Supplemental material

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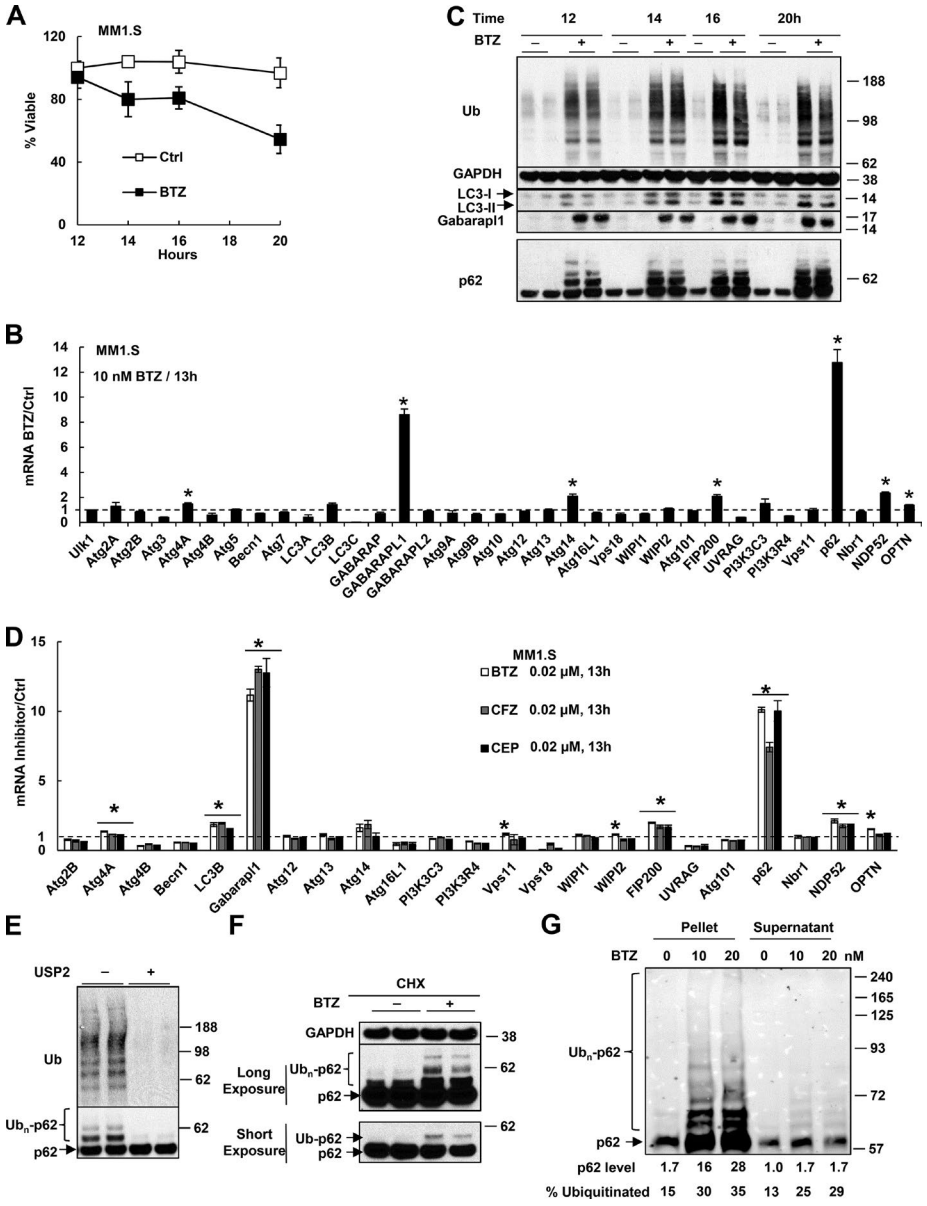

Figure S1. **Treatment of myeloma MM1.S cells with BTZ rapidly induces p62 and GABARAPL1 mRNA and protein and also causes the ubiquitination of p62 before they lose viability. (A)** After MM1.S cells were treated with 10 nM BTZ for 12, 14, 16, or 20 h, their viability was measured by MTS assay. BTZ treatment did not cause >50% loss of cell viability until 20 h of treatment. Four wells of cells were assayed for each condition. **(B)** MM1.S cells were treated with 10 nM BTZ for 13 h, and the mRNAs of all autophagy proteins (Atg proteins) or autophagy poly-Ub receptors in untreated or BTZ-treated cells were measured by real time RT-PCR. **(C)** From MM1.S cells treated with BTZ as in B, the levels of GABARAPL1, p62, LC3, and Ub conjugates were measured by Western blotting. BTZ treatment induced expression of p62 and GABARAPL1 at 12 h but did not cause LC3-II buildup until 20 h of treatment. Molecular masses are given in kilodaltons. **(D)** mRNAs from MM1.S cells treated for 13 h with 20 nM BTZ, CFZ, or CEP18770 (CEP) were measured by real time RT-PCR. These inhibitors all caused dramatic induction of *p62* and *GABARAPL1* but not most other Atg genes. **(E)** In lysate from BTZ-treated MM1.S cells, p62 antibody reacted with a ladder of higher-molecular-weight bands. After incubation with a recombinant deubiquitinase Usp2 (active domain; 4°C for 2 h), which completely removed poly-Ub chains, those high-molecular-weight species were also removed, indicating that they are polyubiquitinated p62. **(F)** Treatment of MM1.S cells with BTZ (1 μM for 4 h) in the presence of cycloheximide (100 μg/ml) still caused p62 polyubiquitination. **(G)** MM1.S cells treated for 14 h with 10 or 20 nM BTZ were fractionated into supernatant (soluble) and pellet (aggregated) fractions, and the same amounts of total proteins were loaded on SDS-PAGE. The levels of total and ubiquitinated p62 were measured by Western blotting and quantified by densitometry. The amount of total p62 in the supernatant of untreated cells was set as 1. *, $P < 0.05$. Error bars indicate SD.

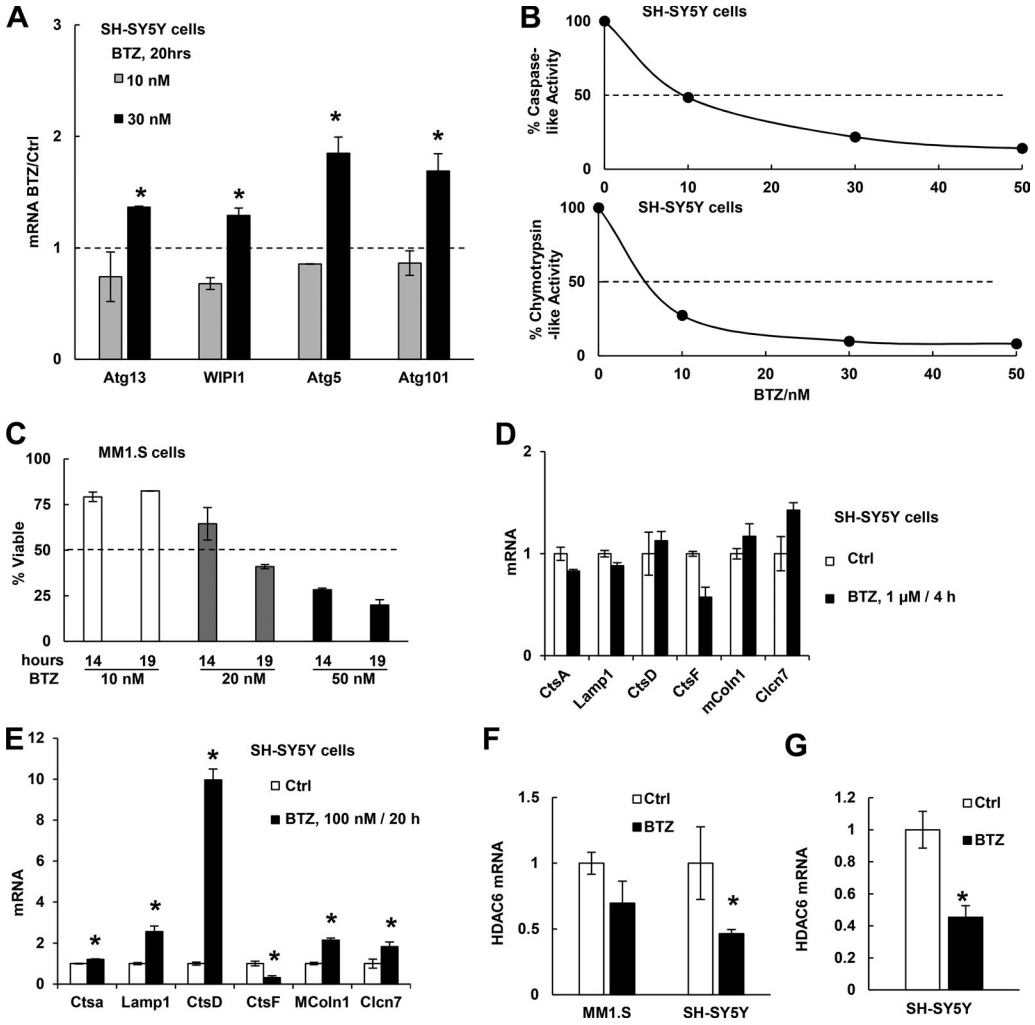

Figure S2. **Effect of BTZ treatment on mRNAs for Atg genes, autophagy poly-Ub receptors, lysosomal genes, and *HDAC6* in SH-SY5Y or MM1.S cells.** **(A)** Treatment of SH-SY5Y cells for 20 h with 30 nM BTZ caused the induction of mRNAs for indicated Atg genes or autophagy poly-Ub receptors, but they were not induced by treatment with 10 nM BTZ. **(B)** To quantify the extent of proteasome inhibition by different concentrations of BTZ, the caspase-like (upper) or chymotrypsin-like (lower) proteasome activities in lysates of untreated (normalized as 100%) or BTZ-treated cells were measured with small fluorogenic peptide substrates. 30 nM is the minimal concentration that inhibits both activities by >50%. **(C)** Viability of MM1.S cells treated for 14 or 19 h with 10, 20, or 50 nM BTZ was measured by MTS assay and presented as percentage of untreated cells. Cell viability reduced by >50% with 20 nM BTZ for 19 h or 50 nM BTZ for 14 h. Two wells of cells were assayed for each condition. **(D and E)** After SH-SY5Y cells were treated with BTZ as indicated, the mRNAs of lysosomal proteins (Cathepsin [Cts] A, D, and F; Lamp1; mColn1; and Clcn7) were measured. None of these genes were induced by treatment with 1 μM BTZ for 4 h (D), but most were induced by treatment with 100 nM BTZ for 20 h (E). **(F and G)** HDAC6 mRNA was suppressed in MM1.S or SH-SY5Y cells treated for 13 h with 10 nM BTZ (F) or SH-SY5Y cells treated for 20 h with 100 nM BTZ (G). *, $P < 0.05$. Error bars indicate SD.

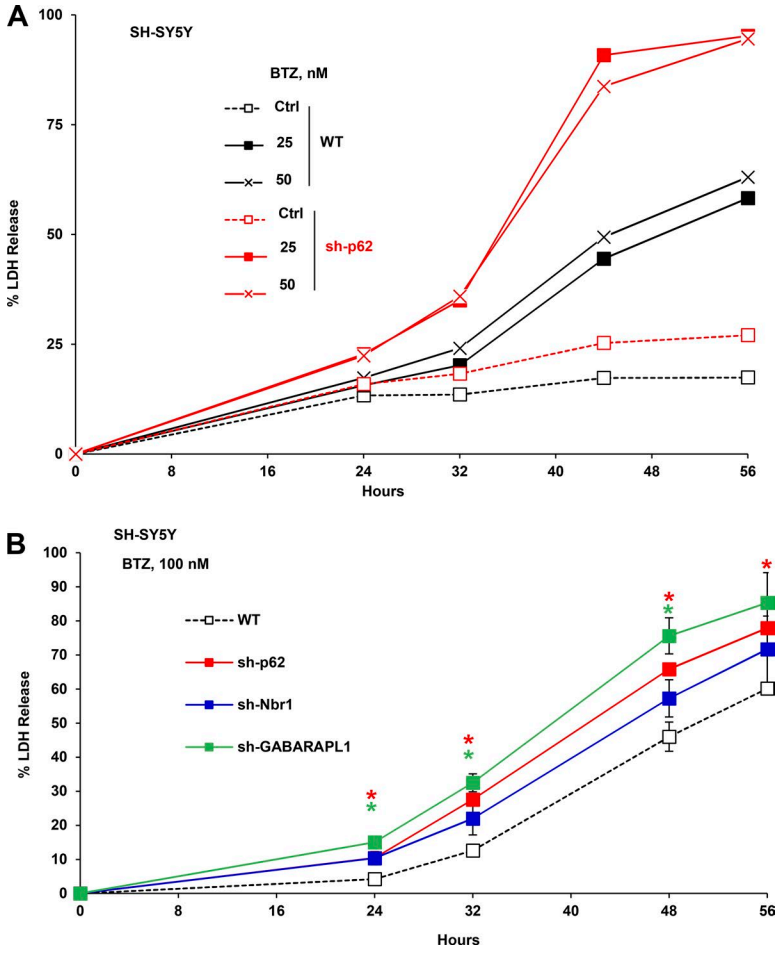

Figure S3. **In SH-SY5Y cells, knockdown of *p62* or *GABARAPL1* but not *Nbr1* increases sensitivity to killing by BTZ. (A and B)** WT SH-SY5Y cells or cells with stable knockdown of *p62* (A and B), *Nbr1* (B), or *GABARAPL1* (B) were treated for up to 56 h with indicated concentrations of BTZ. Cell death was quantified by measuring the percentage of cells that lost plasma membrane integrity and released intracellular LDH. One well of cells was assayed in A to obtain qualitative results, and two wells were assayed in B to determine statistical significance. The color of asterisk was used to indicate which knockdown cell line was killed more than WT cells. *, $P < 0.05$. Error bars indicate SD.

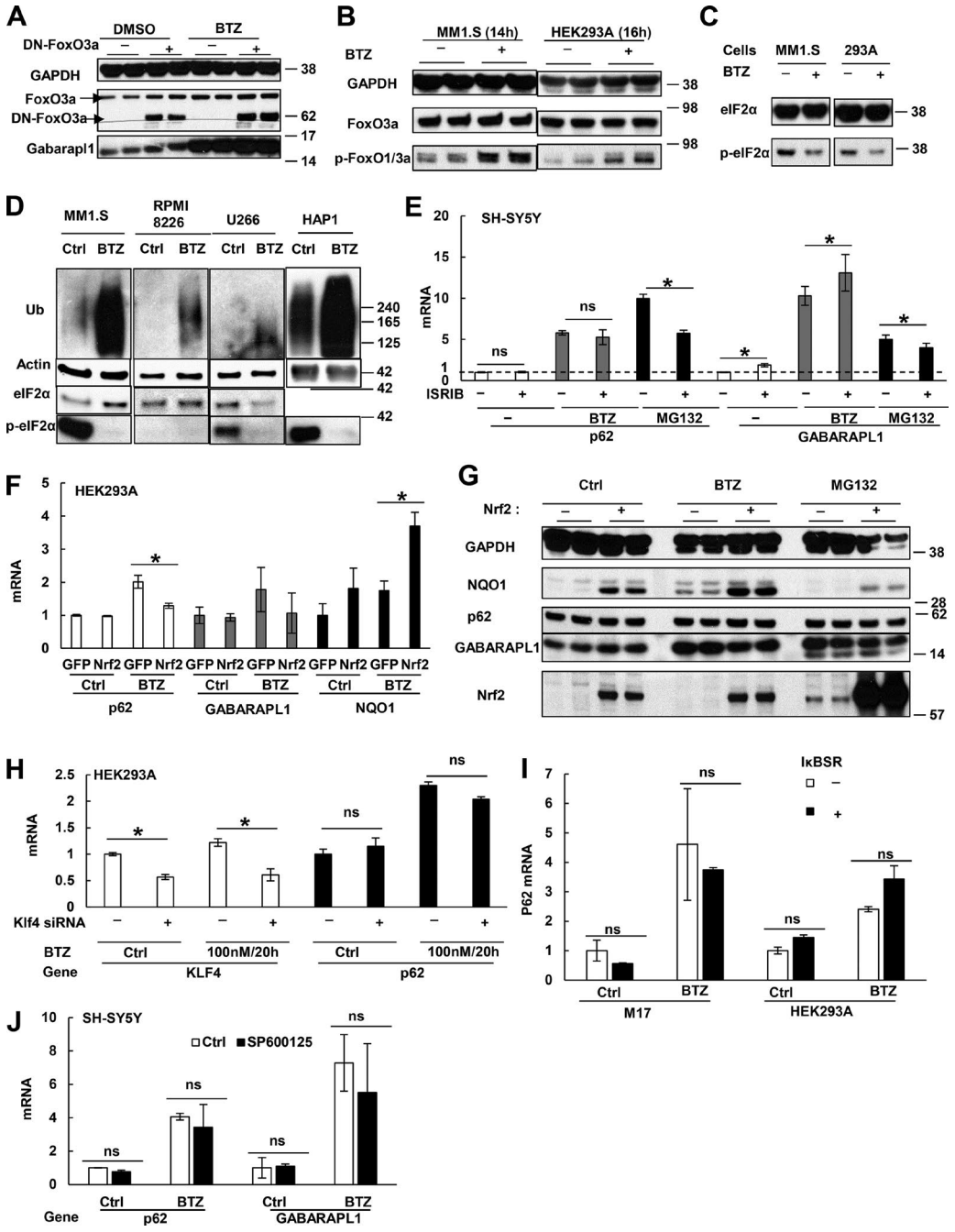

Figure S4. **FoxO3a, p-eIF2α, ATF4, Nrf2, or KLF4 are not responsible for the induction of *p62* and *GABARAPL1* upon rapid proteasome inhibition, although ATF4 is capable of inducing *p62* upon treatment with a very high concentration (10 µM) of MG132. (A)** Overexpression of DN-FoxO3a in HEK293A cells was unable to suppress induction of GABARAPL1 protein upon BTZ treatment (10 nM for 16 h). **(B)** In MM1.S (left) and HEK293A (right) cells, BTZ treatment (10 nM) increased the phosphorylation of FoxO1 (Thr24) and FoxO3a (Thr32; without changing total FoxO3a level), which inactivates these transcription factors. **(C)** Treatment of MM1.S or HEK293A cells with BTZ (10 nM for 16 h) suppressed eIF2α phosphorylation without affecting total eIF2α level. **(D)** Three myeloma cell lines (MM1.S, RPMI8226, and U266) were treated for 12 h with 20 nM BTZ, and HAP1 cells were treated for 16 h with 100 nM BTZ. BTZ treatment reduced eIF2α phosphorylation in MM1.S, U266, and HAP1 cells and did not cause detectable increase of eIF2α phosphorylation in RPMI 8226 cells. **(E)** The induction of *p62* and *GABARAPL1* in SH-SY5Y cells treated for 14 h with 10 µM MG132 was suppressed by cotreatment with ISRIB, which blocks translation of ATF4. ISRIB did not reduce the induction of *p62* upon 10-nM BTZ treatment for 14 h and, curiously, increased GABARAPL1 mRNA in control cells or cells treated with 10 nM BTZ. **(F and G)** Overexpression of Nrf2 in HEK293A cells activated the expression of NQO1 mRNA (F) and protein (G) but did not increase the mRNA (F) and protein (G) levels of p62 or GABARAPL1 in untreated cells or cells treated for 16 h with 10 nM BTZ or 10 µM MG132. Control transfection was done to express GFP. Molecular masses are given in kilodaltons. **(H)** *KLF4* was knocked down from HEK293A cells with siRNA and validated by measuring KLF4 mRNA. Knockdown of *KLF4* did not affect the mRNA of p62 in untreated cells or cells treated for 20 h with 0.1 µM BTZ. **(I)** Expression of IκBSR in HEK293A cells or M17 cells did not affect the mRNA of p62 in untreated cells or cells treated with BTZ (100 nM for 16 h). **(J)** SH-SY5Y cells were treated with 10 µM JNK inhibitor SP600125 for 30 min, and then 1 µM BTZ was added for 4 h. SP600125 did not affect the mRNA of p62 or GABARAPL1 in untreated or BTZ-treated cells. *, $P < 0.05$. Error bars indicate SD.

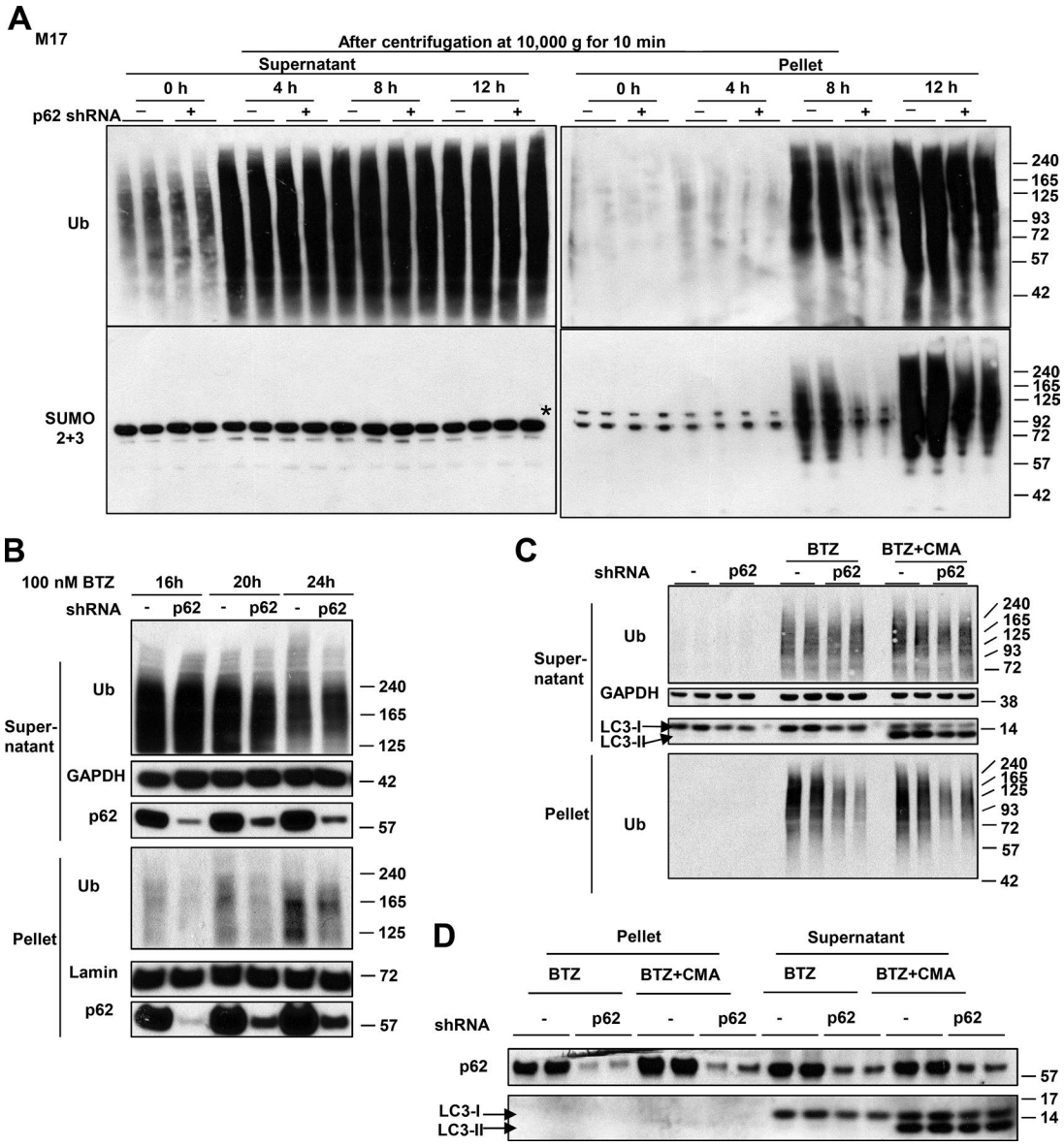

Figure S5. **In neuroblastoma cells, autophagy-mediated degradation of Ub conjugates and p62 is hardly detectable, and *p62* knockdown reduces formation of inclusions containing Ub and SUMO conjugates without affecting their turnover by autophagy. (A)** WT or sh-p62 SH-SY5Y cells were treated with 0.1 µM BTZ for 0, 4, 8, or 12 h. Cell lysates were fractionated as in Fig. 8 B, and the amounts of Ub conjugates and SUMO2/3 conjugates in the supernatant and pellet fractions were measured by Western blotting. BTZ treatment for 4 h caused accumulation of Ub conjugates in the supernatant, and by 8 h, there was p62-dependent accumulation of both Ub and SUMO conjugates in the pellet. Asterisk indicates a nonspecific band recognized by our anti SUMO2/3 antibody (not depicted). **(B)** WT or *p62* knockdown (by shRNA) SH-SY5Y cells were treated with 100 nM BTZ for 16, 20, and 24 h. Proteins were measured by Western blotting after fractionation. At every time point, *p62* knockdown only reduced the amount of Ub conjugate in the pellet but did not increase conjugate level in the supernatant. **(C and D)** WT or sh-p62 SH-SY5Y cells were treated with BTZ (100 nM) and/or concanamycin A (CMA; 10 nM; to block lysosomal degradation) for 16 h. The accumulation of LC3-II in CMA-treated cells indicates that lysosomal degradation was efficiently inhibited. Blocking lysosomal degradation did not cause a detectable increase in the level of Ub conjugates in both supernatant and pellet fractions (C) or the level of p62 (D). Molecular masses are given in kilodaltons.