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Supplemental Materials

β-Catenin-Dependent Lysosomal Targeting of Internalized Tumor Necrosis Factor-α Suppresses Caspase-8 Activation in Apoptosis-Resistant Colon Cancer Cells

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Content:

Antibodies and Chemicals

Mouse anti-β-catenin (610153), mouse anti-FADD (556402), mouse anti-RIPK1 (610458) and mouse anti-total EGFR (610016) were from BD Biosciences. Mouse anti-caspase-8 (#9746), rabbit anti-cleaved caspase-3 (#9664), rabbit anti-cleaved caspase-8 (#9496), rabbit anti-PARP1 (#9542), rabbit anti-FLIP (#3210), rabbit anti-cIAP (#4952), rabbit anti-XIAP (#2042) and rabbit anti-phospho-EGFR Tyr1173 (#4407) were from Cell Signaling Technology. Rabbit anti-TNFRI (ab19139) was from Abcam. Mouse anti-GAPDH (MAB374) was from Millipore. Rabbit anti-TRADD (sc-7868) and rabbit anti-IKB-α (SC-371) were from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology. The TUNEL staining kit was obtained from BD Biosciences; recombinant human and mouse TNF were from Peprotech; BafilomycinA1 and concanamycin A were from Sigma-Aldrich. E64D and Pepstatin were from Enzo Life Sciences. Lysotracker-red DND-99 was from Invitrogen.

Immunoblotting

Proteins from cell lines were extracted in RIPA buffer (25 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1% SDS, 150 mM NaCl, 1% NP-40, 1 % Sodium Deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail) and measured by Lowry protein assay. Equal amounts of total proteins were loaded on SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with primary antibodies overnight at 4°C. HRP conjugated secondary antibodies were

incubated for 1 hour at room temperature, and HRP signal were detected by chemiluminescence (Thermo).

Immunofluorescence

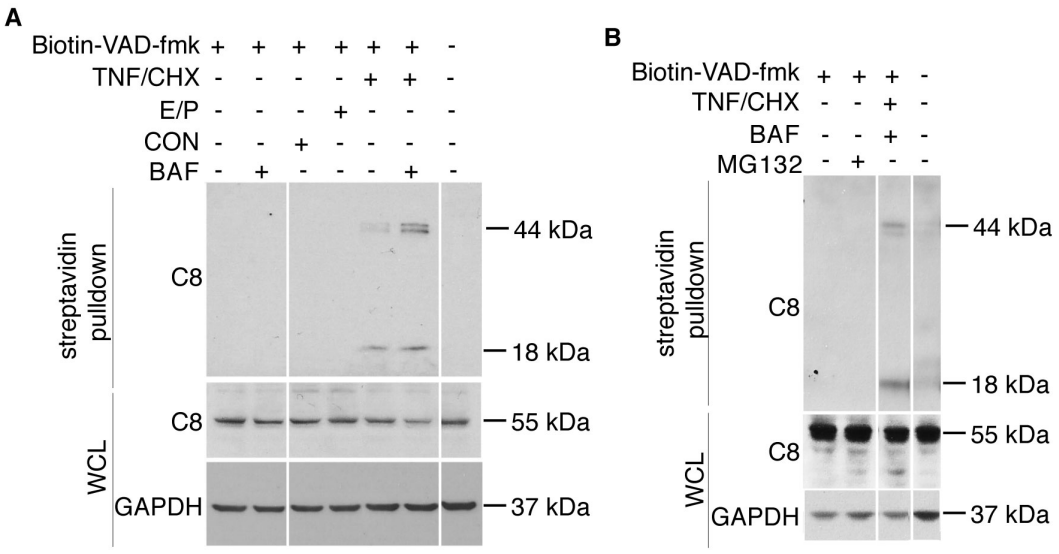
Cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton x-100 and blocked with 5% bovine serum albumin. Incubated with primary antibodies for 2 hr at 37 °C. Cells were then incubated with anti-mouse ALEXA fluor-594 and anti-rabbit ALEXA fluor-488 (Invitrogen) for 1 hour at 37 °C. DNA was stained with DAPI. Fluorescence images were captured by Zeiss LSM Confocal microscopy.

Down-regulation of EGF receptor (EGFR) in HCT116 and LIM1899 cells

HCT-116 and LIM 1899 cells were seeded and 24 hours later pre-treated with 200nM bafilomycin and/or 50μM sodium ortho-vanadate for 2 hours. Cells were then treated with 100nM EGF at 4° for one hour, and then warmed to 37° to induce endocytosis. At the indicated time points after temperature shift, cells were collected and lysed in RIPA buffer (25 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1% SDS, 150 mM NaCl, 1% NP-40, 1% Sodium Deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). Equal amounts (30 μg) of total proteins were loaded on SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with primary antibodies overnight at 4°. HRP conjugated secondary antibodies were incubated for 1 hour at room temperature. Proteins

were visualized by chemiluminescence as recommended by the manufacturer (Thermo).

Supplemental Figure 1

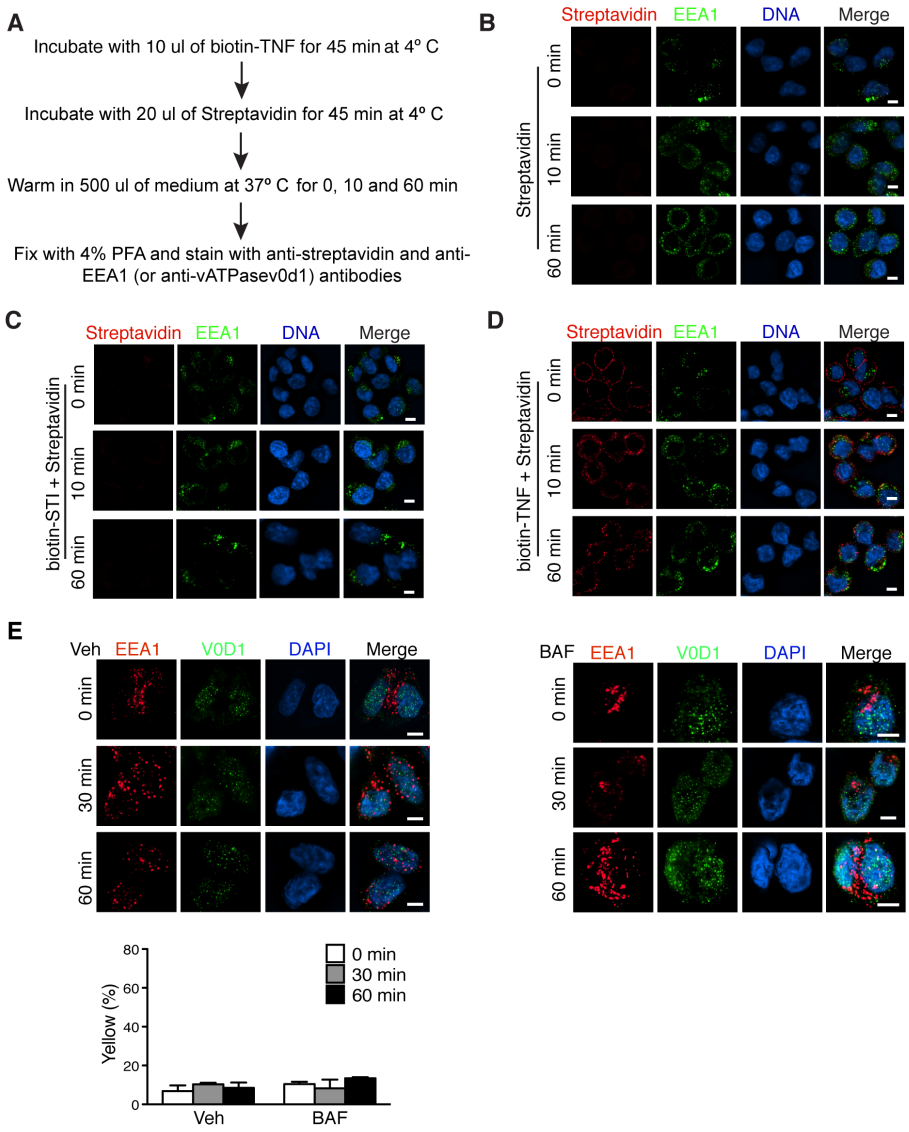


Supplemental Figure 1. V-ATPase, lysosomal and proteasomal inhibitors do not activate caspase-8.

A. *In vivo* affinity labeling of activated caspase-8 in LIM1899 cells, treated with bafilomycin (BAF: 200 nM), concanamycin (CON: 100 nM), E64D/Pepstatin (E/P: 10 μg/ml each), TNF-α (TNF: 10 ng/mL) and cycloheximide (CHX: 2.5 μg/ml). Cells were harvested after incubation with the drugs for 6 hr, lysed and whole cells lysates were incubated with streptavidin beads and caspase-8 was detected in the bound fractions by immunoblotting. Samples were run on the same gel, but were non-contiguous.

B. *In vivo* affinity labeling of activated caspase-8 in LIM1899 cells, treated with the indicated drugs as in B. MG132: 10 μM. Samples were run on the same gel but were non-contiguous.

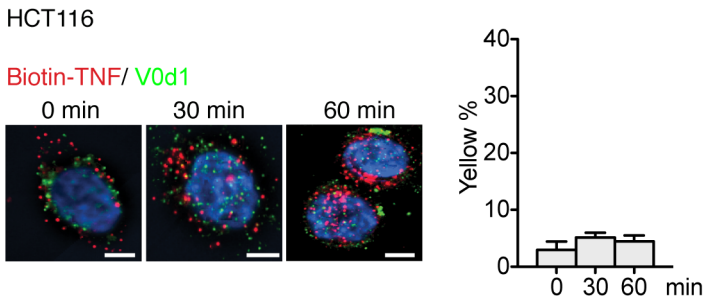
Supplemental Figure 2



Supplemental Figure 2. Subcellular localization of internalized TNF.

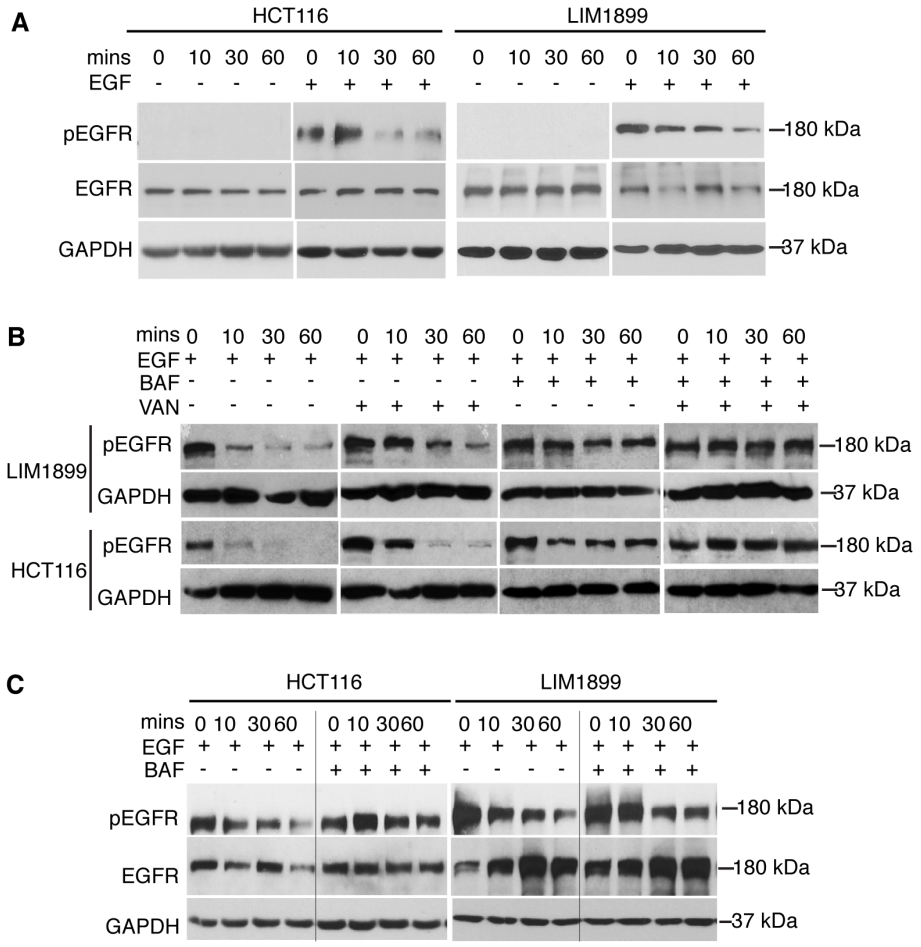
(A) Flow chart of experimental strategy to synchronize the internalization of streptavidin-biotin-TNF. (B-D) LIM1899 cells on coverslips were incubated with streptavidin alone (B), biotin-soy been trypsin inhibitor (biotin-STI) (C), or biotin-TNF (2.5 µg/mL) plus CHX (2.5 µg/ml) (D) at 4° for 45 min, followed by another 45 min incubation with streptavidin (5 ng/mL) at 4°, and then warmed to 37°. Cells were fixed at indicated time points and stained with anti-streptavidin (red) and anti-EEA1 (green). (E) LIM1899 cells were labeled and fixed as in A and stained with anti-EEA1 (red) and anti-v0d1 of V-ATPase (V0D1) (green). Histogram shows co-localization of EEA1 and V0D1 determined with the software Colocalizer Pro.

Supplemental Figure 3



Supplemental Figure 3. Internalized biotin-TNF did not co-localize with V0D1 in HCT116 cells. Co-localization of biotin-TNF with V0D1 in HCT116 cells was determined as in Figs2, Fig. 3B and see Methods. Scale bar: 10 μm.

Supplemental Figure 4

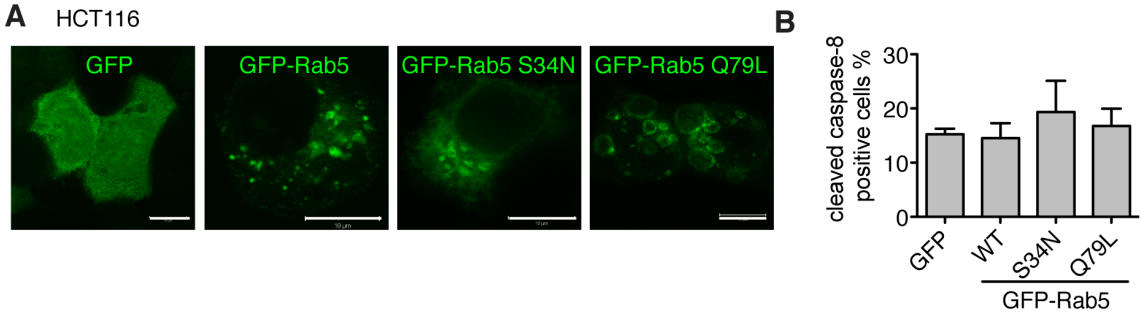


Supplemental Figure 4. EGF receptor down-regulation and its rescue by Bafilomycin A are similar in HCT116 and LIM1899 cell lines.

(A) The indicated cell lines were treated with or without EGF (100 ng/ml) at 4° for 1 hr. Cells were harvested at the indicated time points after warming at 37°. Western blots show EGFR and phosphorylated EGFR (pEGFR) level. GAPDH was used as loading control.

(B) & (C) The indicated cell lines were pretreated with BAF and or VAN for 2 hr at 37° (BAF - Bafilomycin A: 200 nM; VAN - sodium orthovanadate: 50 μM), followed by incubation with EGF for 1 hr at 4°. Cells were harvested at the indicated time points after warming at 37°. Western blots show EGFR and pEGFR level. GAPDH was used as loading control.

Supplemental Figure 5



Supplemental Figure 5. Ectopic expression of GFP-Rab5, dominant-negative GFPRab5 S34N or dominant-active GFP-Rab5-Q79L did not affect TNF/CHX induced caspase-8 cleavage in HCT116 cells

(A) Localization of GFP tagged WT, dominant-negative Rab5 (S34N) or dominant activate Rab5 (Q79L) in HCT116 cells. Scale bar: 10 μm.

(B) TNF/CHX-induced caspase-8 cleavage in HCT116 cells transiently transfected with the indicated constructs were fixed and stained with anti-ΔC8 antibody. TNF/CHX treatment began at 48 hr post-transfection and cells were treated for 4 hours. Values shown are mean and standard deviation from 5 fields with at least 1000 cells counted for each sample.