Supplementary Material for

Ubiquitination-deubiquitination by the TRIM27-USP7 Complex Regulates TNF-α-induced Apoptosis

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Supplementary Table 1

Supplementary Figure Legends

FIG S1 Generation of *Trim27*-deficient (*Trim27*$^{-/-}$) mice. (A) Schematic representation of the mouse *Trim27* gene, the targeting vector, and the predicted disrupted allele. The probes used for Southern blot analyses are shown together with the predicted sizes of the hybridizing fragments. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; N, *Nco*I; P, *Pst*I; X, *Xho*I. Black and shaded boxes indicate the exon 1 region encoding the 5'-untranslated region and the N-terminal region of Trim27, which covers the RING finger, B box, and a part of the coiled-coil domain. In the targeting vector, the region encoding the N-terminal portion of Trim27, which includes the RING finger domain, was replaced by the PGK-neo cassette indicated by the gray box. (B) Southern blot analysis of genomic DNA extracted from the tails of wild-type (WT), *Trim27*$^{+/-}$, and *Trim27*$^{-/-}$ mice. (Left panel) Genomic DNA digested with *Nco*I was hybridized with the 5' probe to yield 6.6-kb and 8.8-kb bands, representing the WT and targeted alleles, respectively. Note that one *Nco*I site surrounded by parenthesis in the targeting vector was disrupted. (Right panel) Genomic DNA digested with *Hind*III was hybridized with the 3' probe to yield 8.0-kb and 1.5-kb bands, representing the WT and targeted alleles, respectively. (C) Immunodetection of Trim27. Whole cell lysates from xx of WT, *Trim27*$^{+/-}$, and *Trim27*$^{-/-}$ mice were used for immunoblotting with anti-Trim27 antibody. Equal amounts of total protein were loaded in each lane.

FIG S2 Effect of TNF-α treatment on *Trim27* and *Usp7* expression. Primary MEFS from WT or *Trim27*$^{-/-}$ mice were treated with TNF-α (20 ng/ml), and expression levels

of *Trim27* (A) or *Usp7* (B) were analyzed by qRT-PCR. Mean values ± SD (n = 3) is shown.

FIG. S3 FAS-, TRAIL-, or etoposide-induced apoptosis. WT and *Trim27*$^{-/-}$ MEFs were treated with ant-Fas antibody (5 μg/ml) and CHX (1 μg/ml) (A), Recombinant human TRAIL (100 ng/ml in conjugation with 2 μg/ml enhancer antibody) (B) or etoposide (200 μM) (C). Twenty-four hours (TRAIL/FAS) or 36 hr (Etoposide) after treatment, cell viability was measured. Average of values relative to the non-treated cells is shown with SD.

FIG S4 Localization of TRIM27 in the mitochondria of MEFs. **(A)** Immortalized MEFs were transfected with FLAG-TRIM27 expression vector, and treated with TNF-α (20 ng/ml) for indicated time. Cells were incubated with Mitotracker, fixed with 1% paraformaldehyde, and incubated with anti-FLAG antibody. After the treatment of the secondary antibodies, fluorescence signals in cells were visualized under the laser confocal microscope. The signals for Mitotracker (red) and TRIM27 (green) were merged on the right most panel. (B) Immortalized MEFs were transfected with the T7-TRIM27 and FLAG-USP7 expression vectors, fixed with 1% paraformaldehyde, and incubated with anti-T7 and anti-FLAG antibodies. After treatment with the corresponding secondary antibodies, fluorescence signals in cells were visualized with a laser confocal microscope. The far right panel shows the merged signals for TRIM27 (green) and USP7 (red).

FIG S5 Localization of TRIM27 on mitochondria. (A) Mitochondria were purified from HepG2 cells transfected with the FLAG-TRIM27 expression vector, and treated with digitonin or proteinase K. Fractions such as the mitoplast were separated by centrifugation. (B) Equivalent amounts of each fraction were analyzed by SDS-PAGE, followed by Western blotting with anti-FLAG antibody. (C) The localization of TRIM27 in mitochondria, which was suggested by the data, is schematically shown below.

FIG S6 TRIM27 ubiquitination of a USP7 mutant that lacks deubiquitinating activity. (A and B) The RFP-USP7 complex transiently interacts with RIP1. HEK 293T cells were co-transfected with the T7-RFP and FLAG-RIP1 expression vectors and the WT (A) or catalytic domain mutant (C223A) (B) of FLAG-USP7. Upper panel: lysates were immunoprecipitated with anti-T7 or control IgG and analyzed by Western blotting against anti-FLAG. Lower panel: lysates were subjected to Western blotting against anti-T7. Asterisk indicates a non-specific band. (C) Ubiquitination of USP7 by TRIM27 was examined as described in Figure 5B, except that the USP7 mutant, in which Cys-223 in the catalytic domain was mutated to Ala, was used instead of WT USP7. The degree of ubiquitination of the C223A mutant was similar to that of WT (compare with the data in Figure 5A), suggesting that USP7 does not remove ubiquitin from USP7 itself. (D) Co-immunoprecipitation of USP7 and various forms of TRIM27. HEK293T cells were transfected with a vector expressing the indicated form of

FLAG-TRIM27, and cell lysates were immunoprecipitated with anti-FLAG antibody or control IgG. The immunocomplexes were subjected to Western blotting using anti-FLAG or anti-USP7 antibodies.

FIG S7 Effect of USP7 on TRIM27 ubiquitination and TRIM27 autoubiquitination. (A) HEK293T cells were transfected with the FLAG-TRIM27 expression vector or control empty vector, together with the Myc-Ub expression vector and increasing amounts of the USP7 expression vector. (Left) Upper panel: Cell lysates were immunoprecipitated with anti-FLAG antibody, and the immunocomplexes were subjected to Western blotting with anti-Myc antibody. Lower panel: Lysates were analyzed by Western blotting with anti-FLAG antibody. (Right) The amount of sample per lane was adjusted to contain similar level of FLAG-TRIM27, and analyzed as described above. (B) HEK293T cells were transfected with the vector to express WT or RING finger mutant (RF), or control empty vector, together with the Myc-Ub expression vector. Upper panel: cell lysates were immunoprecipitated with anti-FLAG antibody, and the immunocomplexes were subjected to Western blotting with anti-Myc antibody. Lower panel: lysates were analyzed by Western blotting with anti-TRIM27 antibody. (C) HepG2 cells were transfected with the vector to express His-tagged WT or 4KR mutant of TRIM27, in which four ubiquitination sites, Lys-79, Lys-304, Lys-380, and Lys-382, were replaced by Arg, or control empty vector, together with the Myc-Ub expression vector. Upper panel: His-tagged TRIM27 was purified using cobalt-resin, and was subjected to Western blotting with anti-Myc antibody. lower

panel: Lysates were analyzed by Western blotting with anti-TRIM27 antibody. (D) Co-localization of TRIM27 mutants and USP7. HepG2 cells were transfected with vectors expressing T7-tagged TRIM27 mutants and FLAG-USP7, and immunostained with anti-T7 and anti-FLAG antibodies. After treatment with the corresponding secondary antibodies, fluorescence signals were visualized under a laser confocal microscope. Merged signals for T7-TRIM27 and FLAG-USP7 are shown on the far right panel. (E) Co-immunoprecipitation of TRIM27 mutants with USP7. HEK293T cells were transfected with the vector to express FLAG-tagged TRIM27 mutants indicated. In the Ala mutant, putative phosphorylation sites (Ser-476, 478, and 482) of TBK-1 and IKKε were replaced by Ala. Whole cell lysates from transfected cells were immunoprecipitated with anti-FLAG, and the immunocomplexes were analyzed by Western blotting with anti-USP7 or anti-FLAG.

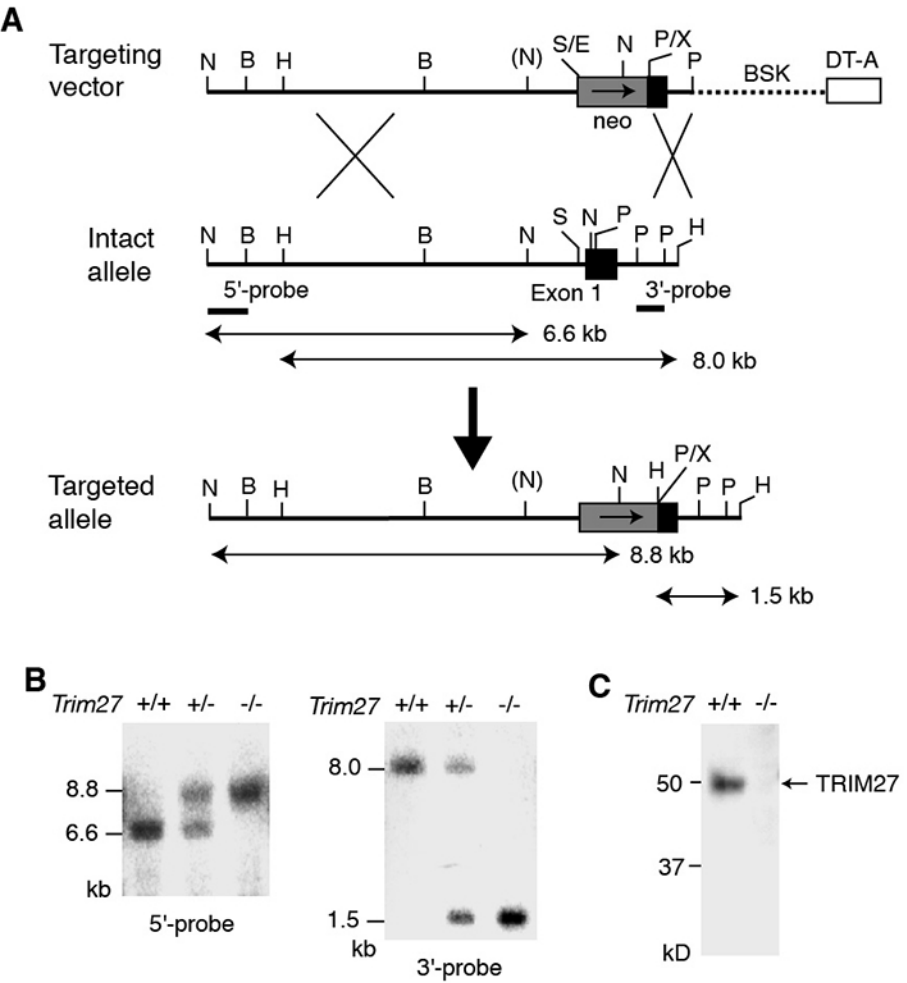
FIG. S8. USP7 is involved in TNFa/CHX-induced apoptosis. (A) Cell viability was examined as described in Fig. 1H using WT immortalized MEFs and WT cells pretreated with the USP7 inhibitor HBX 41,108 (20 mM) for 4 h. Experiments were repeated three times, and average value relative to the non-treated cells is shown with SD. **, $p < 0.01$. (B) Cleaved caspase-3 was determined by immunoblot analysis of MEFs lysates prepared at 2.5 h after TNF-a/CHX addition. WT immortalized MEFs and WT cells were pretreated with the USP7 inhibitor HBX 41,108 (20 mM) for 4 h.

FIG. S9. Comparison of ubiquitination of endogenous USP7 between WT and

Trim27$^{-/-}$ MEFs. MEFs were treated with TNF-α (10 ng/ml) and CHX (1μg/ml) for the indicated time. Endogenous USP7 was immunoprecipitated and analyzed by immunoblotting with anti-Ub. Lysates were also analyzed by Western blotting with anti-USP7 antibodies.

Table S1 Sequences of primers used.

qRT-PCR		
Gene	Forward	Reverse
A20	GAACAGCGATCAGGCCAGG	GGACAGTTGGGTGTCTCACATT
cFLIP	GACTCTAAGCCCCTGCAACC	GGAGGGCTTCTCCAAGTGAG
cIAP2	ACGCAGCAATCGTGCATTTTG	CCTATAACGAGGTCACTGACGG
ICAM	CAGATCCTGGAGACGCAGAG	GAACCACCTTCGACCCACTG
Usp7	GCGTGGGACTCAAAGAAGC	GAATCATCGCCCTCTGTTGG
Trim27	CTTCGTGGAGCCTATGATGC	CTGCGGACACGACACGTTAG
Mouse genotype by PCR		
Trim27	CTGCTTCACCAGCTGGGTCA CGTTG	GGAAACTCAGCAGAAGACTACGGG CCCACAGACGC


A
Targeting vector
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neo
Intact allele
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5'-probe
Exon 1
3'-probe
6.6 kb
8.0 kb
Targeted allele
N B H B (N) N H P/X P P H
8.8 kb
1.5 kb
B
Trim27 +/+ +/- -/-
8.8
6.6
kb
5'-probe
Trim27 +/+ +/- -/-
8.0
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3'-probe
C
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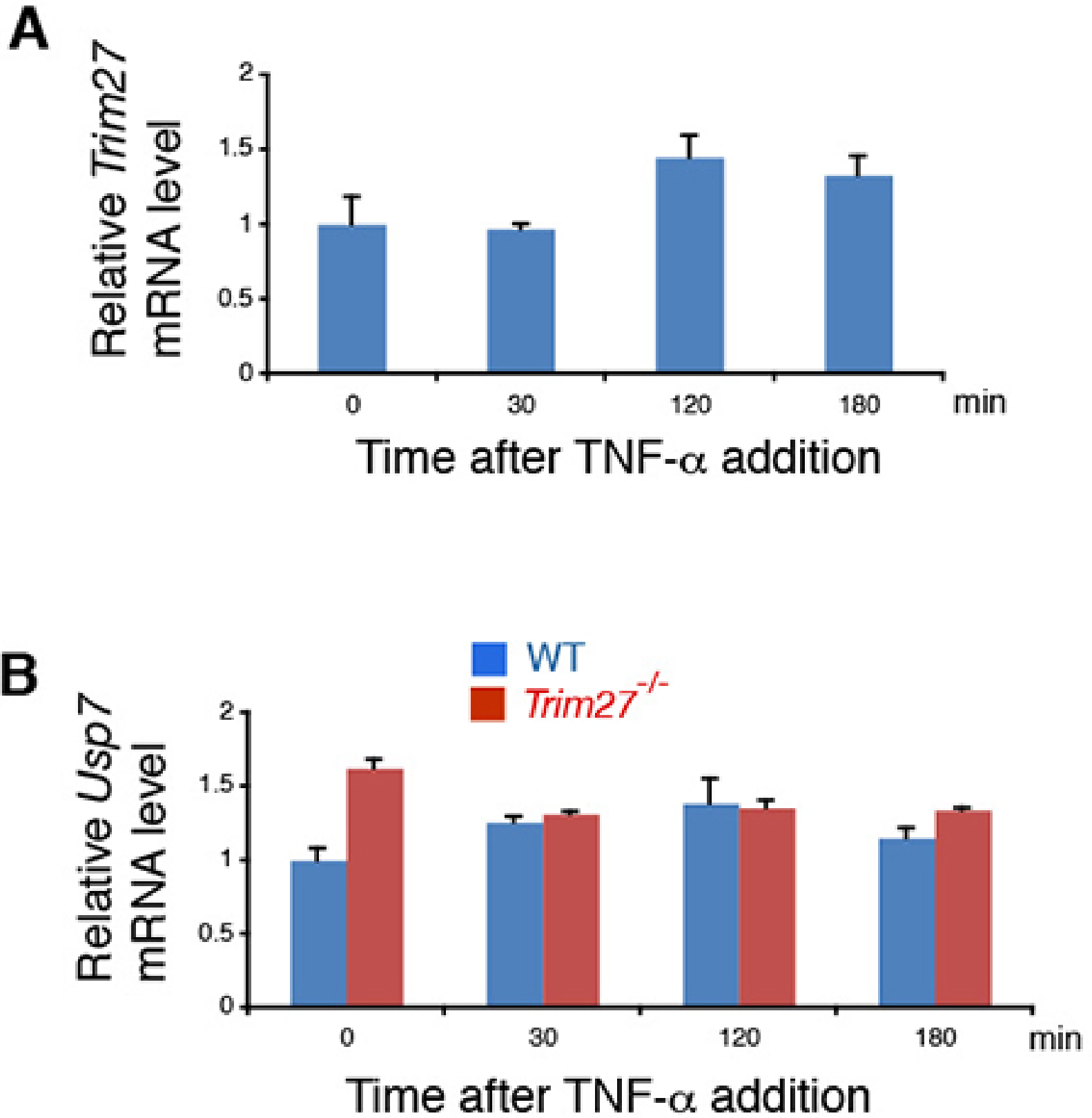
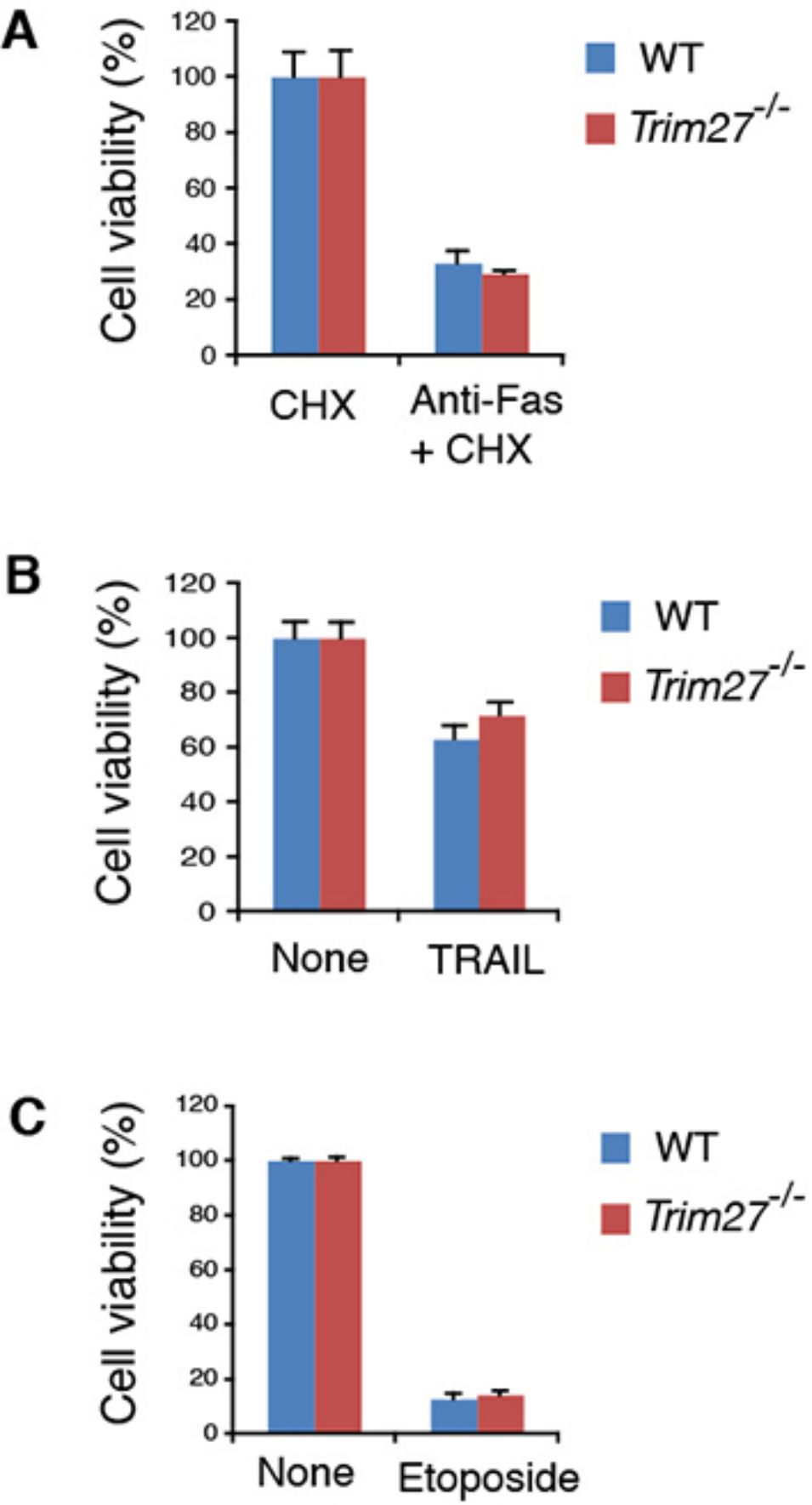
50
37
kD
TRIM27

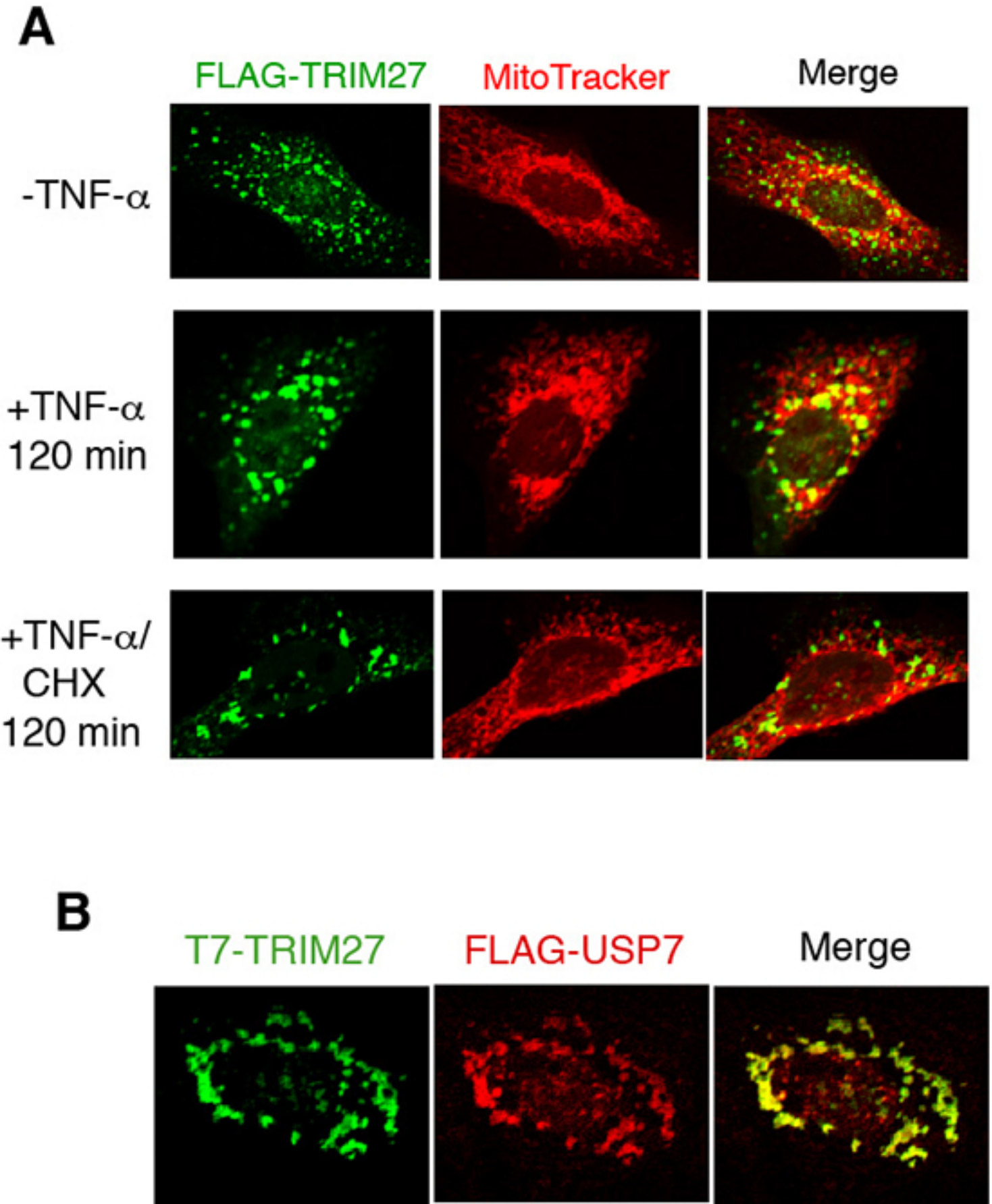
Supplementary Figure S2. Zaman, M.M. *et al.*



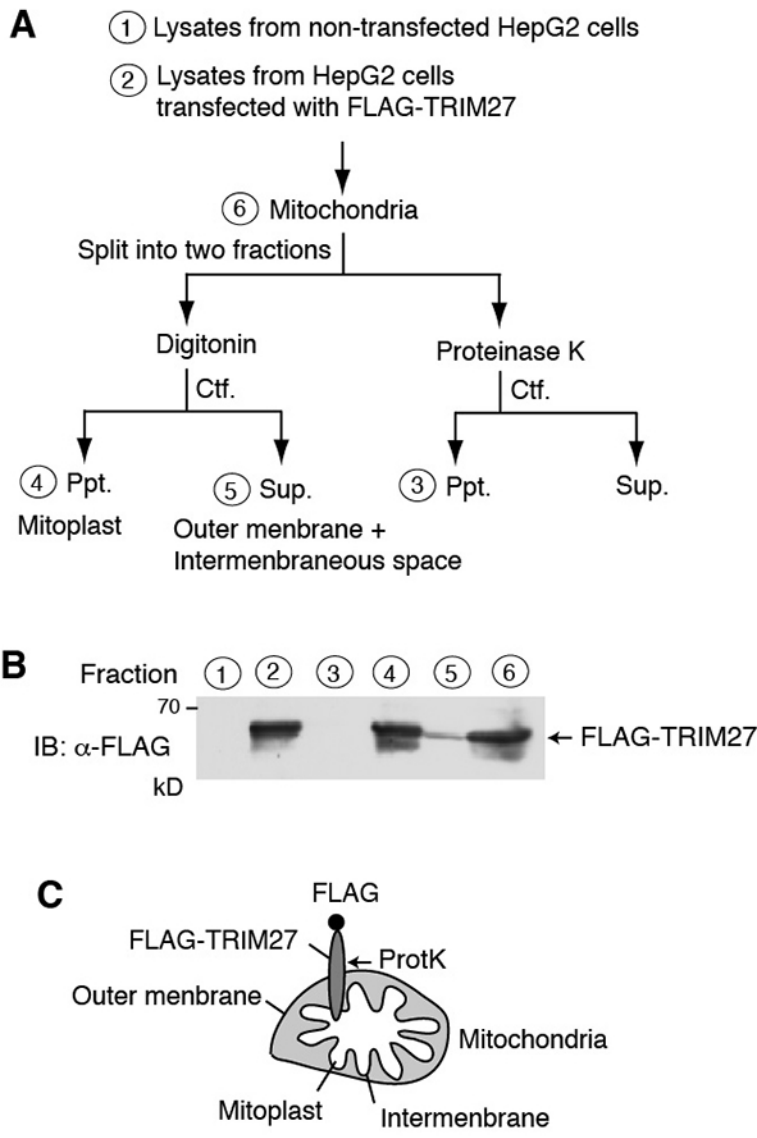
Supplementary Figure S3. Zaman, M.M. *et al.*



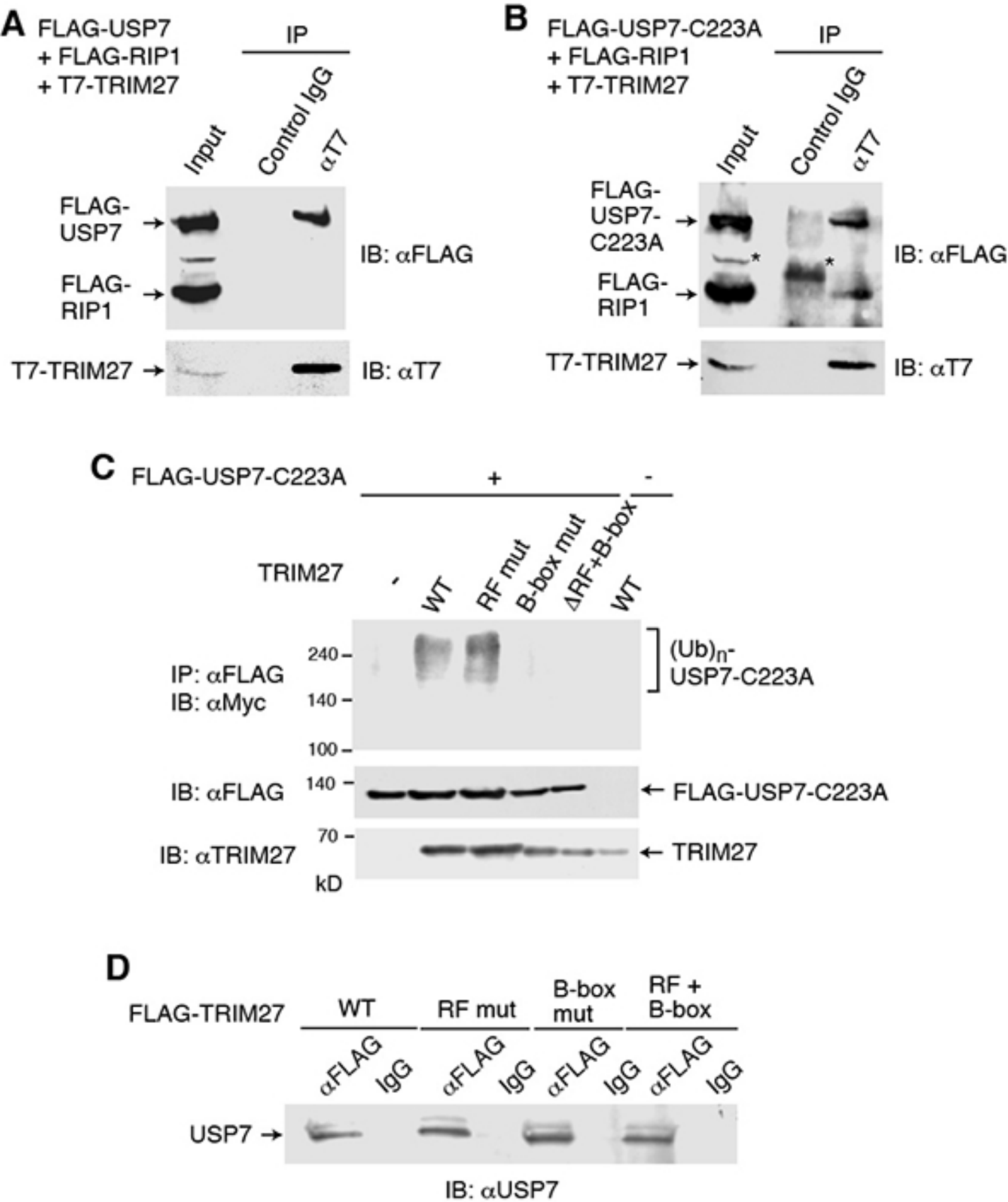
Supplementary Figure S4. Zaman, M.M. *et al.*

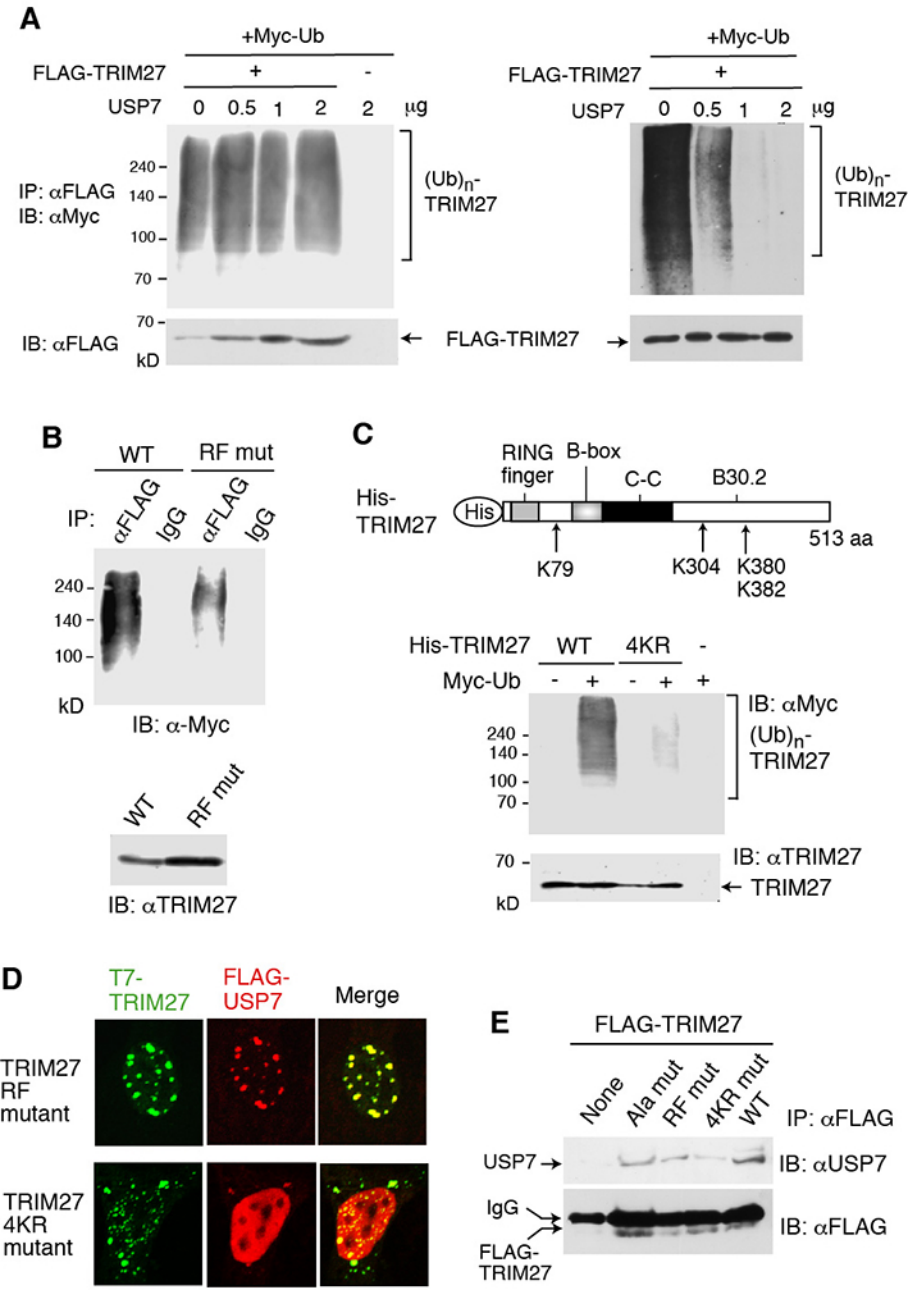


Supplementary Figure S5. Zaman, M.M. *et al.*



Supplementary Figure S6. Zaman, M.M. *et al.*

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+Myc-Ub
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+
-
USP7
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IB: αMyc
240
140
100
70
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IB: αFLAG
70
kD
FLAG-TRIM27
+Myc-Ub
FLAG-TRIM27
+
USP7
0 0.5 1 2 µg
(Ub)n-TRIM27
FLAG-TRIM27
B
WT
RF mut
IP:
αFLAG
IgG
αFLAG
IgG
240
140
100
kD
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WT
RF mut
IB: αTRIM27
C
His-TRIM27
RING finger
B-box
C-C
B30.2
His
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K79
K304
K380
K382
His-TRIM27
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4KR
-
Myc-Ub
- + - + +
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140
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70
70
kD
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Merge
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TRIM27 4KR mutant
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RF mut
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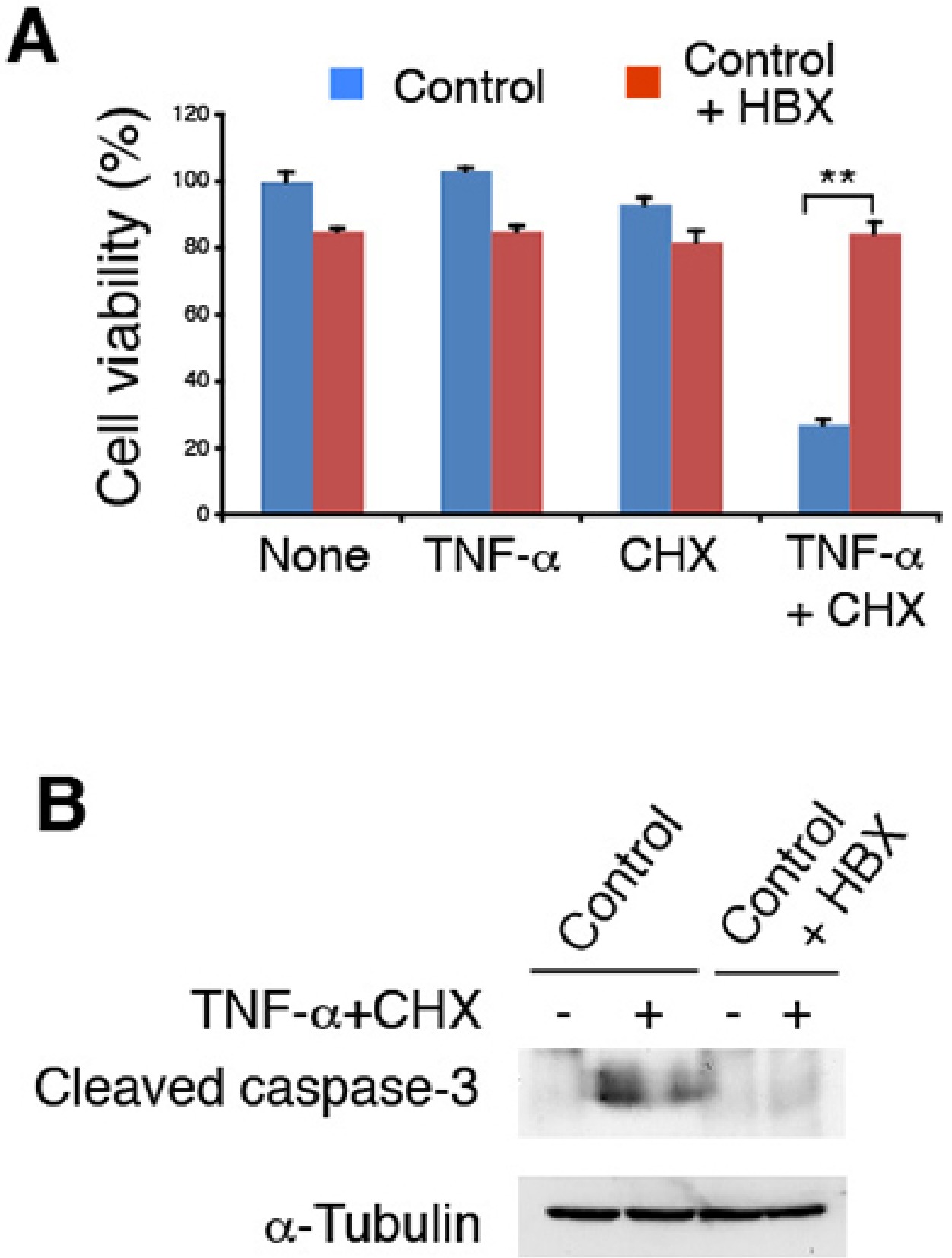
Supplementary Figure S8. Zaman, M.M. *et al.*



Supplementary Figure S9. Zaman, M.M. *et al.*