SUPPLEMENTARY DATA

Supplementary Material and methods

Cell culture

Mouse embryonic fibroblasts (MEF), HT1080, and HEK293 cells were grown in DMEM medium containing 10% inactivated fetal bovine serum. HOIL-deficient, *cpdm* and wild-type MEFs, a kind gift from Dr. K Iwai (Osaka University), were reported previously (Nakamura et al, 2006; Tokunaga et al, 2011). The 70Z/3 murine pre-B cell line and derived NEMO deficient 1.3E2 cell line were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.05 mM β-mercaptoethanol, 1,250 U of penicillin G, and 0.5 mg of streptomycin sulfate per ml in a 5% CO_2 humidified incubator.

Generation of stable transfectants in 1.3E2 cells

All the NEMO constructs have been described previously (Huang et al, 2003; Wu et al, 2006) or generated as above. 1.3E2 cells were reconstituted with NEMO wild-type and mutant constructs by electroporation as described previously (Huang et al, 2002). For electroporation, briefly, $2x10^7$ cells were washed once with culture media and then resuspended in 750 μl of media with 20% fetal bovine serum and transferred directly to a 0.4-cm electrode gap Gene Pulser cuvette (Bio-Rad, Hercules, CA). Then, 50 μg of expression DNA plasmid was preincubated with the cells on ice for 10 min. Cells were then electroporated at 280 V and 975 μF in a Gene Pulser apparatus with capacitance extender (Bio-Rad). After the pulse, cells were incubated on ice for 5 min, resuspended in culture media and placed in the 37℃ incubator. After 24 h of incubation, G418 was added to a final concentration of 1mg/ml to

select for resistant pools. Stable clones may be further derived.

Electrophoretic mobility shift assay (EMSA)

The Igκ-κB oligonucleotide probe and conditions for EMSA were previously described (Miyamoto et al, 1998). The Oct-1 site oligo used for control EMSA reactions was obtained from Promega. Gels were exposed to phosphoscreen and quantified with Cyclone phosphoimager (Perkin-Elmer, Shelton, CT). The intensity of NF-kB activation band was first normalized to the corresponding Oct-1 signal, then the normalized value were used to calculate fold induction while the untreated sample was arbitrary set as 1.

Luciferase assay

The dual Luciferase Assay System and passive lysis buffer were purchased from Promega (Madison, WI). 293 cells were transfected with κB-FLuc reporter, RLuc-TK and indicated plasmids. After 48 h, cells were treated and lysed with passive lysis buffer and the activity of *Firefly* Luciferase and *Renilla* Luciferase in the supernatants was assayed with the dual Luciferase Assay System using a TD-20/20 bioluminometer (Promega).

Semi-quantitative PCR (RT-PCR)

Total RNA was isolated using Trizol (Invitrogen) and RNA was reverse-transcribed into cDNA using a Reverse Transcription kit (Invitrogen). The housekeeping gene β-actin and GAPDH was used as an internal control. The gene-specific primers used for PCR and the length of products were as follows: forward, 5'-gagaacggcggctgggacac-3'and reverse, 5'-

agttgtggtgggggcagggt-3’ (Bcl-xL, 208 bp); forward, 5’-ggagtctggctcgtgctggc-3’ and reverse, 5’-ggcatggtgctcatcgccgt-3’ (cIAP1, 223 bp); forward, 5’-tgtggcctgatgttggataac-3’and reverse, 5’-ggtgacgaatgtgcaaatctact-3’ (cIAP2, 164 bp); forward, 5’-cgagctgggtttctttataccg-3’ and reverse, 5’-gcaatttggggatattctcctgt-3’ (XIAP, 126 bp); forward, 5’-ccgcaggaggcgccgctg-3’ and reverse, 5’-ggtatttcctcgaaagtctcg-3’ (IκBα, 273 bp); forward, 5’-ttcttgggactgatgctg-3’ and reverse, 5’-ctggctttgtctttcttgtt-3’ (IL-6, 380 bp); forward, 5’-gcattgctgacaggatgcag-3’ and reverse, 5’- cctgcttgctgatccacatc-3’ (β-actin, 159 bp). forward, 5’- ggtcgcattgtggccttttc -3’and reverse, 5’- tgctgcattgttcccatagag -3’ (human Bcl-xL, 189 bp); forward, 5’-gtttcaggtctgtcactggaag -3’ and reverse, 5’-tggcatactaccagatgacca-3’ (human cIAP1, 122bp); forward, 5’- tcctggatagtctactaactgcc -3’and reverse, 5’- gcttcttgcagagagtttctgaa -3’ (human cIAP2, 160 bp); forward, 5’- ccatatacccgaggaaccct -3’ and reverse, 5’- tgtccttgaaactgaacccc -3’ (human XIAP, 711 bp); forward, 5’- gaagaaggagcggctactgg -3’ and reverse, 5’-gctcacaggcaaggtgtagg -3’ (human IκBα, 401 bp); forward, 5’-gcattgctgacaggatgcag-3’ and reverse, 5’- cctgcttgctgatccacatc-3’ (human GAPDH, 205bp). PCR products were analyzed by electrophoresis on 1.5% agarose gel and visualized with Gel Logic Image system (Kodak).

Enzyme-Linked Immunosorbent Assay (ELISA)

The human TNFα ELISA Kit was purchased from eBioscience (San Diego, CA). Cells were seeded in 6-well plates, and conditioned media was collected after the indicated treatments. ELISA was performed in triplicate according to manufacturer's instruction. The absorbance was measured at 450 nm using Synergy H4 microplate reader (Winooski, VT).

Statistical analysis

The results were presented as Mean ± SD, and analyzed with Student's *t*-test. $P<0.05$ was taken to denote statistical significance.

Supplementary Figure legends

Supplementary Figure 1. (**A**) HT1080 cells were transfected with control or siRNAs targeting HOIP. After 48 h, cells were treated with etoposide (Etop, 10 μM) for 2 h, TNFα (10 ng/ml) for 30 min or left untreated (-). NF-κB activation was analyzed by EMSA using Igκ probe. Total cell lysates were analyzed by Western blotting using antibodies as indicated. **(B)** HT1080 cells were mock transfected or transfected with LUBAC-WT (HA-HOIL-1/HOIP-Myc), LUBAC-CS (HA-HOIL-1/HOIP^{CS}-Myc) mutant constructs as shown. Cells were treated and analyzed as in (A). **(C)** U2OS cells were transfected with LUBAC-WT, LUBAC-CS construct or HOIP siRNA. After 48 h, cells were treated with etoposide (10 μM) for 2 h, TNFα (10 ng/ml) for 30 min or left untreated (-). NF-κB activation was analyzed by EMSA. Cell lysates were probed with the indicated antibodies. **(D-F)** HOIL+/+ and HOIL-/- MEFs were treated with CPT (D), Dox (E) or Etop (F) for time as indicated. NF-κB activation was analyzed by EMSA using Igκ probe. Total cell lysates were analyzed by Western blotting using antibodies as indicated.

Supplementary Figure 2. (A) HEK293 cells were transfected with control or siRNAs targeting HOIP. After 48 h, cells were treated with etoposide (Etop, 10 μM) for time as indicated. Total cell extracts were subjected to immunoblotting with antibodies as shown. **(B)**

HEK293 cells stably expressing Myc-NEMO were transfected with control or siRNAs targeting HOIP. After 48 h, cells were treated with etoposide (Etop, 10 μM) for 1.5h or left untreated. DNA damage-induced NEMO sumoylation and phosphorylation were analyzed with IP followed by immunoblotting. **(C)** HEK293 cells were transfected with control or siRNAs targeting HOIP. After 48 h, cells were treated with etoposide (Etop, 10 μM) for time as indicated. Subcellular fractions were prepared and subjected to immunoblotting as shown. **(D)** HEK293 cells were transfected and treated as in (B) and ELKS ubiquitination were examined. (**E**) HEK293 cells were transfected with HA-Ub-NEMO S85A mutant along with LUBAC. Cells were treated with Dox (10 μM, 2 h) in the presence or absence of Ku55933 (10 μM). NEMO linear ubiquitination was examined.

Supplementary Figure 3. (**A**) HEK293 cells were treated with Etoposide (10 μM) for time as indicated. TNFα released into culture media were quantified with ELISA. Data from three independent experiment were pooled and shown as Mean ± SD. **(B)** HEK293 cells were treated with etoposide (10 μM), Dox (2 μg/ml), CPT (10 μM) for 2 h, TNFα (10 ng/ml) for 30 min or left untreated (-), in the presence of control IgG or neutralizing antibody against TNFα. NF-κB activation was analyzed by EMSA. **(C)** HEK293 cells were treated with etoposide (10 μM, 2 h) or left untreated (-), in the presence of control IgG or neutralizing antibody against TNFα. NEMO linear ubiquitination was examined.

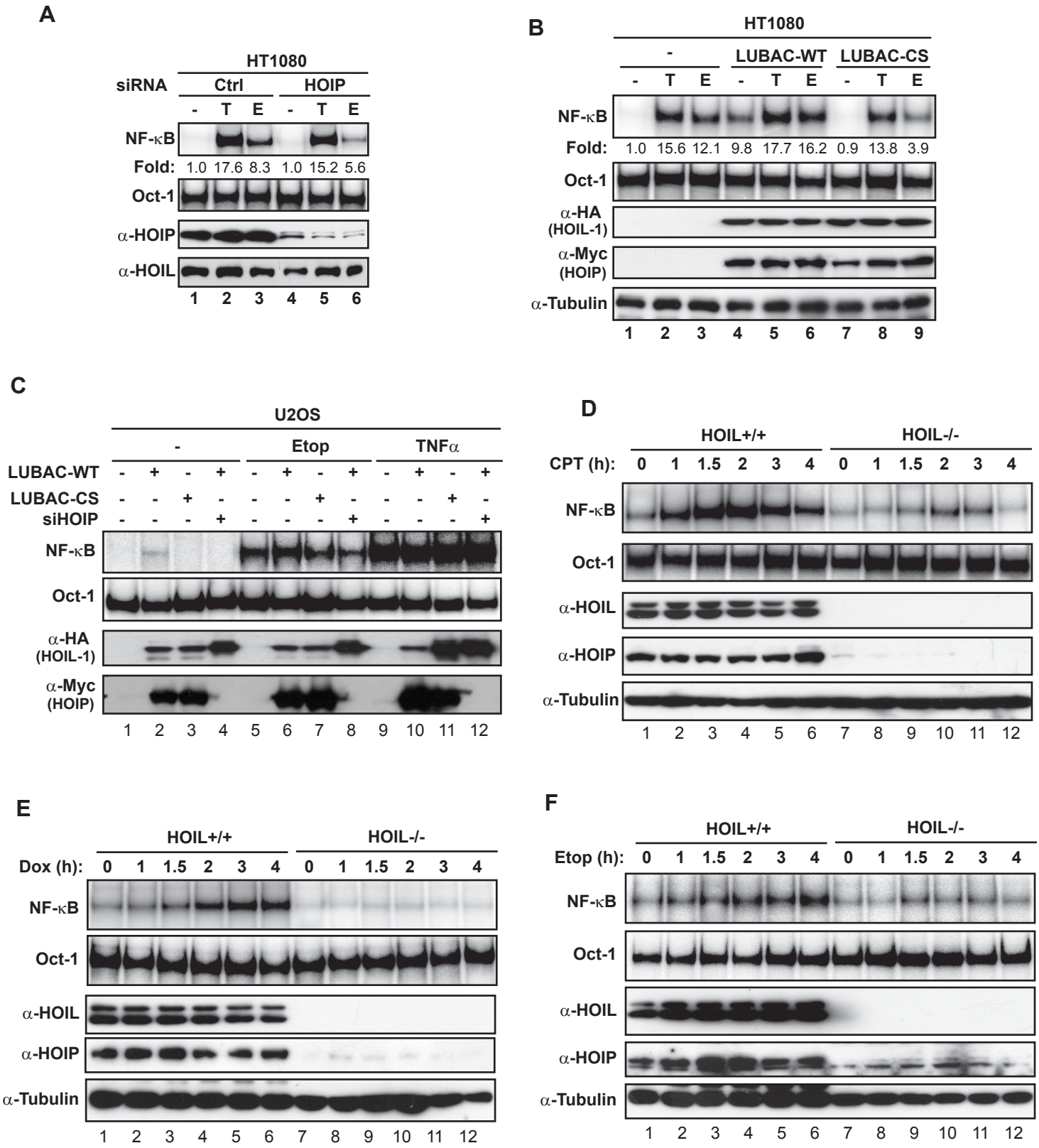
Supplementary Figure 4. (A) HOIL+/+ and HOIL-/- MEFs were treated with TNF, Dox or UV, JNK activation was analyzed with immunoblotting as shown. **(B)** HEK293 cells were

transfected with NEMO-WT, NEMO-K285/309R or NEMO-K277/309A mutant, and treated with etoposide (10 μM, 1.5 h) for left untreated. Whole cell lysates were boiled in the presence of 1% SDS and further diluted for IP with anti-Myc antibody. Immunoprecipitates and input samples (5%) were analyzed with immunoblotting using antibodies as shown. **(C)** 1.3E2 cells stably expressing Myc-NEMO, Myc-NEMO F312A or Myc-NEMO L329P mutant were treated with etoposide (10 μM, 2 h) or left untreated. Whole cell lysates were boiled in the presence of 1% SDS and further diluted for IP with anti-Myc antibody. Immunoprecipitated samples were analyzed with immunoblotting using antibodies against ubiquitin and NEMO. **(D)** ELKS+/+ and ELKS-/- MEFs were treated with etoposide (10 μM, 2 h) or left untreated. Whole cell lysates were subjected to IP with anti-NEMO antibody followed by immunoblotting using antibodies as shown.

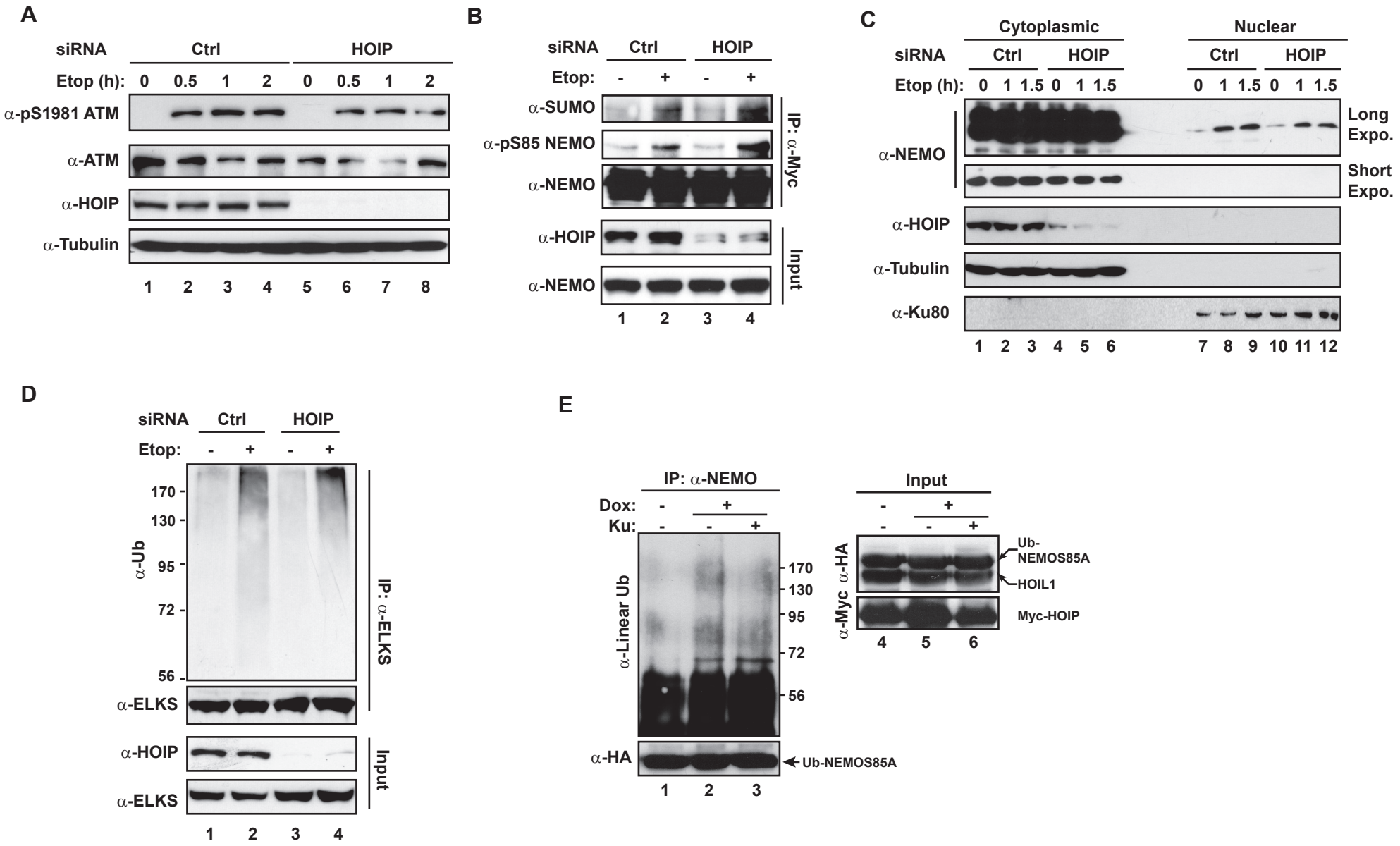
Supplementary Figure 5. (A) Illustration of SUMO- and/or Ub-fused NEMO K277/309 mutants. **(B)** 1.3E2 cells stably expressing SUMO-NEMO K277/309R, Ub-NEMO K277/309R or SUMO-NEMO K277/309R-Ub mutant were treated with LPS (L, 10 μg/ml, 30 min) or etoposide (E, 10 μM, 2 h) as indicated. NF-κB activation was analyzed with EMSA and NEMO expression was assayed with immunoblotting. **(C)** HT1080 cells were transfected with NEMO-WT, NEMO K285/309R or Ub-NEMO K285/309R mutant. Transfected cells were immunostained with anti-NEMO antibody and visualized with FITC-conjugated secondary antibody. DAPI was used for nuclei-staining. **(D)** NEMO-WT, NEMO-K285/309R, and Ub-NEMO-K285/309R reconstituted 1.3E2 cells were treated with Etop (10 μM) or left untreated for 4 h. Total RNA was isolated and the mRNA of specific genes was

analyzed by semi-quantitative RT-PCR. PCR products were visualized by electrophoresis on 1.5% agarose gel. **(E)** NEMO-WT, NEMO-K285/309R, and Ub-NEMO-K285/309R reconstituted 1.3E2 cells were treated with Etop (20 μM, 6 h), stained with Propidium Iodide (PI), and analyzed by flow cytometry to detect the percentage of apoptotic cells (subG1 population). Histogram bars represent the mean of three independent experiments and were shown as Mean+SD.

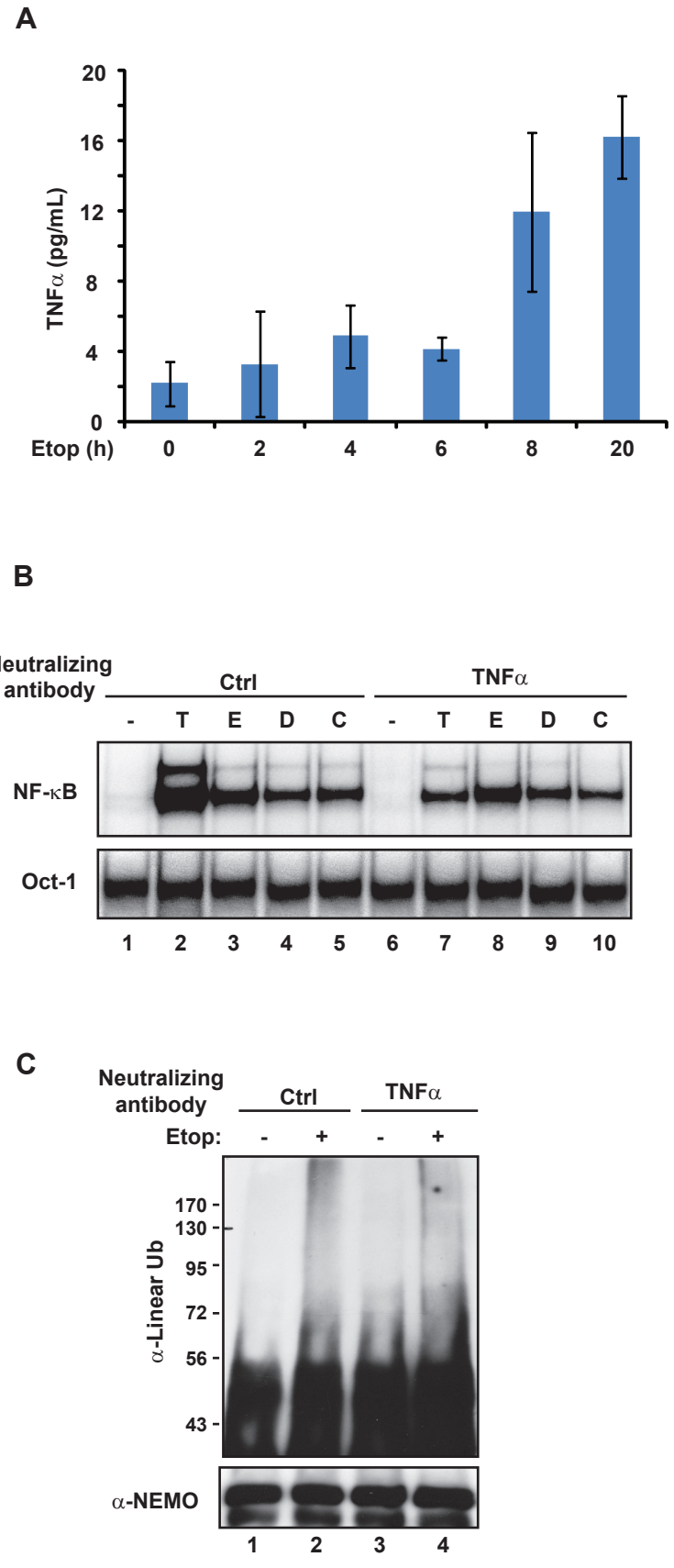
Supplementary Figure 1



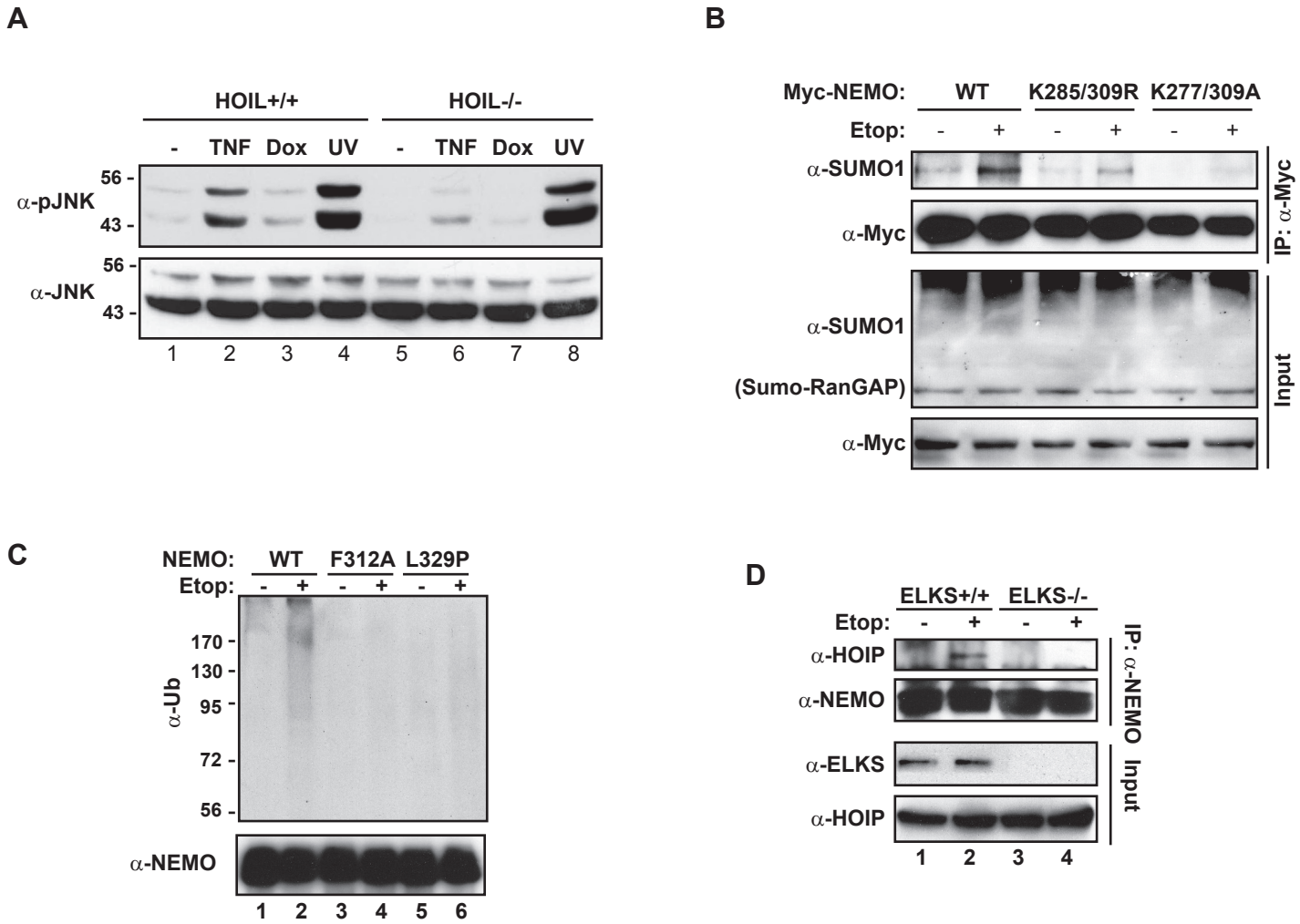
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5