# The E3 ligase Itch and deubiquitinase Cyld co-operatively regulate Tak1 and inflammation

Neesar Ahmed[1], Minghui Zeng[1], Indrajit Sinha[1], Lisa Polin[1], Wei-Zen Wei[1], Chozhavendan Rathinam[2], Richard Flavell[3], Ramin Massoumi[4] & K Venuprasad[1]

[1]Karmanos Cancer Institute, Departments of Oncology, Immunology & Microbiology, Wayne State University School of Medicine, Detroit, MI 48201, USA. [2]Department of Genetics & Development, Columbia University Medical Center, New York, NY 10032, USA. [3]Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520, USA. [4]Department of Laboratory Medicine, Lund University, Malmö, Sweden.

**Correspondence should be addressed to K.V.** (poojaryv@karmanos.org)

## SUPPLEMENTARY METHODS

### Immunoprecipitation, immunoblotting and precipitation assays

These standard assays were described earlier[35]. In brief, cell lysis was performed using NP-40 buffer containing 50 mM Tris [pH8], 150 mM NaCl, 1% NP-40, EDTA free protease inhibitor cocktail (Roche), 2 mM sodium fluoride (Boston Bioproducts), 10 mM β-glycerophosphate (Boston Bioproducts), 1 mM sodium orthovanadate (Sigma) and protein estimations were done using the Bio-Rad protein assay kit according to manufacturer's protocol. Whole cell lysates were precleared with 20 μl of Protein A/G plus agarose beads (Santa Cruz) for 1 h at 4°C. Lysates were first incubated with 1 μg of the desired antibody for 2 h at 4°C followed by a further 1 h of incubation at 4°C with 25 μl of Protein A/G beads. The immunocomplexes were washed five times with NP-40 buffer, denatured using 4x Laemmli buffer and separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted with the indicated antibodies and visualized with Amersham ECL or ECL plus western blotting detection system (GE). For reprobing of membranes, membranes were stripped by incubation in a stripping buffer (62.5 mM TRIS-HCl, pH6.7; 100 mM 2-mercaptoethanol and 2% SDS) at 70°C for 45 min and washed thoroughly before reprobing. GST-Itch fusion protein was expressed and purified from *E. coli* using the GST bulk kit (GE) according to manufacturer's instructions. The bound proteins were separated by SDS-PAGE and immunoblotted with antibody against Cyld.

### Ubiquitination assay

HEK293T cells were transfected with Flag-Tak1 and various constructs as indicated. Cells were washed three times in PBS and lysed in NP-40 buffer, immunoprecipitation

was performed using antibody against Flag. Tak1 associated ubiquitin was analyzed by immunoblotting using antibody against HA.

**TAMs**

The lung nodules were collected under a dissection microscope and crushed between two glass slides in chilled PBS to obtain a cell suspension. RBCs were lysed (RBC lysis buffer, Biolegend) and cells were thoroughly washed and resuspended in prechilled MoAM (RPMI 1640 supplemented with 7% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin). The cell suspension was then passed through a sterile cell strainer (70 μm, Falcon) and kept on ice till cells were counted. $4x10^6$ cells were incubated in each well of a 6 well tissue culture plate for 45 min at 37°C in a humidified incubator. Non-adherent cells were gently aspirated out and the loosely adherent cells were washed with prewarmed MoAM to obtain only the strongly adherent macrophages. Cells were directly lysed and RNA was collected for gene expression analysis.

**Gene expression analysis**

Total RNA was isolated using RNeasy Mini Kit (Qiagen) and subjected to cDNA synthesis using Verso cDNA kit (Thermo Scientific). Real-time quantitative PCR was performed in Mastercycler ep Realplex (Eppendorf) using the LightCycler 480 SYBR Green I Master reaction mix (Roche) in a 20 μl PCR reaction. The expression of individual genes was normalized to the expression of actin. Cycling conditions were: 95°C for 2 min, followed by 50 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 20 sec. The gene-specific primer sets (all for murine genes) were:

TNF sense 5’ GAACTGGCAAAAGGATGGTGA 3’

TNF antisense 5’ TGTGGGTTGTTGACCTCAAAC 3’

IL-6 sense 5’ TAGTCCTTCCTACCCCAATTTCC 3’

IL-6 antisense 5’ TTGGTCCTTAGCCACTCCTTC 3’

IL-1β sense 5’ AACCTGCTGGTGTGTGACGTTC 3’

IL-1β antisense 5’ CAGCACGAGGCTTTTTTGTTGT 3’

Ahmed N et al. Supplementary Figure 1

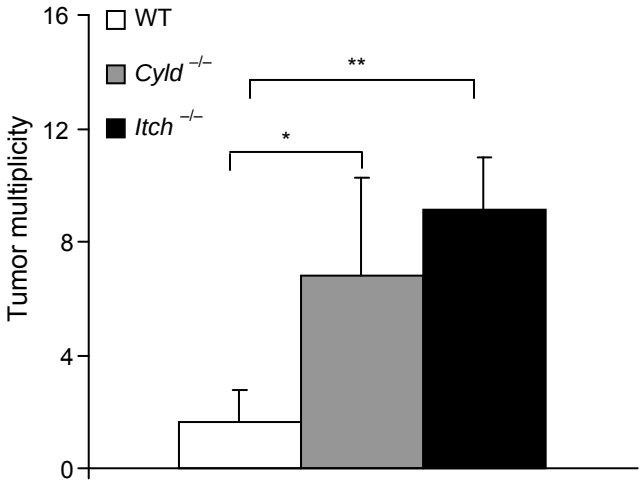



**Figure 1** Elevated lung metastasis in *Itch*$^{-/-}$ and *Cyld*$^{-/-}$ mice. 2x10$^6$ LLC cells were injected subcutaneously on the left flank of WT (n=6), *Itch*$^{-/-}$ (n=4) and *Cyld*$^{-/-}$ (n=5) mice. When the primary tumors reached a size of 2000 mm$^3$, spontaneous lung metastasis was evaluated by counting the number of tumors in the H&E stained histological slides. *P<0.05 and **P<0.01(paired t-test). Data represent two independent experiments.

Ahmed N et al. Supplementary Figure 2

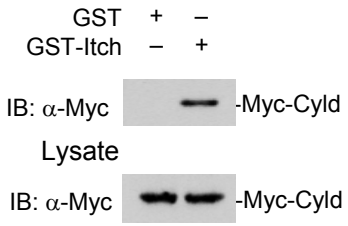



**Figure 2** GST-Itch precipitates Cyld expressed in 293T cells. Immunoblot of cellular lysate from 293T cells transfected with Myc-Cyld precipitated with GST or GST-Itch purified from *E. coli*. Data represent three independent experiments.

Ahmed N et al. Supplementary Figure 3

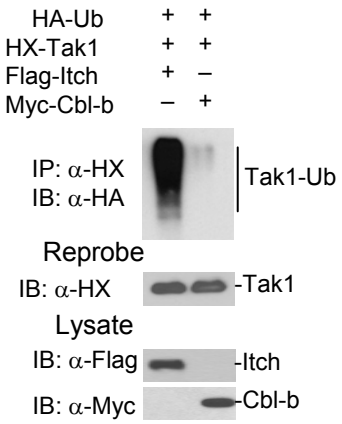



**Figure 3** Cbl-b fails to ubiquitinate Tak1. 293T cells were transfected with HA-Ub, HX-Tak1, Flag-Itch (lane1) or Myc-Cbl-b (lane2). The cell lysate was immunoprecipitated using antibody against HX and probed with antibody against HA. The membrane was reprobed using antibody against HX. The cell lysate was blotted with antibodies against Flag and Myc. Data represent two independent experiments.

Ahmed N et al. Supplementary Figure 4

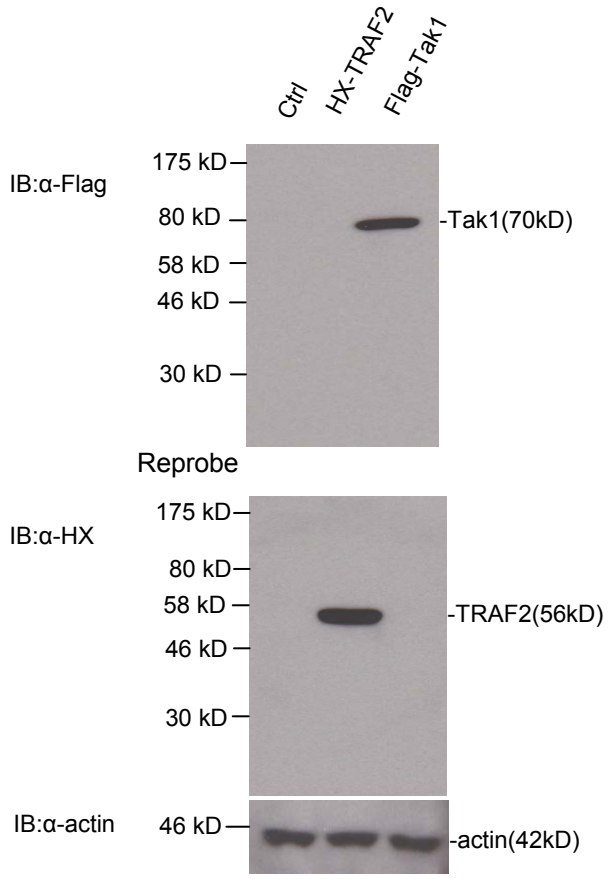



**Figure 4** Antibodies against Flag and HX do not cross-react during immunoblotting of Flag-Tak1 and HX-TRAF2. 293T cells were transfected with HX-TRAF2 (lane2) or Flag-Tak1 (lane3), cell lysate was immunoblotted using antibody against flag. The same membrane was reprobed with antibody against HX. Data represent two independent experiments.

Ahmed N et al. Supplementary Figure 5

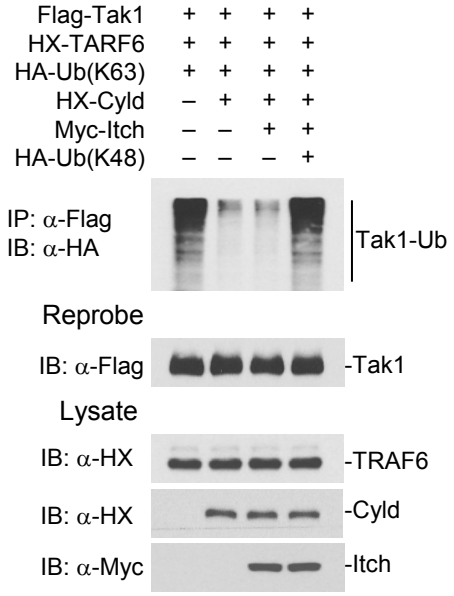



**Figure 5** Itch and Cyld cooperatively switch K63 to K48-linked ubiquitination of Tak1. 293T cells were transfected with Flag-Tak1 with HX-TRAF6 and HA-Ub(K63), HA-Ub(K48), HX-Cyld and Myc-Itch in different combinations as indicated. The cell lysate was immunoprecipitated using antibody against Flag and the membrane was blotted with antibody against HA. Data represent three independent experiments.

Ahmed N et al. Supplementary Figure 6

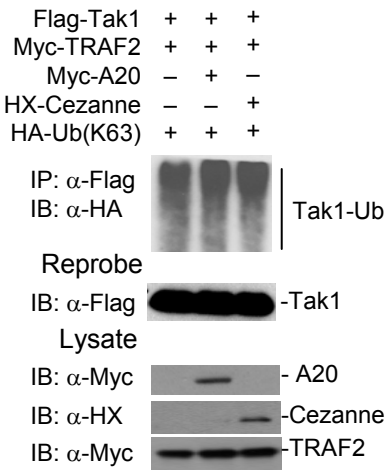
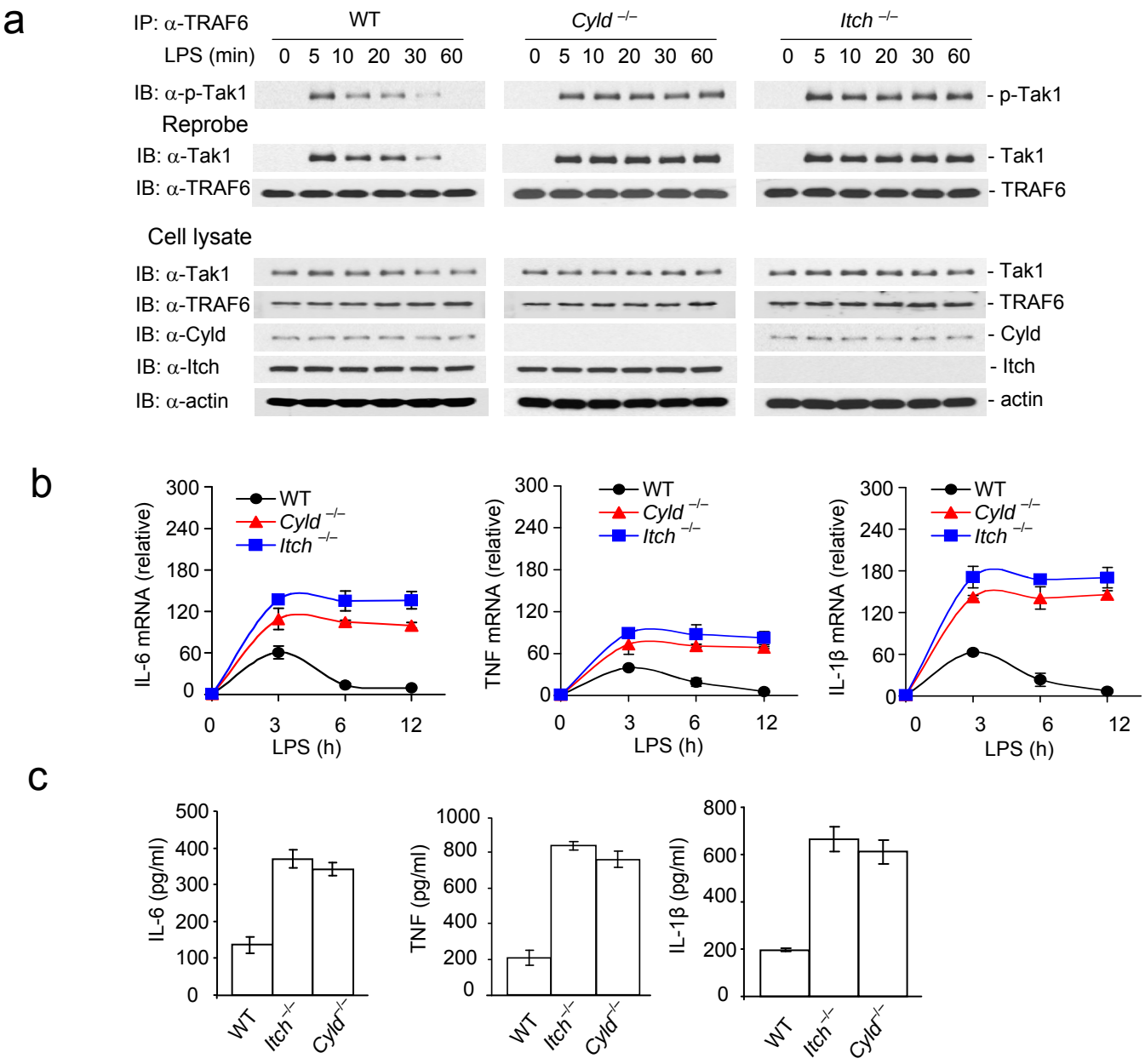



**Figure 6** Cezanne and A20 do not deubiquitinate Tak1. 293T cells were transfected with Flag-Tak1, Myc-TRAF2, HA-Ub(K63) (lane1) along with either Myc-A20 (lane2) or HX-Cezanne (lane3). The cell lysate was immunoprecipitated using antibody against Flag and immunoblotted using antibody against HA. The membrane was reprobed with antibody against Flag. The total cell lysate was immunoblotted with antibodies against Myc, HX and Myc. Data represent two independent experiments.

Ahmed N et al. Supplementary Figure 7



Ahmed N et al. Supplementary Figure 7

**Figure 7** Sustained Tak1 activation results in elevated and chronic production of pro-inflammatory cytokines by *Itch*$^{-/-}$ and *Cyld*$^{-/-}$ BMDMs. (a) BMDMs stimulated with LPS and lysed at the indicated time points. The lysate was immunoprecipitated with antibody against TRAF6 and immunoblotted with antibody against p-Tak1. The membranes were reprobed with antibodies against Tak1 and TRAF6. The total cell lysate was blotted using antibodies against TRAF6, Cyld, Itch and actin. (b) *Cyld*$^{-/-}$ and *Itch*$^{-/-}$ BMDMs were stimulated with LPS, total RNA was isolated at the indicated time points and expression of IL-6, TNF and IL-1β was analyzed using real-time PCR. (c) IL-6, TNF and IL-1β concentration were assayed by ELISA. Data represent three independent experiments. (**b-c** mean and s.d. of triplicate wells).

Ahmed N et al. Supplementary Figure 8

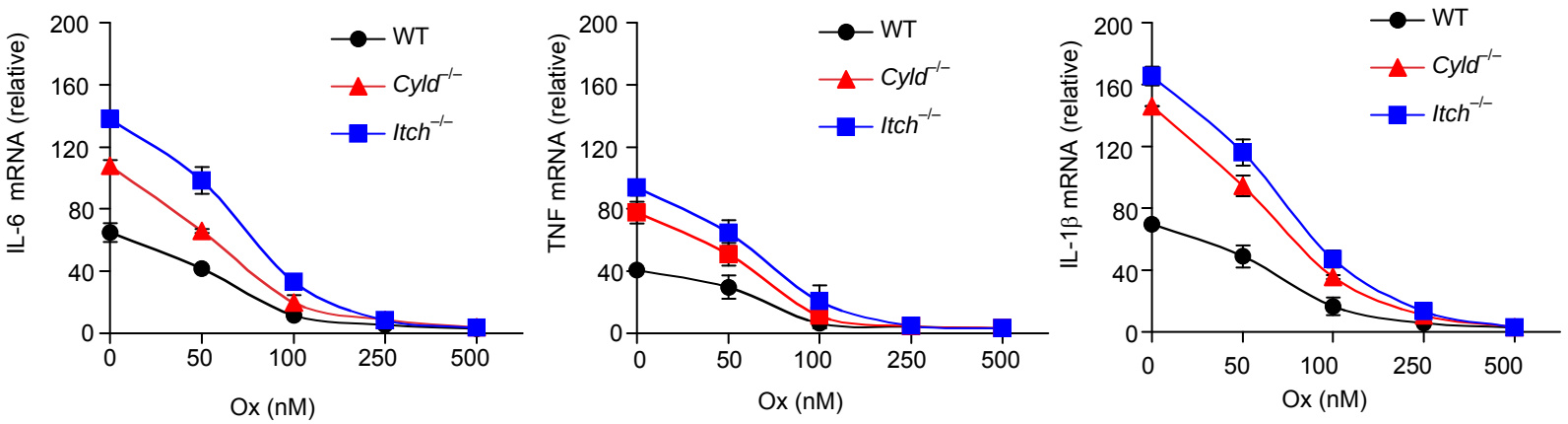



**Figure 8** Tak1 inhibitor, (5Z)-7-Oxozeaenol, inhibits LPS induced cytokine production by *Itch*$^{-/-}$ and *Cyld*$^{-/-}$ BMDMs. BMDMs were pretreated with indicated concentrations of (5Z)-7-Oxozeaenol, total RNA was isolated after 6 hours of stimulation and the abundance of IL-6, TNF and IL-1β expression was analyzed by real-time PCR. Data represent two independent experiments (mean and s.d. of triplicate wells).