Supplementary information

Specific recognition of linear polyubiquitin by A20 zinc finger 7 is involved in NF-κB regulation

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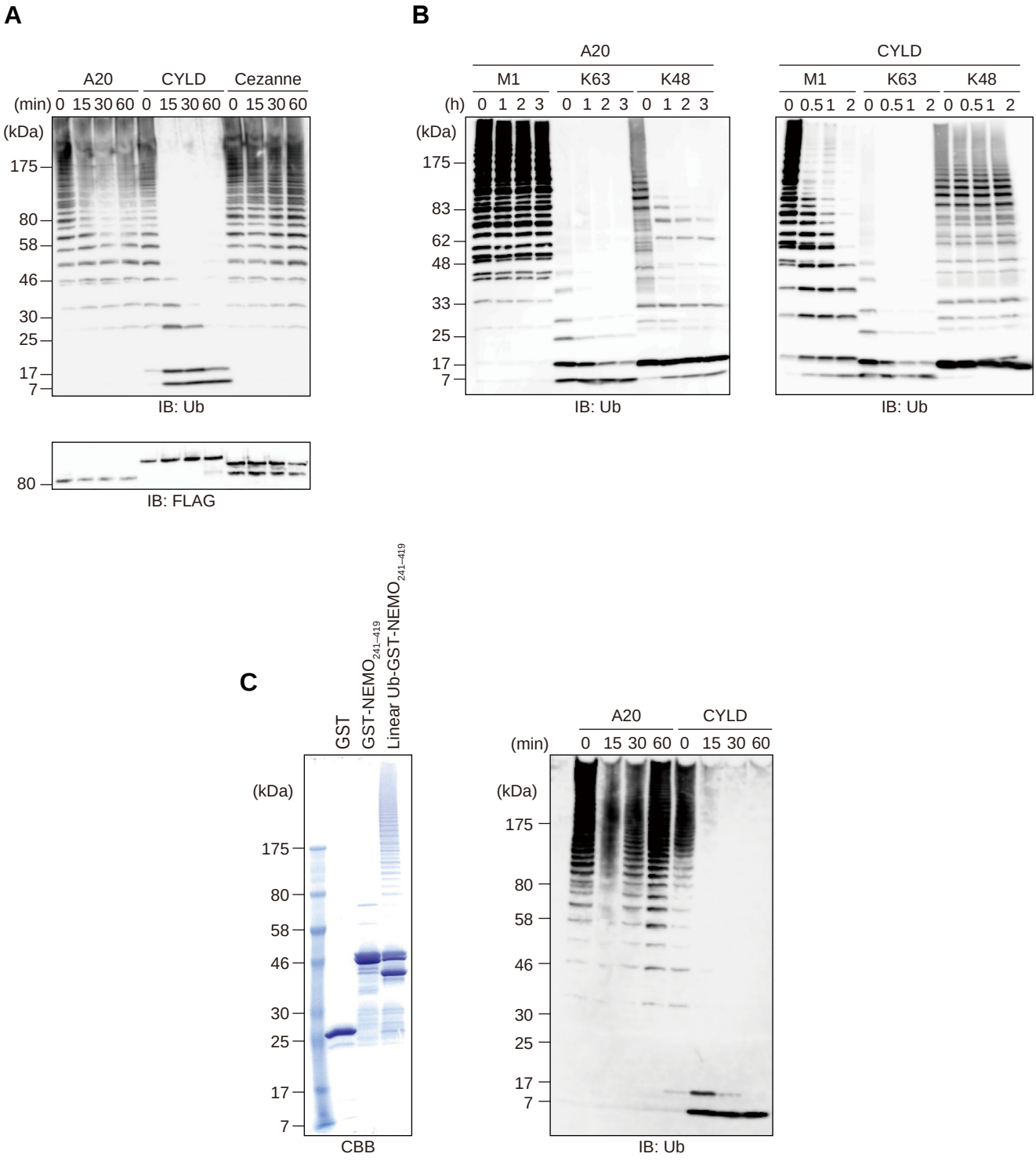
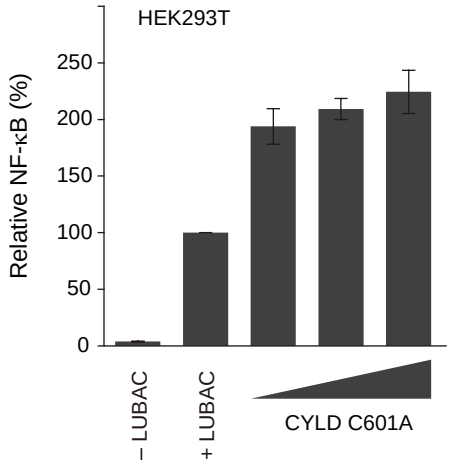
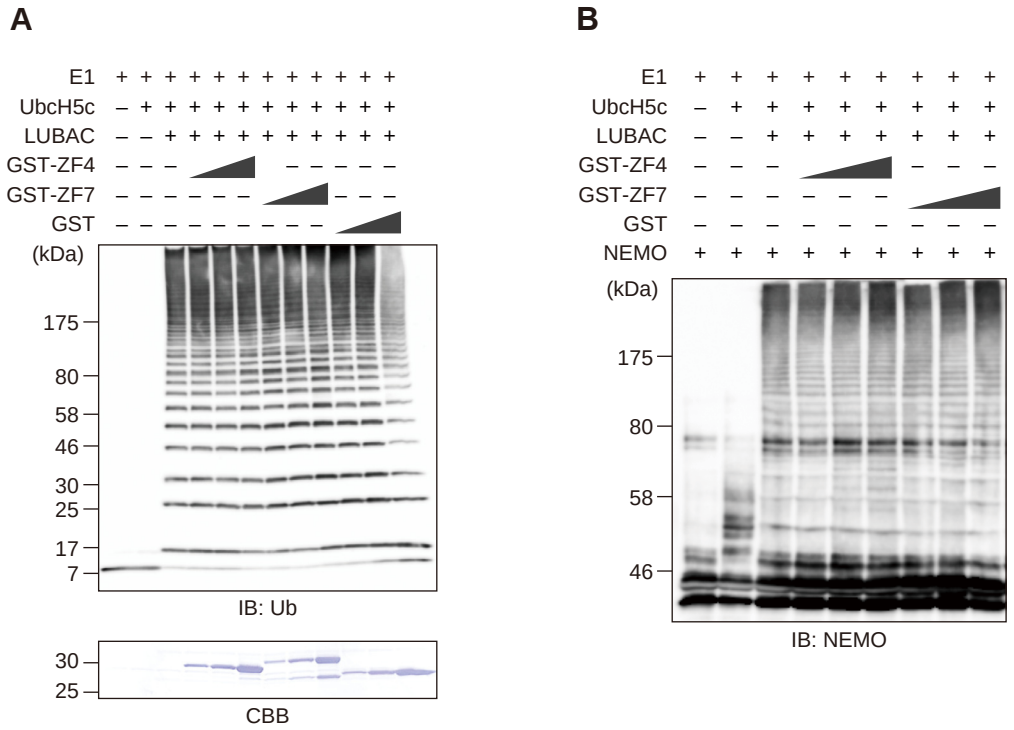
This document includes: Supplementary Figures S1–6



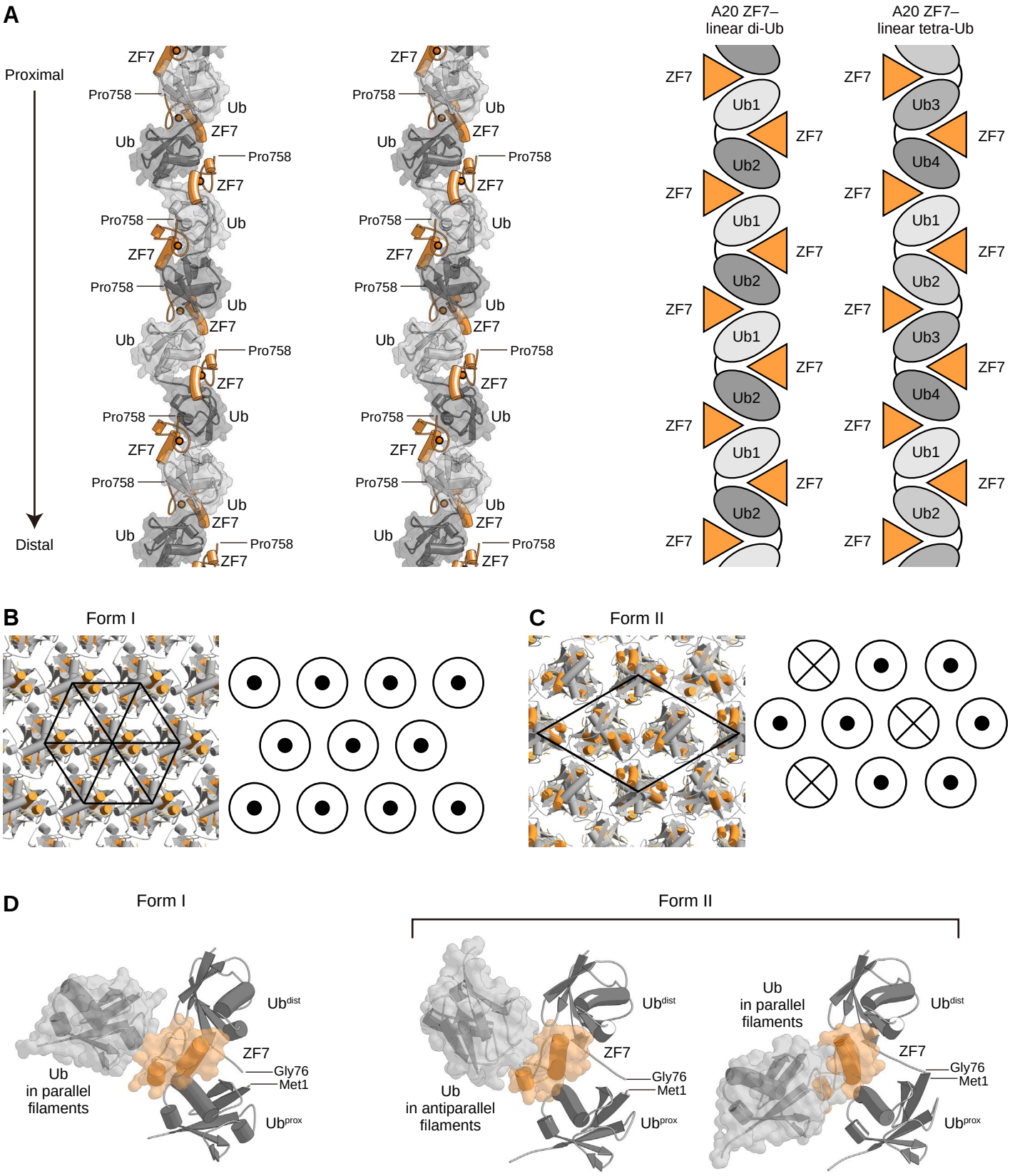
Supplementary Figure S1 DUB activities of A20, CYLD and Cezanne. (**A**) DUB activities of A20, CYLD and Cezanne for linear polyubiquitin chains. FLAG-tagged A20, CYLD and Cezanne were expressed in HEK293T cells, and the cell lysates were incubated with anti-FLAG beads to capture the proteins. LUBAC-generated linear polyubiquitin chains were reacted with the beads for the indicated times. Linear polyubiquitin degradation was detected by western blotting, using an anti-ubiquitin antibody. (**B**) *In vitro* DUB activities of A20 and CYLD for linear, K63- and K48-linked polyubiquitin chains. Baculovirally expressed recombinant A20 and CYLD were reacted with linear (M1)-, K48- or K63-linked polyubiquitin, and analyzed as in (**A**). (**C**) DUB activities of A20 and CYLD for NEMO-conjugated linear polyubiquitin chains. The GST-tagged C-terminal region of NEMO (residues 241–419) was linearly ubiquitinated by LUBAC and purified using glutathione Sepharose beads (left panel). DUB activities of A20 and CYLD for NEMO-conjugated linear polyubiquitin chains were examined as in (**A**) (right panel).



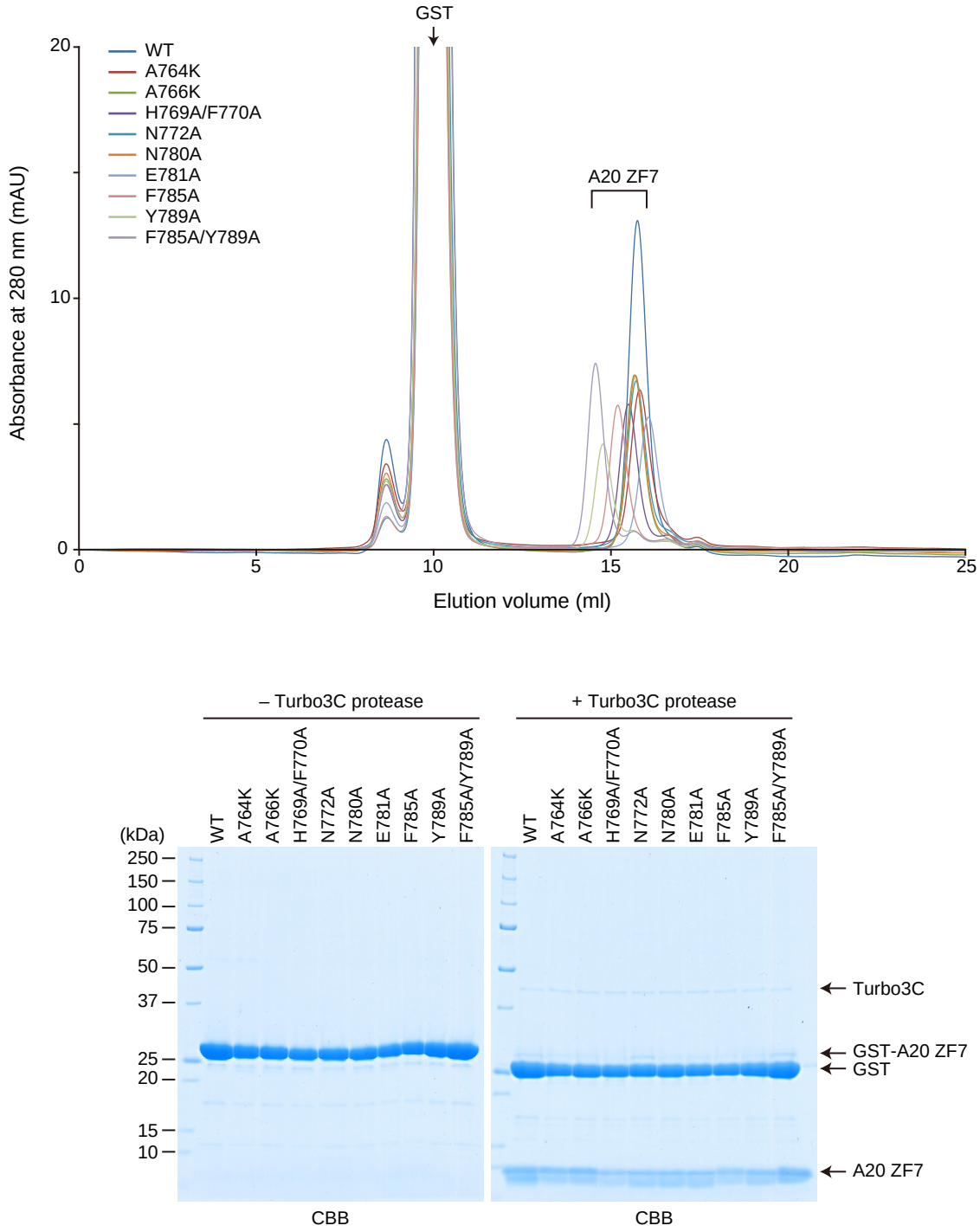
Supplementary Figure S2 Expression of the catalytically inactive CYLD C601A mutant enhances LUBAC-mediated NF-κB activation. Luciferase reporter assays were performed as in Figure 1B, and the relative luciferase activities are shown as mean ± s.d. (n = 3).



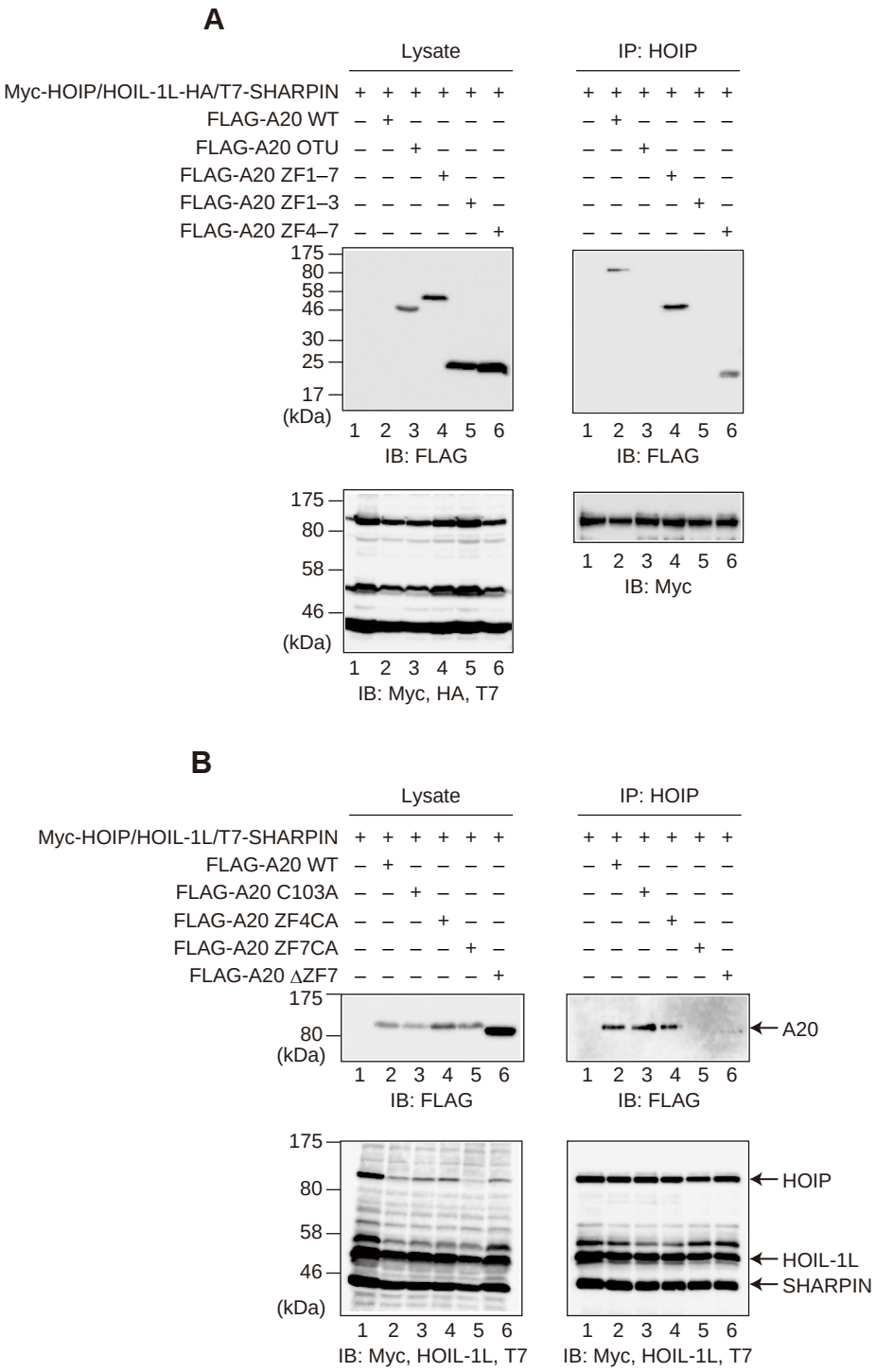
Supplementary Figure S3 A20 ZF7 does not inhibit LUBAC E3 activity. (**A**, **B**) A20 ZF7 does not inhibit the LUBAC-catalysed generation of either free linear polyubiquitin (**A**) or NEMO-conjugated linear polyubiquitin (**B**). *In vitro* ubiquitination experiments were performed using E1 (100 ng), UbcH5c (200 ng), recombinant LUBAC (1 μg) and increasing amounts of GST proteins (0.3, 1, 3 μg), in the absence (**A**) and presence (**B**) of recombinant FLAG-His6-tagged full-length human NEMO. Polyubiquitin chains were detected by western blotting, using the indicated antibodies.



Supplementary Figure S4 Two types of crystal packing of A20 ZF7 in complex with linear ubiquitin chains. (**A**) In both Forms I and II, A20 ZF7 (orange) molecules intercalate between each pair of two adjacent ubiquitin molecules (grey) in the same manner, forming a straight, filamentous structure (stereoview). The N-terminal Pro758 of A20 ZF7 projects outward from the filamentous structure, suggesting that full-length A20 can bind linear polyubiquitin in a manner similar to that of A20 ZF7 alone. The schematic drawings show the crystal packing in the diubiquitin and tetraubiquitin complexes. (**B**, **C**) All of the filaments formed by A20 ZF7 (orange) and ubiquitin (grey) molecules run parallel in Form I (**B**), whereas one filament is surrounded by six filaments that run antiparallel to it in Form II (**C**). The filamentous structures run perpendicular to the paper, and the unit cells are indicated by black lines. (**D**) A20 ZF7 (orange) interacts with three and four adjacent ubiquitin molecules (grey) in Forms I and II, respectively. Forms I and II commonly contain one A20 ZF7–linear diubiquitin complex with a virtually identical conformation.



Supplementary Figure S5 Size-exclusion chromatography of WT and mutants of A20 ZF7. GST-tagged A20 ZF7 proteins were expressed in *E. coli* and purified by glutathione Sepharose chromatography. The purified GST-tagged A20 ZF7 proteins (0.1 mg) were treated with Turbo3C protease (0.6 μg) (Nacalai Tesque) overnight at 4ºC to cleave the GST-tag, and then were analyzed by size-exclusion chromatography on a Superdex75 10/300 column (GE Healthcare), equilibrated with PBS buffer supplemented with 1 mM TCEP. The proteins before and after the protease treatment were analyzed by 10–20% SDS-PAGE, and stained with SimplyBlue SafeStain (Invitrogen).



Supplementary Figure S6 A20 ZF7 is crucial for association with LUBAC. (**A**) A20 ZF4–7 participates in the association with LUBAC. (**B**) Intact A20 ZF7 is required for the association with LUBAC. Cell lysates and immunoprecipitates from HEK293T cells expressing the indicated proteins were detected by western blotting, using the indicated antibodies (**A** and **B**).