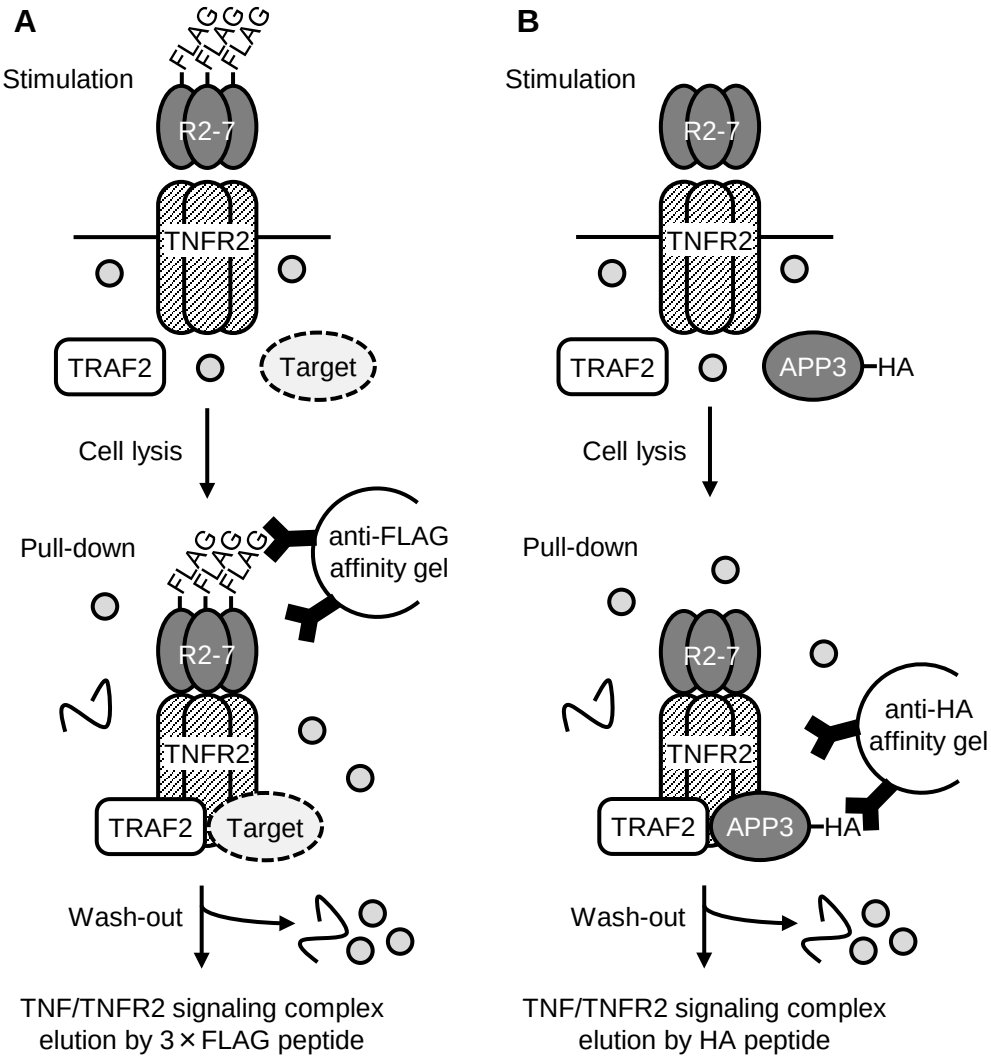

Fig. S1. Purification of the TNFR2 signaling complex by immunoprecipitation
(A) Scheme of TNFR2 signaling complex purification by FLAG-tag. R2-7-FLAG or wtTNF-FLAG binding to TNFR2 recruits target protein (dotted circle) that forms part of the TNFR2 signaling complex. After cell lysis, the TNFR2 signaling complex is pulled down using an anti-FLAG affinity gel. Finally, impurities such as cell membranes and nonspecifically bound proteins are removed by washing and the complex is eluted by competition with a 3 × FLAG peptide. (B) Scheme of TNFR2 signaling complex purification by HA-tag. In APP3m-HA transfected cells, APP3m-HA is recruited to intracellular domain of TNFR2 by R2-7 stimulation. After cell lysis, the TNFR2 signaling complex is pulled down using an anti-HA affinity gel. Finally, impurities such as cell membranes and nonspecifically bound proteins are removed by washing and the complex is eluted by competition with an HA peptide.

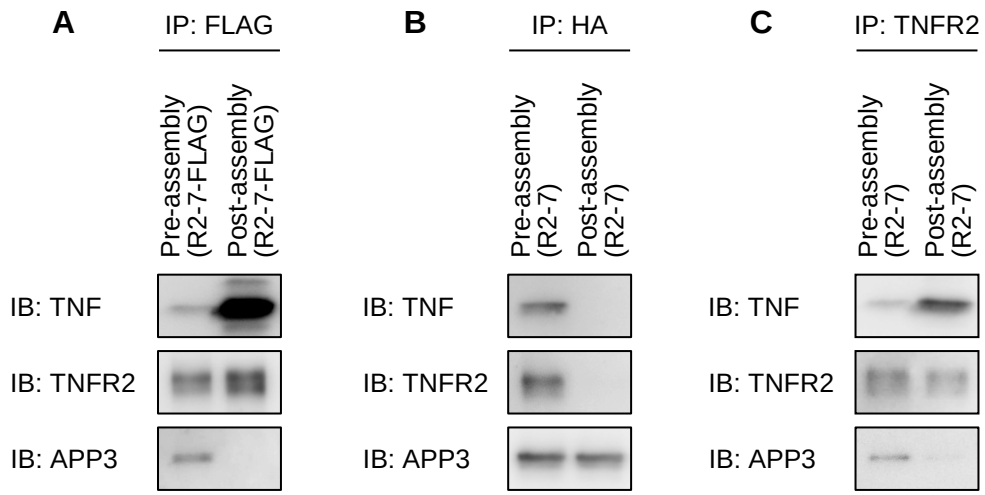

Fig. S2. Formation of the TNFR2 signaling complex in cell lysates

(A) HEK293T-TNFR2 cells were stimulated by R2-7-FLAG (100 ng/ml) for 30 min. The cells were lysed, and the cell lysate was recovered as supernatant (pre-assembly). Unstimulated HEK293T-TNFR2 cells were also lysed in the same way, and the same amount of R2-7-FLAG as used in pre-assembly was added to the cell lysate and incubated for 30 min (post-assembly). Anti-FLAG IP was performed on each lysate. The lysate was analyzed by western blotting with anti-TNF, anti-TNFR2 or anti-APP3 antibody. It showed that TNFR2 and APP3 were present with R2-7 in the pre-assembly sample. On the other hand, only TNFR2, but not APP3, was present with R2-7 in the post-assembly sample, and a large amount of R2-7-FLAG was required to detect the same level of TNFR2. Thus, the interaction between TNF and TNFR2 was increased incidentally in cell lysate, but the TNFR2 complex involving APP3 was not generated as an artifact. (B) APP3m-HA was overexpressed in HEK293T-TNFR2 cells. The cells were treated with R2-7 (100 ng/ml) for 30 min and cell lysate was prepared (pre-assembly). Unstimulated cells were also lysed, and the same amount of R2-7 as used in pre-assembly was added to the lysate and incubated for 30 min (post-assembly). Anti-HA IP was performed on each lysate. The lysate was assessed by western blotting with anti-TNF, anti-TNFR2 or anti-APP3 antibody. It showed that R2-7 and TNFR2 were present only in the pre-assembly sample. This result also suggested that the TNFR2 complex was not formed as an artifact. (C) HEK293T-TNFR2 cells were stimulated by R2-7 (100 ng/ml) for 30 min. The cells were lysed, and the cell lysate was recovered as supernatant (pre-assembly). Unstimulated HEK293T-TNFR2 cells were also lysed in the same way, and the same amount of R2-7 as used in pre-assembly was added to the cell lysate and incubated for 30 min (post-assembly). Immunoprecipitation was performed with each lysate using anti-TNFR2 antibody (MR2-1; Abcam) and protein G affinity gel (Sigma Aldrich). Western blotting showed that R2-7 and APP3 were present with TNFR2 in the pre-assembly sample. On the other hand, only R2-7, but not APP3, was present with TNFR2 in the post-assembly sample even though the amount of R2-7 detected in the post-assembly sample was larger than that detected in the pre-assembly sample. This result also supported the idea that the TNFR2 complex was not generated as an artifact.

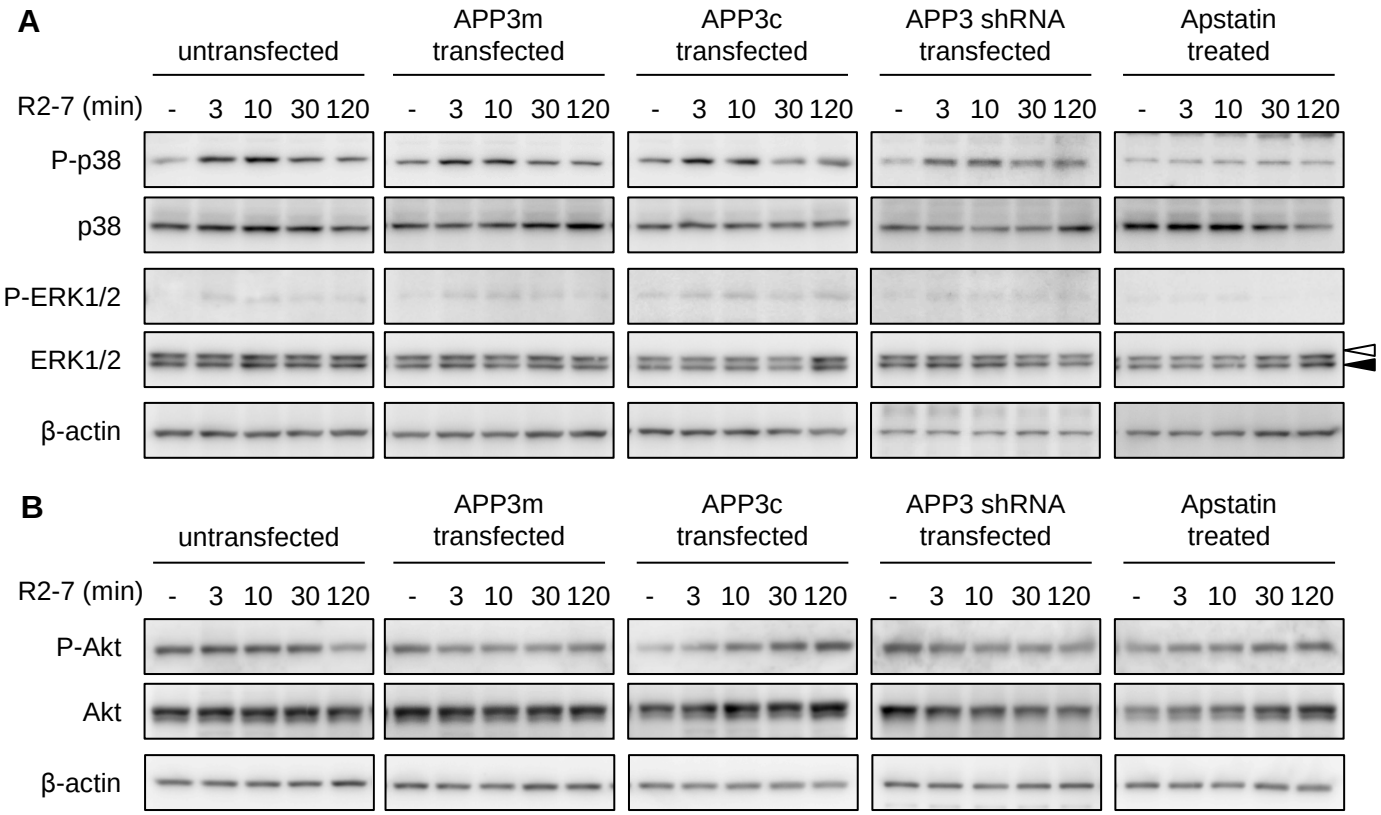

Fig. S3. Phosphorylation of p38, ERK1/2 and Akt induced by APP3 in TNFR2 signaling
(A) HEK293T-TNFR2 cells transfected with APP3m-, APP3c-, or APP3 shRNA were prepared. To inhibit aminopeptidase P enzymatic activity, HEK293T-TNFR2 cells were pre-treated with 100 μM apstatin. Untransfected cells were used as a reference. The cells were stimulated with R2-7 (100 ng/ml) at the indicated time. After cell lysis, western blotting was performed to confirm phosphorylation of p38 and ERK1/2. ERK1 and ERK2 are indicated by the open arrow and filled arrow, respectively. β-actin served as a loading control. (B) Akt and phospho-Akt were also detected in each HEK293T-TNFR2 cell line.

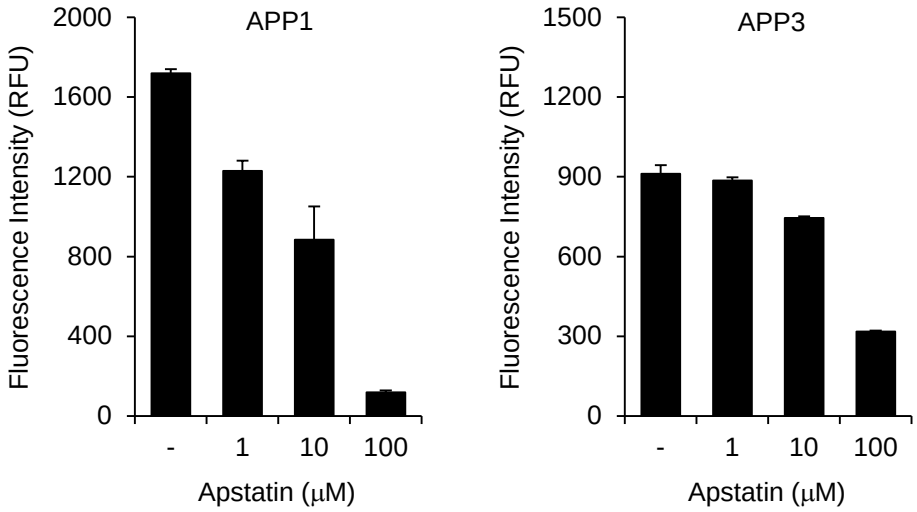

Fig. S4. Inhibition of APP1 and APP3 by apstatin

APP3m-HA was overexpressed in HEK293T cells. After cell lysis, APP3m-HA was purified by anti-HA IP. Recombinant APP1 (R&D Systems) and affinity-purified APP3m were treated with or without apstatin (1, 10, or 100 μM) for 30 min. The inhibition of enzymatic activity was then measured by fluorescence substrate digestion. Results are mean±s.d., n=3.

Table S1. Proteins identified in HEK293T-TNFR2 cells with R2-7-FLAG stimulation.

Download Table S1

Table S2. Proteins identified in HEK293T-TNFR2 cells without R2-7-FLAG stimulation.

Download Table S2