Supplementary Information

K33-linked polyubiquitination of TCR-ζ regulates T cell activation by modulating endocytosis-independent TCR phosphorylation

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Inventory of Supplemental Information

The manuscript proper is accompanied by the following supplemental items:

1. Supplemental Figure S1-S6

2. Legends to the Supplemental Figures

As a ‘roadmap’ to the supplemental figures,

Fig. S1 links to and embellishes Figure 1

Fig. S2 links to and embellishes Figure 1

Fig. S3 links to and embellishes Figures 2 and 3

Fig. S4 links to and embellishes Figure 4

Fig. S5 links to and embellishes Figure 4

Fig. S6 links to and embellishes Figure 6

3. Supplemental Results

Supplemental Text and Figures

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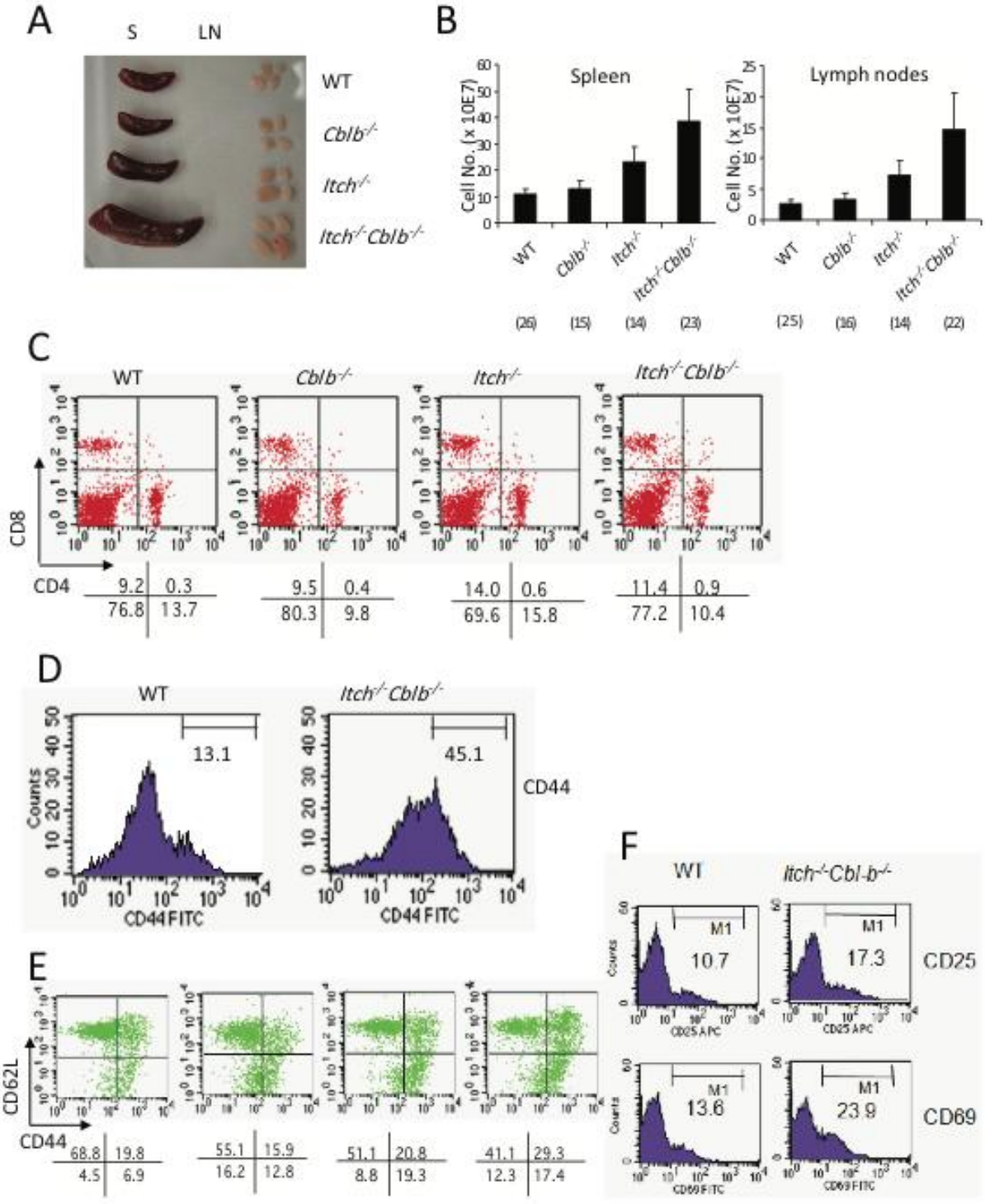
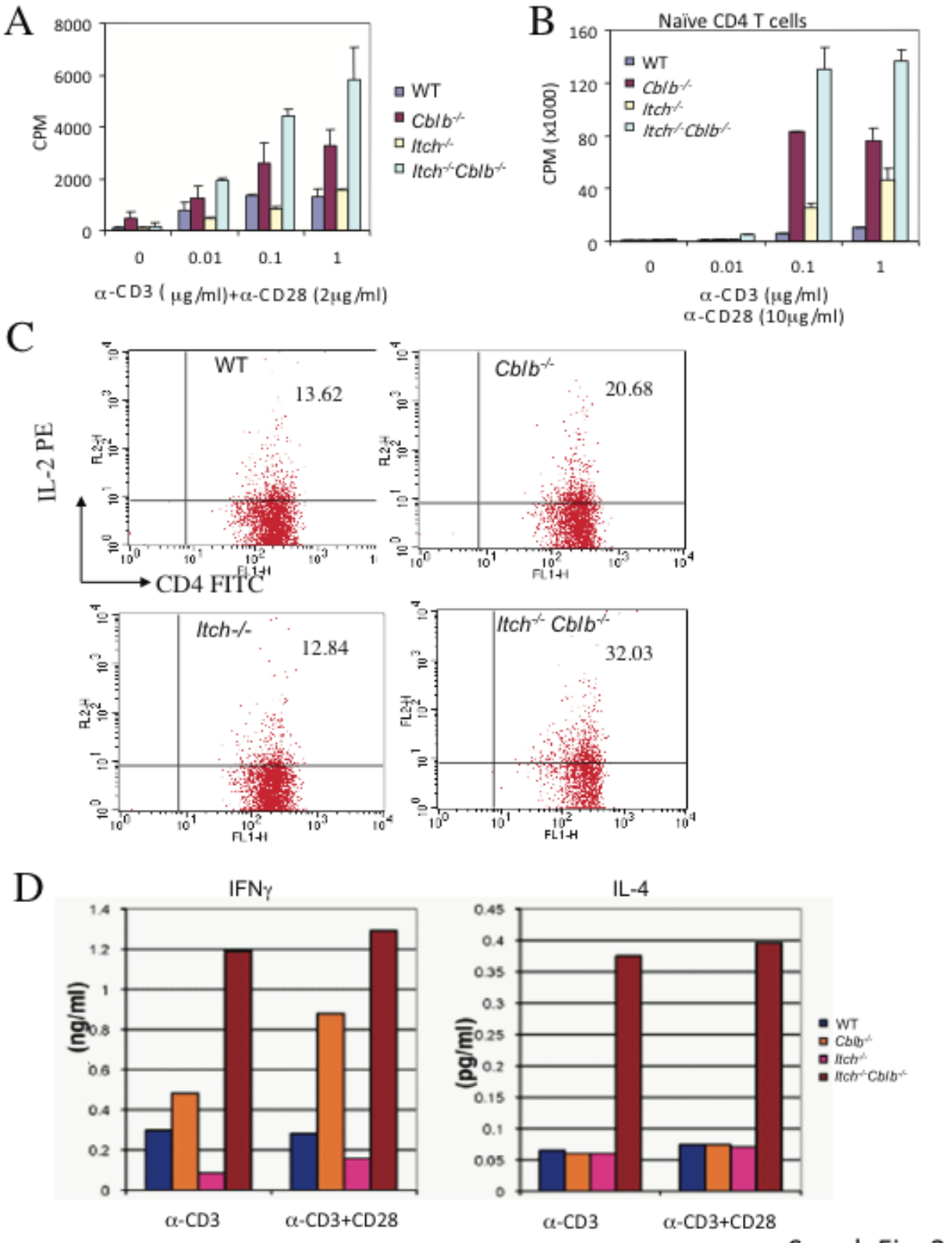
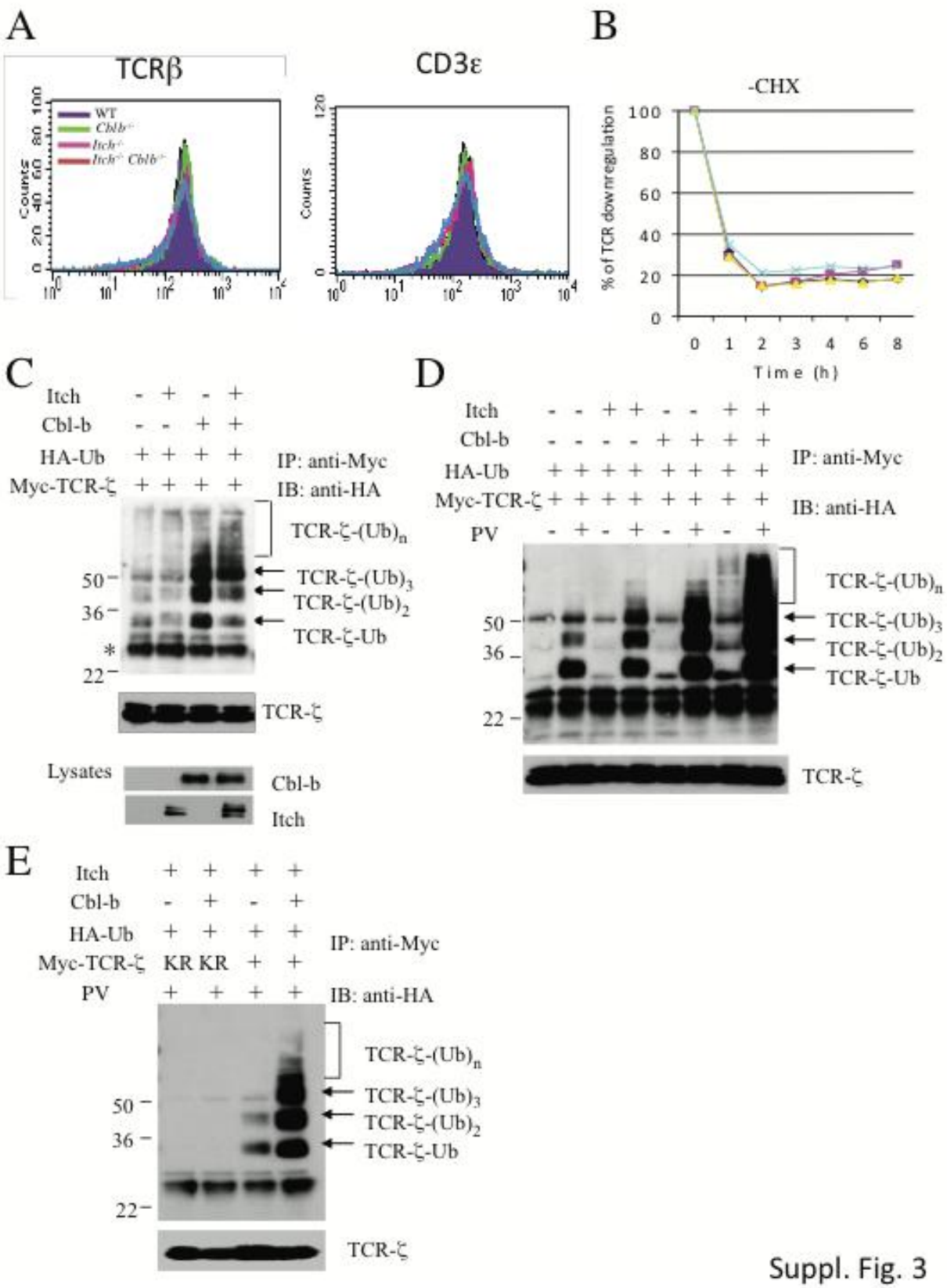
Supplementary Figures



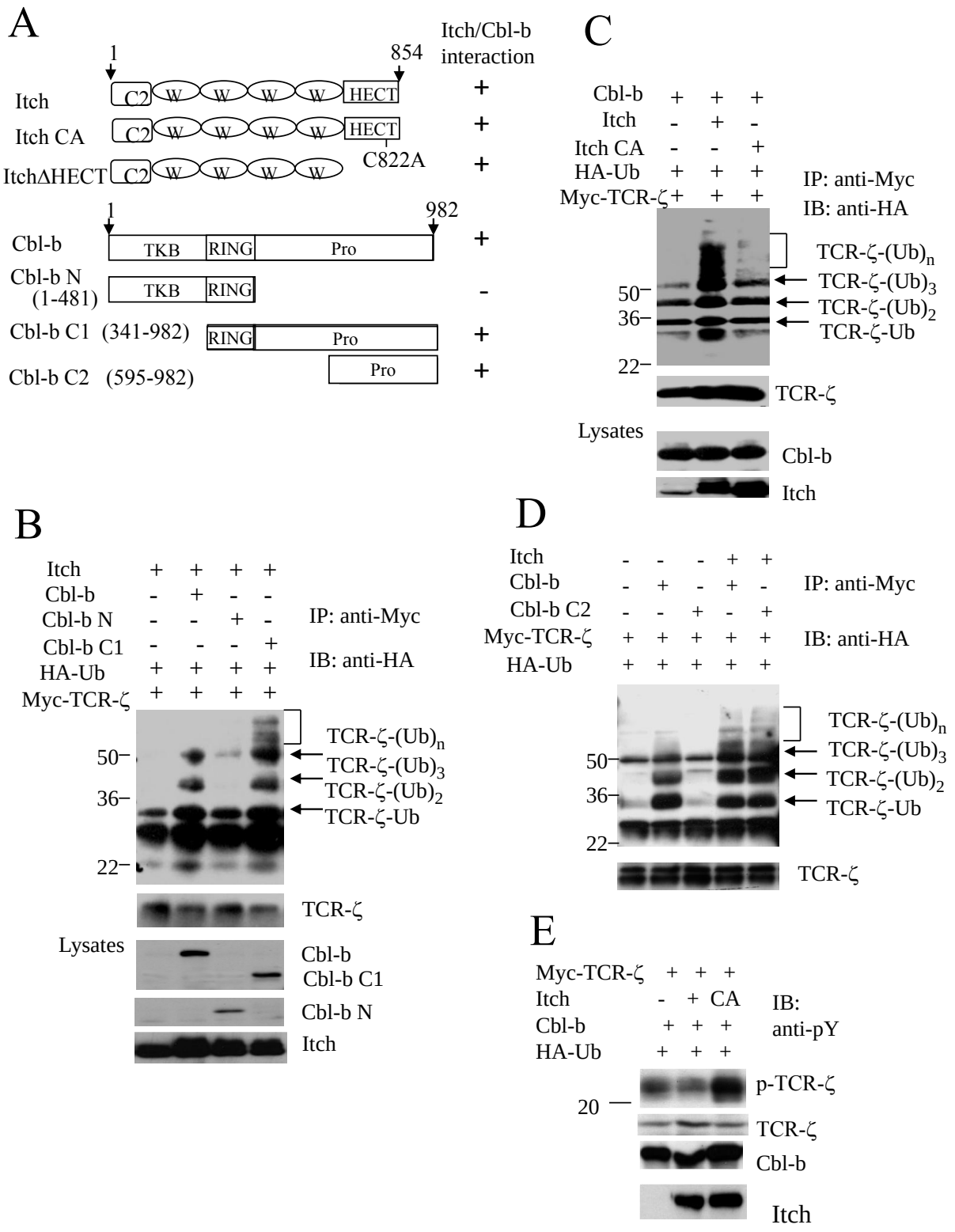
Suppl. Fig. 1


A
8000
6000
4000
2000
0
CPM
WT
Cblb-/-
Itch-/-
Itch-/-Cblb-/-
0
0.01
0.1
1
α-CD3 (μg/ml)+α-CD28 (2μg/ml)
B
Naïve CD4 T cells
160
120
80
40
0
CPM (x1000)
WT
Cblb-/-
Itch-/-
Itch-/-Cblb-/-
0
0.01
0.1
1
α-CD3 (μg/ml)
α-CD28 (10μg/ml)
C
IL-2 PE
CD4 FITC
WT
13.62
Cblb-/-
20.68
Itch-/-
12.84
Itch-/- Cblb-/-
32.03
D
IFNγ
(ng/ml)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
α-CD3
α-CD3+CD28
IL-4
(pg/ml)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
α-CD3
α-CD3+CD28
WT
Cblb-/-
Itch-/-
Itch-/-Cblb-/-

Suppl. Fig. 2

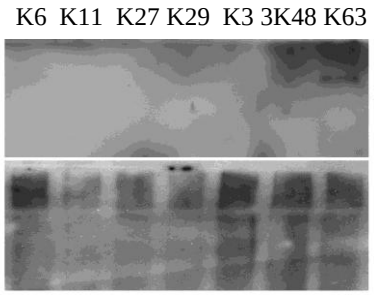

A
TCRβ
CD3ε
WT
Cblb-/-
Itch-/-
Itch-/- Cblb-/-
Counts
B
-CHX
% of TCR downregulation
Time (h)
C
Itch
Cbl-b
HA-Ub
Myc-TCR-ζ
IP: anti-Myc
IB: anti-HA
TCR-ζ-(Ub)n
TCR-ζ-(Ub)3
TCR-ζ-(Ub)2
TCR-ζ-Ub
TCR-ζ
Lysates
Cbl-b
Itch
D
PV
E
KR KR

Suppl. Fig. 3


A
Itch/Cbl-b interaction
1
854
Itch
C2
W
HECT
Itch CA
C822A
ItchΔHECT
1
982
Cbl-b
TKB
RING
Pro
Cbl-b N (1-481)
Cbl-b C1 (341-982)
Cbl-b C2 (595-982)
B
Itch
Cbl-b
Cbl-b N
Cbl-b C1
HA-Ub
Myc-TCR-ζ
IP: anti-Myc
IB: anti-HA
TCR-ζ-(Ub)n
TCR-ζ-(Ub)3
TCR-ζ-(Ub)2
TCR-ζ-Ub
50
36
22
TCR-ζ
Lysates
Cbl-b
Cbl-b C1
Cbl-b N
Itch
C
Cbl-b
Itch
Itch CA
HA-Ub
Myc-TCR-ζ
IP: anti-Myc
IB: anti-HA
TCR-ζ-(Ub)n
TCR-ζ-(Ub)3
TCR-ζ-(Ub)2
TCR-ζ-Ub
50
36
22
TCR-ζ
Lysates
Cbl-b
Itch
D
Itch
Cbl-b
Cbl-b C2
Myc-TCR-ζ
HA-Ub
IP: anti-Myc
IB: anti-HA
TCR-ζ-(Ub)n
TCR-ζ-(Ub)3
TCR-ζ-(Ub)2
TCR-ζ-Ub
50
36
22
TCR-ζ
E
Myc-TCR-ζ
Itch
CA
Cbl-b
HA-Ub
IB: anti-pY
20
p-TCR-ζ
TCR-ζ
Cbl-b
Itch

Suppl. Fig. 4

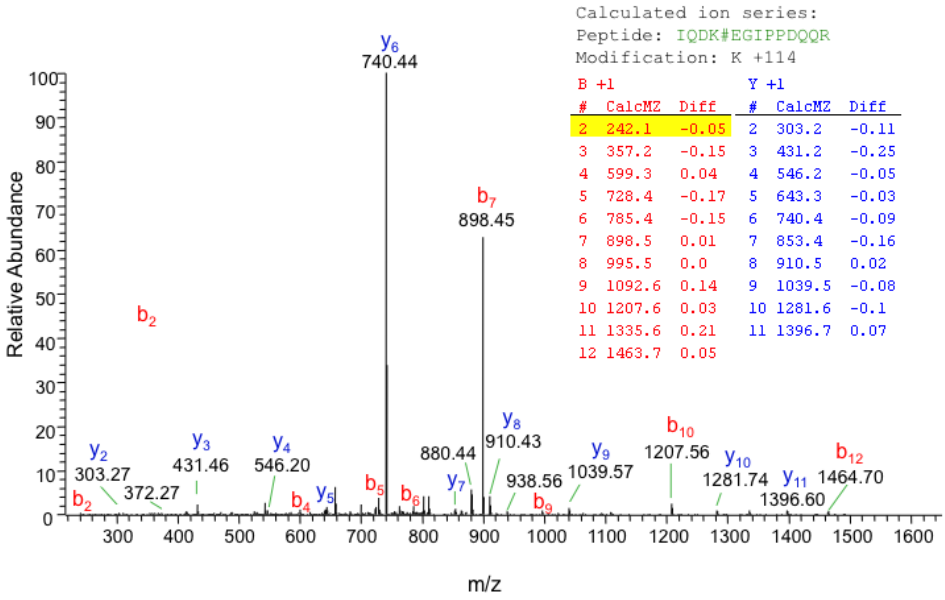
A



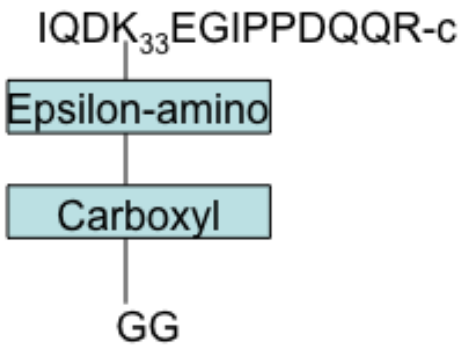
B

	6	11	27	29	33	48	63	75	76
Wt	K	K	K	K	K	K	K	G	G
K6	K	R	R	R	R	R	R	G	G
K11	R	K	R	R	R	R	R	G	G
K27	R	R	K	R	R	R	R	G	G
K29	R	R	R	K	R	R	R	G	G
K33	R	R	R	R	K	R	R	G	G
K48	R	R	R	R	R	K	R	G	G
K63	R	R	R	R	R	R	K	G	G
K48/63R	K	K	K	K	K	R	R	G	G
K33R	K	K	K	K	R	K	K	G	G
ΔGG	K	K	K	K	K	K	K	(stop)	

C



D



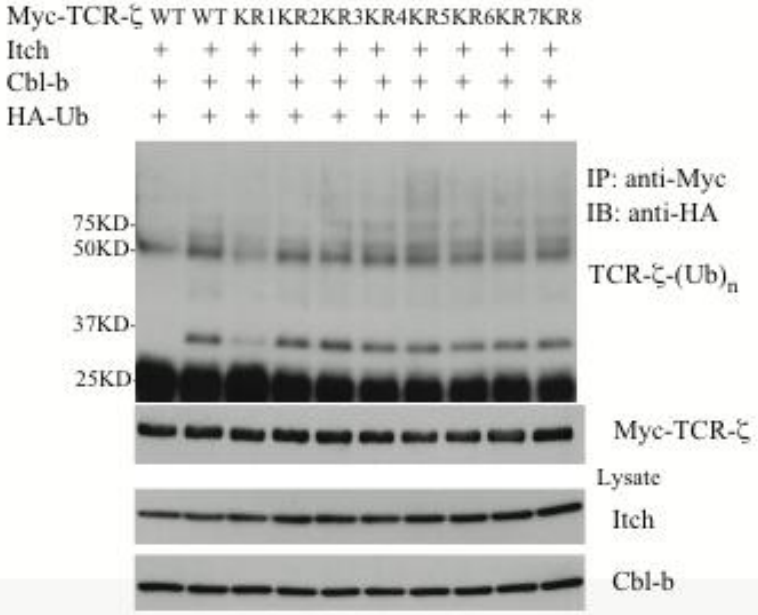
Suppl. Fig. 5

A

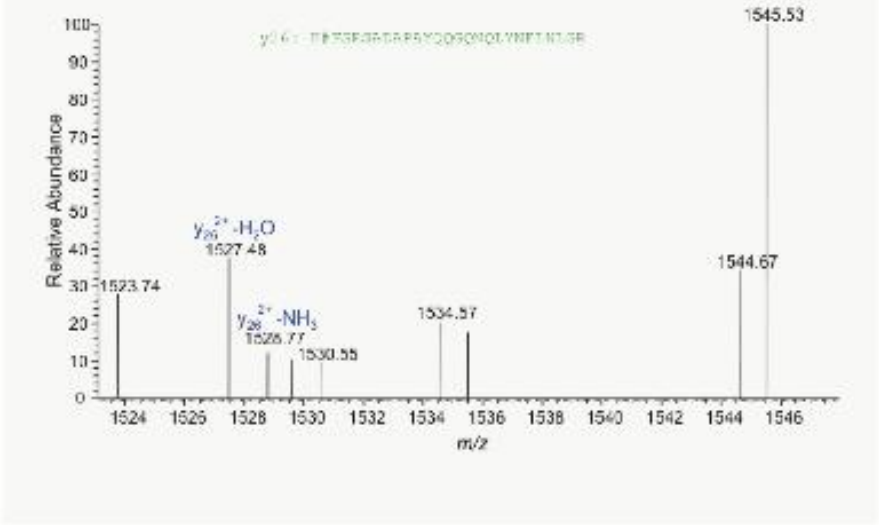
TCR-ζ protein sequence:

MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLRVKFSRSADAPAY
QQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQK
DKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

B



C



Suppl. Fig. 6

Supplementary Figure Legends

Figure S1, related to Figure 1. Chronic T cell activation in double-deficient mice. (A) Spleen and lymph nodes from 8 week-old wild-type (WT), *Cblb*$^{-/-}$, *Itch*$^{-/-}$, or double-deficient mice were isolated and photographed. (B) Total cell numbers of spleen and lymph nodes in wild-type (WT) and double-deficient mice were counted. The numbers in parenthesis were mouse numbers used for the analysis. (C) Profiles of splenic T cells as revealed by double staining of the cells with FITC-anti-CD4 and PE-anti-CD8. The numbers below each panel represent the percents of cells in each quadrant. (D) Splenic CD4 T cells were also examined for CD44 and CD62L cell surface expression in 8 week-old mice. (E, F) The cell surface expression of CD44 (E) and CD25 or CD69 (F) on gated CD4$^+$ T cells in 3-month-old mice.

Figure S2, related to Figure 1. Enhanced proliferation and IL-2 production in double-deficient T cells**.** (A) Splenic T cells from WT, *Cblb*$^{-/-}$, *Itch*$^{-/-}$, or double-deficient mice were isolated and stimulated anti-CD3 plus anti-CD28 for 24 h. Cell proliferation was measured by ^{3}H-thymidine uptake. (B) Naive CD4$^+$ T cells were purified from spleen and lymph nodes and were stimulated for the measurement of T cell proliferation. (C) The IL-2 production was measured by intracellular staining with anti-IL-2 PE plus cell surface staining with anti-CD4-FITC, and the intracellular IL-2 accumulation was analyzed by FACS analysis. Data are representatives of three repeated experiments. (D) Isolated splenic CD4$^+$ T cells were cultured with soluble anti-CD3 and anti-CD28 (1 μg/ml each). After 24 h stimulation, the culture supernatants were harvested and concentrations of IFN-γ and IL-4 were measured by ELISA.

Figure S3, related to Figures 2 and 3. (A) Cell surface expression of TCRβ and CD3ε in purified $CD4^+$ T cells as measured by flow cytometry. (B) TCR downregulation. Purified $CD4^+$ T cells were stimulated with anti-CD3ε (2 μg/ml) for different time periods, followed by FACS analysis of the cell surface TCR expression. The percentage of TCR downmodulation was shown. Data are representatives of five repeated experiments. (C) Cbl-b and Itch cooperate to induce polyubiquitination of TCR-ζ. 293T cells were transfected with plasmids containing Itch, Cbl-b, HA-Ub, and Myc-TCRζ cDNAs. The cell lysates were prepared in a lysis buffer containing SDS and N-ethylmaleimide to disrupt non-specific protein-protein interactions. The lysates were immunoprecipitated with anti-Myc and blotting with anti-HA. Molecular size markers are shown at left. The numbers of Ub conjugated to TCR-ζ is indicated. TCR-ζ-(Ub)n indicates the polyubiquitinated TCR-ζ. The cell membrane was reprobed with anti-TCR-ζ. The position of Ig light chain was marked with *. Please note that the TCR-ζ-$(Ub)_3$ co-migrates with the Ig heavy chain. Aliquots of cell lysates were detected for the expression of Itch or Cbl-b. (D) Transiently transfected 293T cells were left untreated or treated with pervanadate (PV) for 5 min and the TCR-ζ ubiquitination was analyzed. Molecular size markers are indicated at left. (E) Ub conjugation to TCR-ζ. 293T cells were transfected with either wild-type TCR-ζ or TCR-ζ KR mutant in that all nine lysine residues were mutated to arginine in the intracellular tail plus other indicated plasmids. The cell lysates were subjected to immunoprecipitation with anti-Myc and immunoblotting with anti-HA. Molecular size markers are indicated at left.

Figure S4, related to Figure 4. (A) Itch and Cbl-b interact with each other. Schematic diagrams show the cDNA structures of Itch and Cbl-b plasmids used in this project. The mapping result of Itch and Cbl-b interaction among the different constructs is shown on the left. (B) Cbl-b as an adaptor to regulate Itch-mediated polyubiquitination of TCR-ζ. 293T cells were transfected with wild-type Cbl-b, or its N-terminal region (Cbl-b N) or C-terminal region (Cbl-b C1, amino acids 341-982) plus other indicated plasmids. The ubiquitination of TCR-ζ was analyzed. (C) Requirement of Itch ligase activity for TCR-ζ ubiquitination. 293T cells were transfected with WT Itch, or Itch CA mutant plus other indicated plasmids and the transfected cells were analyzed as in (B). (D) Cells were transfected with WT Cbl-b or a proline-rich region of Cbl-b (Cbl-b C2) plus other indicated plasmids. Molecular size markers are indicated at left. (E) WT Itch or Itch ligase-deficient mutant (Itch CA) was coexpressed with Cbl-b in 293T cells and the effect on TCR-ζ phosphorylation was examined.

Figure S5, related to Figure 4. Analysis of Ub chain formation in TCR-ζ**.** (A) Detection of the specificity of Ub K63 antibody. Seven different HA-Ub mutant constructs were overexpressed in Jurkat cells and immunoprecipitated with anti-HA antibody, followed by immunoblotting with Ub K63 antibody (upper panel). The same membrane was probed with anti-HA (bottom panel). (B) Schematic representation of Ub and its mutants. (C) proteomics dentification of Ub K33-linkage in TCR-ζ. Jurkat T cells were expressed with His-HA-K33 Ub together with Itch and Cbl-b. Cell lysates were purified using Ni-NTA beads, followed by mass spectrometry. Tandem mass spectrum of the ubiquitin peptide derived from K33 Ub conjugates is shown. The full tryptic peptide IQDK#EGIPPDQQR with K33 modified by GG was unambiguously identified by the

almost complete ion series, including the site localization ions. The precursor ion mass was 819.84, XCorr 4.497 and dCn 0.445. "#" denotes the residue on its left had a mass addition of 114. (D) Illustration of the K33 linkage between one Ub at the K33 site with the last two GG residues of another Ub. Upon trypsin digestion, a branched peptide is formed in which the G-76 –COOH terminus of one Ub peptide ($G^{75}G^{76}$) is attached to the ε-amine of K33 in the tryptic peptide ($I^{30}QDKEGIPPDQQR^{42}$) of another Ub. That gives the K33 residue a mass of addition of 114 dalton.

Figure S6, related to Figure 6. Mapping of the Ub conjugation site(s) in TCR-ζ. (A) Protein sequence of TCRζ. Small lettered region is the signal peptide. Underlined region is the transmembrane domain. Lysine 54 is highlighted in red. (B) Point-mutation at the conserved lysine residues in TCR-ζ (KR1, K54, KR2, K88, KR3, K99; KR4, K104, K5, K116/K118; KR6, K129; KR7 ,K136; KR8, K150, respectively) was performed using site-directed mutagenesis. Myc-tagged wild-type TCR-ζ or its mutants were coexpressed with Itch, Cbl-b, and HA-Ub in Jurkat T cells. The lysates from anti-CD3-stimulated cells were immunoprecipitated with anti-Myc and blotted with anti-HA. Molecular size markers are indicated at left. Aliquots of the lysates were blotted with Itch or Cbl-b antibodies. (C) A zoom-in view of the tandem mass spectrum at 1520 to 1546 m/z, showing the site-localization ion y26 that covers K54 of TCR-ζ. The y26 ion with neutral loss of H_2O and NH_3 are shown.

Supplementary Results

Cbl-b and Itch cooperate to promote TCR-ζ ubiquitination

To avoid the interference of other T cell-associated components in Jurkat T cells, we used human embryonic kidney 293T cells for the transfection study. This is quite critical, since other subunits of the TCR complex may be also modulated by ubiquitination. By using this system, we showed that coexpression of Cbl-b, but not Itch, with TCR-ζ increased the ubiquitination of TCR-ζ (Fig. S3C). Remarkably, the TCR-ζ ubiquitination was augmented by coexpression of both Itch and Cbl-b, which caused a shift from lower molecular weight bands to higher molecular weight smears.

To assess whether Itch and Cbl-b promote TCR-ζ ubiquitination in an activation-dependent manner, we treated transiently transfected 293T cells with a phosphatase inhibitor, pervanadate, and found that Ub conjugation to TCR-ζ was increased by pervanadate treatment, even in cells without Cbl-b or Itch overexpression (Fig. S3D). Notably, the Ub conjugation to TCR-ζ was markedly increased in the presence of both Cbl-b and Itch.

To further confirm that the Ub conjugation occurs on TCR-ζ itself, but not on its associated proteins, we used a TCR-ζ mutant which lacks all 9 lysine residues on its intracellular tail (TCR-ζ KR) and found that this mutant was not ubiquitinated in the presence of both Cbl-b and Itch even under stimulated conditions (Fig. S3E). The result suggests that TCR-ζ is a substrate for Itch and Cbl-b E3 ligases.

Cbl-b as an adaptor to increase Itch-promoted TCR-ζ ubiquitination

We investigated whether Itch also associates with Cbl-b in cells by using a combination of different constructs and It was found that a C-terminal region (C2, a.a 595 to 982) in Cbl-b is responsible for the interaction with Itch (Fig. S4A). We further studied the structural requirement of Cbl-b in Itch-enhanced TCRζ ubiquitination. Coexpression of Itch and a Cbl-b N-terminal region did not significantly increase the Ub conjugation to TCR-ζ as compared with cells expressed with wild-type Cbl-b (Fig. S4B). However, coexpression of Itch and the Cbl-b-C1 protein, which contains the RING finger and C-terminal portion, promoted even stronger TCR-ζ ubiquitination than the combination of Itch and the wild-type Cbl-b. The E3 ligase activity of Itch was required for such enhancement, since the Ub conjugation to TCR-ζ in cells coexpressed with Cbl-b and the ligase-defective Itch CA mutant decreased to a similar level as in cells expressed with Cbl-b only (Fig. S4C).

It seems that the Cbl-b C-terminal proline-rich region is critical for Itch to enhance TCR-ζ ubiquitination. To further test this hypothesis, we examined a functional role of Cbl-b C2 in TCR-ζ ubiquitination. Surprisingly, the coexpression of Itch with Cbl-b proline-rich sequences, without the presence of the RING finger, induced TCR-ζ ubiquitination to an extent similar as the wild-type Cbl-b, whereas the C2 region alone could not initiate TCR-ζ ubiquitination (Fig. S4D), suggesting that whereas the full-length Cbl-b is required for TCR-ζ ubiquitination in the absence of Itch, this proline-rich region of Cbl-b, which associates with Itch, is sufficient for Itch to induce the ubiquitination of TCR-ζ. In addition, the E3 ligase activity of Itch was required for the inhibition of TCR-ζ phosphorylation, since coexpression of Cbl-b with the ligase activity-

deficient Itch CA mutant rather enhanced the phosphorylation status of TCR-ζ (Fig. S4E).