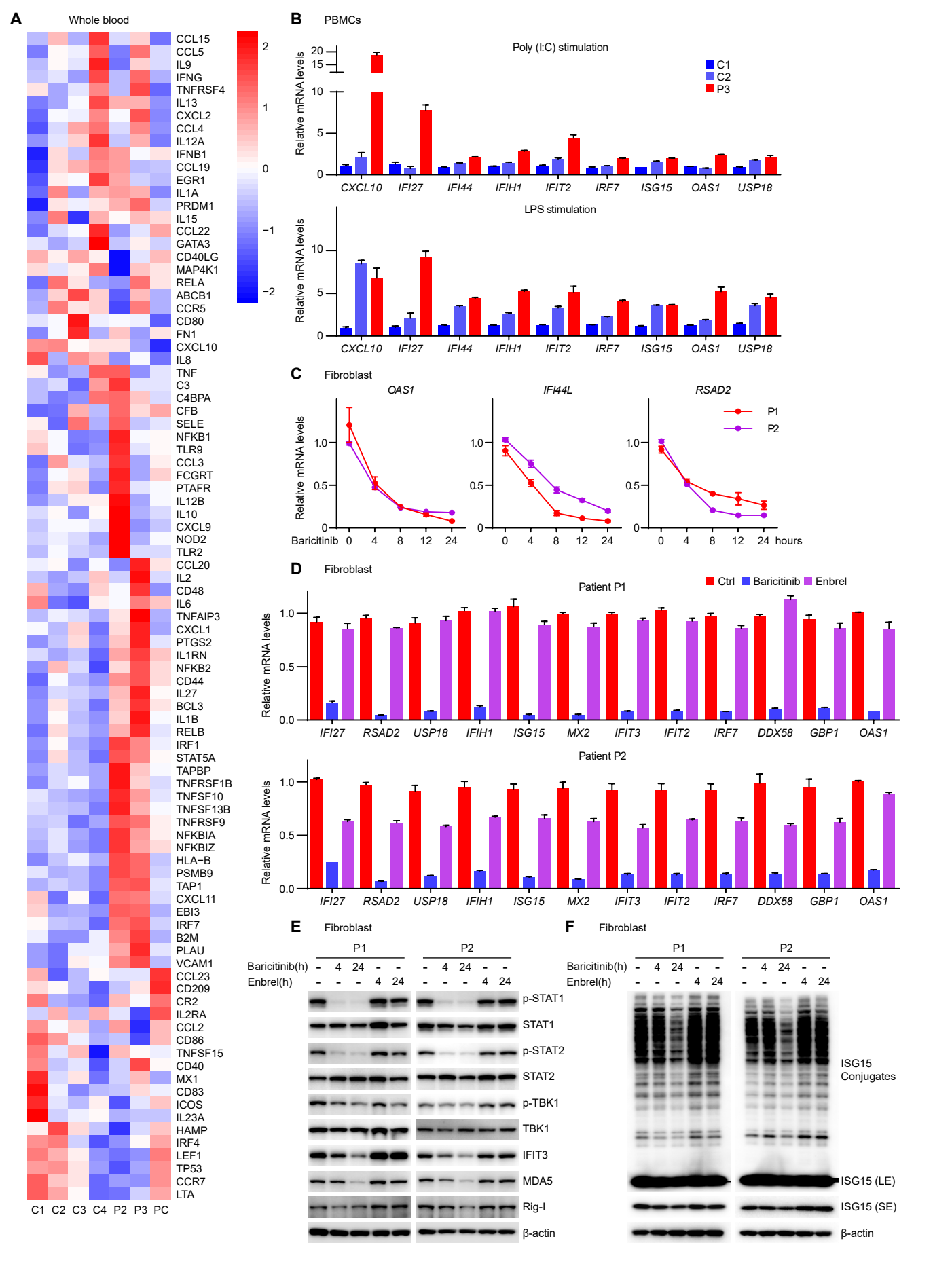

A
Whole blood
CCL15
CCL5
IL9
IFNG
TNFRSF4
IL13
CXCL2
CCL4
IL12A
IFNB1
CCL19
EGR1
IL1A
PRDM1
IL15
CCL22
GATA3
CD40LG
MAP4K1
RELA
ABCB1
CCR5
CD80
FN1
CXCL10
IL8
TNF
C3
C4BPA
CFB
SELE
NFKB1
TLR9
CCL3
FCGRT
PTAFR
IL12B
IL10
CXCL9
NOD2
TLR2
CCL20
IL2
CD48
IL6
TNFAIP3
CXCL1
PTGS2
IL1RN
NFKB2
CD44
IL27
BCL3
IL1B
RELB
IRF1
STAT5A
TAPBP
TNFRSF1B
TNFSF10
TNFSF13B
TNFRSF9
NFKBIA
NFKBIZ
HLA−B
PSMB9
TAP1
CXCL11
EBI3
IRF7
B2M
PLAU
VCAM1
CCL23
CD209
CR2
IL2RA
CCL2
CD86
TNFSF15
CD40
MX1
CD83
ICOS
IL23A
HAMP
IRF4
LEF1
TP53
CCR7
LTA
C1 C2 C3 C4 P2 P3 PC
2
1
0
−1
−2
B
PBMCs
Poly (I:C) stimulation
C1
C2
P3
Relative mRNA levels
CXCL10 IFI27 IFI44 IFIH1 IFIT2 IRF7 ISG15 OAS1 USP18
LPS stimulation
C
Fibroblast
OAS1
IFI44L
RSAD2
P1
P2
Baricitinib 0 4 8 12 24 hours
D
Fibroblast
Patient P1
Ctrl
Baricitinib
Enbrel
IFI27 RSAD2 USP18 IFIH1 ISG15 MX2 IFIT3 IFIT2 IRF7 DDX58 GBP1 OAS1
Patient P2
E
Fibroblast
P1
P2
Baricitinib(h) - 4 24 - -
Enbrel(h) - - - 4 24
p-STAT1
STAT1
p-STAT2
STAT2
p-TBK1
TBK1
IFIT3
MDA5
Rig-I
β-actin
F
Fibroblast
ISG15 Conjugates
ISG15 (LE)
ISG15 (SE)
β-actin

Fig. S1. Excessive inflammation signatures in Otulipenia patients.

(A) NanoString analysis of NF-κB signaling in whole blood samples from two patients and four healthy controls, and a type I interferonopathy patient control (PC) with DNASE2 deficiency.

(B) Gene expression levels of IFN-I pathway in PBMCs. The PBMCs from P3 and two healthy controls were stimulated with poly(I:C) (20 μg/mL) or LPS (1 μg/mL) for 22 hours before RNA extraction.

(**C**) Expression levels of *OAS1*, *IFI44L* and *RSAD2* in patients' fibroblasts. Fibroblasts from P1 and P2 were treated with 0.5 μM Baricitinib for the indicated amount of time.

(**D**) Gene expression levels of IFN pathway in P1's and P2's fibroblasts. Cells were treated with 0.5 μM Baricitinib or 10 μg/mL Enbrel for 24 hours.

(**E**, **F**) Western blots of the inhibition level of type I IFN signaling (**E**) and ISG15 conjugates (**F**) in P1's and P2's fibroblasts. Cells were treated with 0.5 μM Baricitinib or 10 μg/mL Enbrel for 4 or 24 hours. LE, long exposure; SE, short exposure.

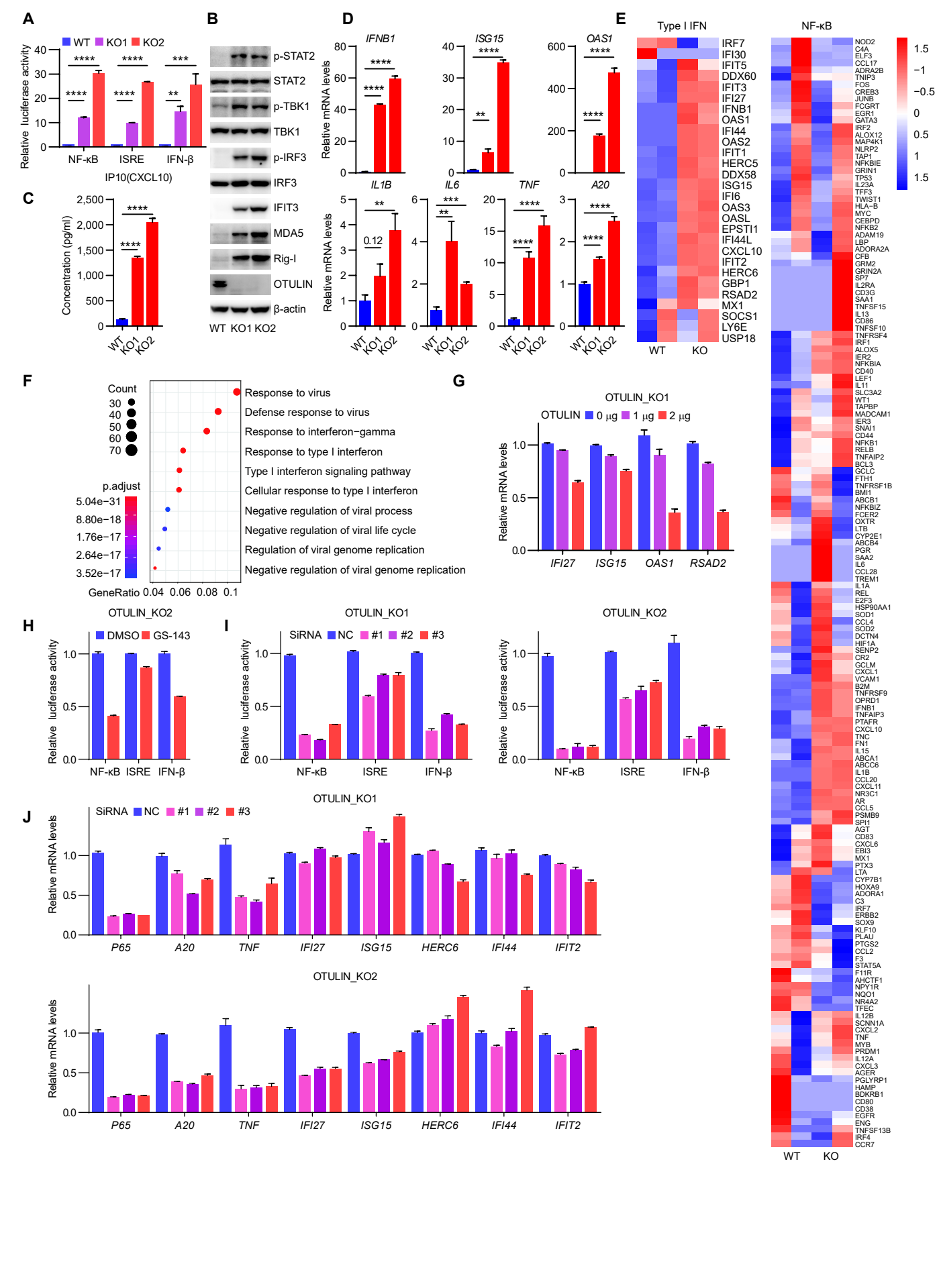

A
WT KO1 KO2
Relative luciferase activity
NF-κB
ISRE
IFN-β
IP10(CXCL10)
B
p-STAT2
STAT2
p-TBK1
TBK1
p-IRF3
IRF3
IFIT3
MDA5
Rig-I
OTULIN
β-actin
WT KO1 KO2
C
Concentration (pg/ml)
D
IFNB1
ISG15
OAS1
IL1B
IL6
TNF
A20
Relative mRNA levels
E
Type I IFN
IRF7
IFI30
IFIT5
DDX60
IFIT3
IFI27
IFNB1
OAS1
IFI44
OAS2
IFIT1
HERC5
DDX58
ISG15
IFI6
OAS3
OASL
EPSTI1
IFI44L
CXCL10
IFIT2
HERC6
GBP1
RSAD2
MX1
SOCS1
LY6E
USP18
WT KO
NF-κB
F
Count
p.adjust
Response to virus
Defense response to virus
Response to interferon−gamma
Response to type I interferon
Type I interferon signaling pathway
Cellular response to type I interferon
Negative regulation of viral process
Negative regulation of viral life cycle
Regulation of viral genome replication
Negative regulation of viral genome replication
GeneRatio
G
OTULIN_KO1
OTULIN 0 μg 1 μg 2 μg
IFI27
ISG15
OAS1
RSAD2
H
OTULIN_KO2
DMSO GS-143
I
SiRNA NC #1 #2 #3
J
P65
A20
TNF
IFI27
ISG15
HERC6
IFI44
IFIT2

Fig. S2. The elevated type I IFN signals in OTULIN deficient cells are NF-κB-independent.

(A) The transcription level of NF-κB, ISRE (Interferon Stimulation Response Element) and IFN-β in wild type (WT) and OTULIN KO HEK293T cells measured by luciferase assay.

(B) Western blots of the activation levels of type I IFN signaling in wild type (WT) and OTULIN KO 293T cell lines.

(C) ELISA analysis of IP10 level in the supernatants of wild type and OTULIN KO 293T cell lines.

(D) Quantitative real-time PCR analysis of expression levels of genes in IFN-I and NF-κB signaling pathways in wild type and OTULIN KO 293T cell lines.

(E) RNA sequencing analysis of the expression levels of genes in the IFN-I pathway (left) and NF-κB pathway (right) in wild type and OTULIN KO 293T cells.

(F) Gene enrichment analysis, using GO database, of differentially expressed genes by RNA sequencing in wild type and OTULIN KO 293T cells reveals enrichment of antiviral-related pathways.

(G) Gene expression levels of IFN pathway in OTULIN KO HEK293T cells complemented with different amounts of wild type OTULIN.

(H) Luciferase assay of the transcription level of NF-κB, ISRE and IFN-β in OTULIN KO HEK293T cells treated with a NF-κB inhibitor. OTULIN_KO2 cells were treated with 10 μM IκBα ubiquitination inhibitor GS-143 for 8 hours.

(I) Luciferase assay of the transcription level of NF-κB, ISRE and IFN-β in both OTULIN KO cell lines after inhibition of the NF-κB by knockdown of p65 using 3 different siRNAs.

(J) The expression levels of genes in IFN and NF-κB signaling pathways. p65 in HEK293T

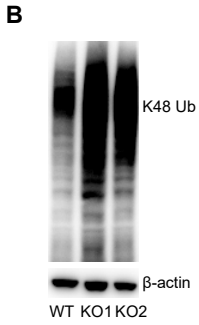
OTULIN KO cells was knocked down by 3 different siRNAs.

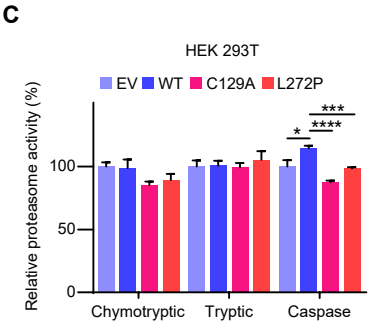
Data in panel A, C, D are shown as mean ± SEM from at least three repeated biological analyses.

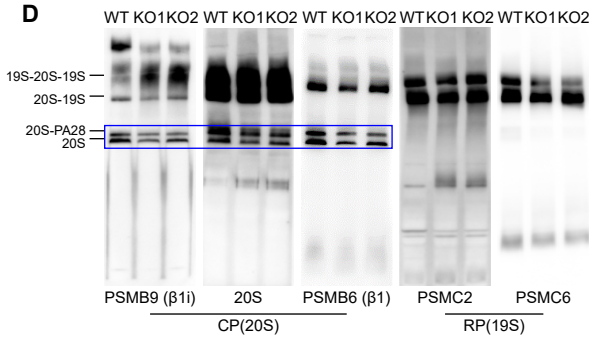
P values were determined by unpaired two-tailed *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

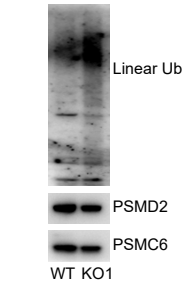
A

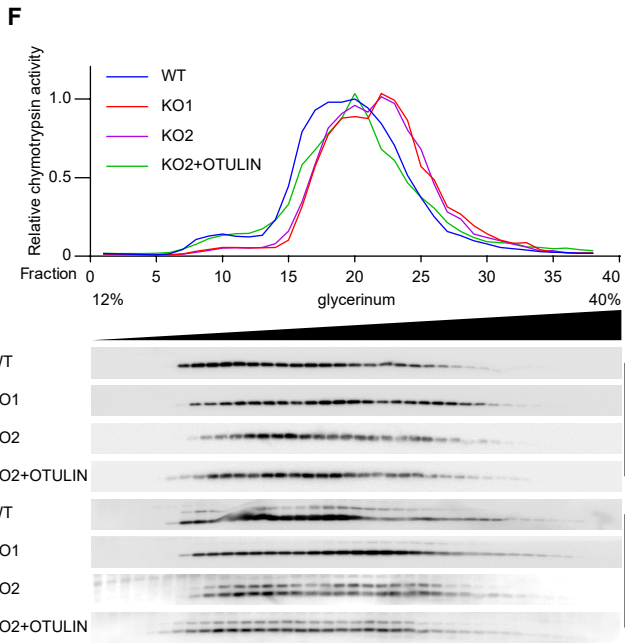
Complex	Gene Name	OTULIN Total	OTULIN Unique	HOIP Total	HOIP Unique
19S base	PSMC1	5	5	4	4
	PSMC2	0	0	9	9
	PSMC3	2	2	4	4
	PSMC4	2	2	7	7
	PSMC5	0	0	6	5
	PSMC6	4	4	2	2
	PSMD1	11	9	0	0
	PSMD2	11	10	6	6
	PSMD4	0	0	2	2
19S lid	PSMD3	7	6	4	4
	PSMD5	0	0	0	0
	PSMD6	0	0	5	5
	PSMD7	4	4	1	1
	PSMD8	2	2	0	0
	PSMD9	2	2	1	1
	PSMD10	3	3	0	0
	PSMD11	0	0	8	7
	PSMD12	0	0	0	0
	PSMD13	3	3	2	2
	PSMD14	2	2	2	2
20S	PSMA1	4	4	5	5
	PSMA2	0	0	0	0
	PSMA3	5	5	6	6
	PSMA4	8	8	4	4
	PSMA5	0	0	0	0
	PSMA6	0	0	1	1
	PSMA7	6	6	2	2
	PSMB1	0	0	1	1
	PSMB2	1	1	3	3
	PSMB3	0	0	0	0
	PSMB4	0	0	0	0
	PSMB5	5	5	6	6
	PSMB6	2	2	2	2
	PSMB7	0	0	1	1
	PSMB8	0	0	0	0
	PSMB9	0	0	0	0
	PSMB10	0	0	0	0











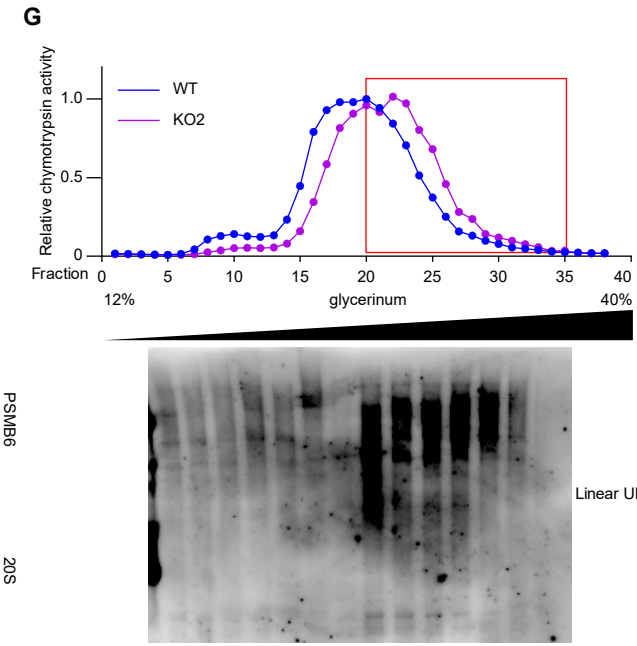


Fig. S3. OTULIN deficiency causes proteasome dysfunction.

(A) Mass spectrometry analysis identified the interactions between OTULIN or HOIP and various proteasome subunits. Numbers represent peptides captured by mass spectrometry.

(B) The K48 ubiquitination levels in wild type (WT) and OTULIN KO 293T cell lines.

(C) Proteasome activities in empty vector (EV), OTULIN wild type (WT), the catalytic-dead variant Cys129Ala (C129A) and the disease-associated variant Leu272Pro (L272P) overexpressed OTULIN KO 293T cells.

(D) Native gel analysis of the proteasome assembly in OTULIN WT and KO (KO1, KO2) 293T cell lines. The assembly of different parts of the proteasome was illustrated using antibodies of different subunits correspondingly.

(E) Western blots analysis of linear ubiquitination levels on purified proteasome from OTULIN KO 293T cells.

(F) Glycerol gradient centrifugation of lysed wild type and OTULIN KO 293T cells (KO1, KO2), and OTULIN complemented OTULIN KO 293T cells (KO2+OTULIN). The fractions were analyzed using proteasome chymotrypsin-like activity (upper panel) and immunoblotting with indicated antibodies (lower panel).

(G) Increased linear ubiquitination of proteasome fraction in OTULIN KO 293T cells as indicated by glycerol gradient centrifugation followed by proteasome chymotrypsin-like activity assay (upper panel) and immunoblotting (lower panel).

Data in panel B is shown as mean ± SEM from four repeated technical replicates. *P* values were determined by unpaired two-tailed *t*-test. $*P < 0.1$, $***P < 0.001$, $****P < 0.0001$.

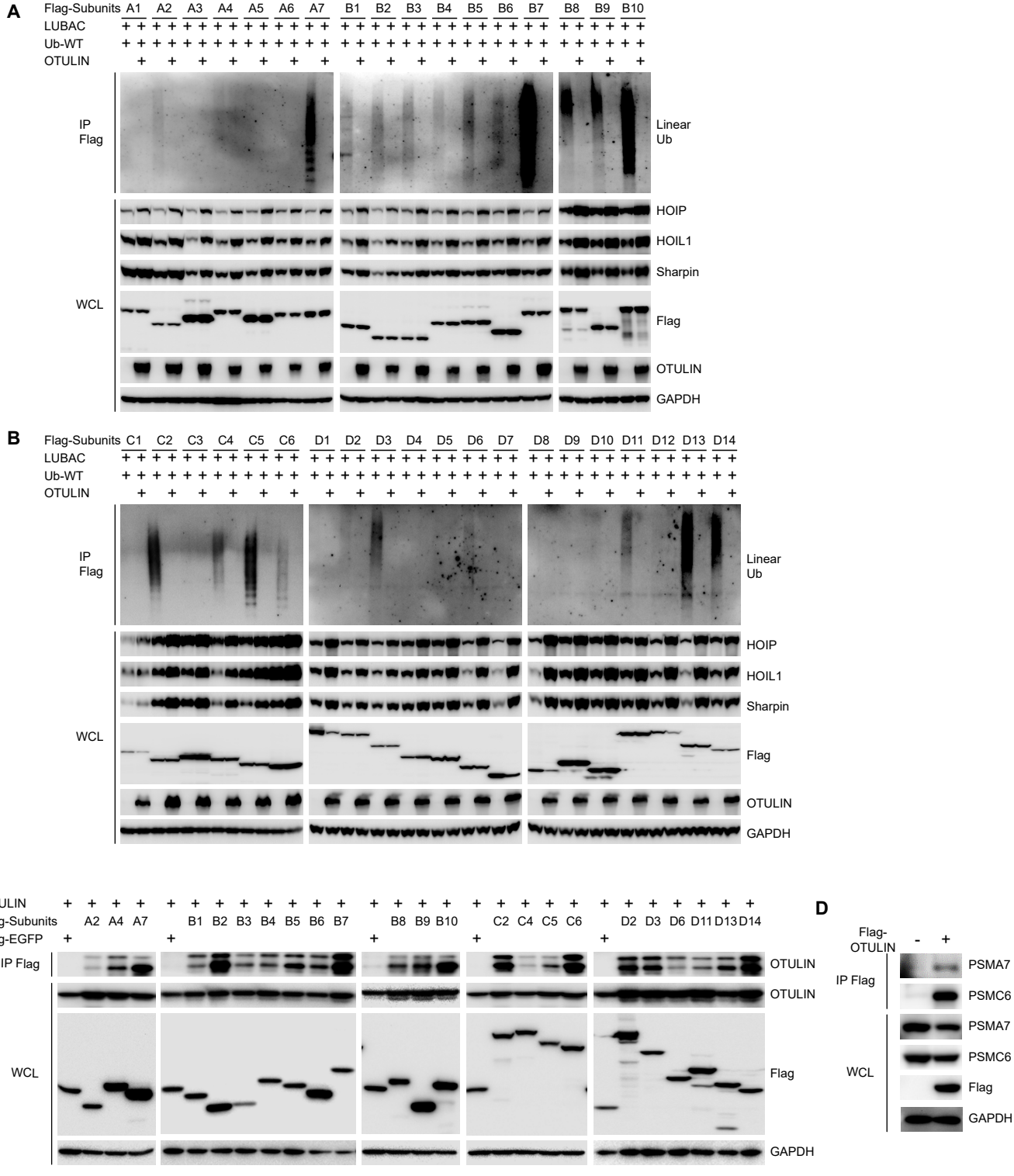

A
Flag-Subunits A1 A2 A3 A4 A5 A6 A7 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10
LUBAC
Ub-WT
OTULIN
IP Flag
Linear Ub
HOIP
HOIL1
Sharpin
WCL
Flag
OTULIN
GAPDH
B
Flag-Subunits C1 C2 C3 C4 C5 C6 D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14
LUBAC
Ub-WT
OTULIN
IP Flag
Linear Ub
HOIP
HOIL1
Sharpin
WCL
Flag
OTULIN
GAPDH
C
OTULIN
Flag-Subunits A2 A4 A7 B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 C2 C4 C5 C6 D2 D3 D6 D11 D13 D14
Flag-EGFP
IP Flag
OTULIN
OTULIN
WCL
Flag
GAPDH
D
Flag-OTULIN - +
IP Flag
PSMA7
PSMC6
WCL
PSMA7
PSMC6
Flag
GAPDH

Fig. S4. Linear ubiquitination on proteasome subunits could be removed by OTULIN deubiquitinase activity.

(A, B) Detection of OTULIN-mediated deubiquitination of the proteasome subunits using immunoprecipitation (IP). LUBAC complex consists of the catalytic subunit HOIP and two accessory proteins: HOIL1 and Sharpin. WCL, whole cell lysate. Ub-WT: wild type Ub plasmid.

(C) Immunoprecipitation confirmed the interactions between OTULIN and proteasome subunits. HEK293T WT cells were transfected with OTULIN and flag tagged proteasome subunits before lysed.

(**D**) The interactions between OTULIN and endogenous proteasome subunits PSMA7 and PSMC6 were indicated by immunoprecipitation in HEK293T cells.