Supporting Information

Lysosome-targeting chimeras containing an endocytic signaling motif trigger endocytosis and lysosomal degradation of cell-surface proteins

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1. Method

General Information: The concentration of proteins was determined by BCA protein assay kit (Thermo Fisher Scientific, USA). Images of Coomassie brilliant blue R250 (Sangon Biotech, China) stained, fluorescently stained bands and chemiluminescent bands were imaged using a Tanon 5200 imaging system (Tanon Science & Technology Co., Ltd., China). The mass of proteins were analyzed by the Electrospray Ionization Mass Spectrometry (Waters, USA). The purity of proteins were analyzed by The Agilent 1260 Infinity II HPLC. All plasmids information has been confirmed via DNA sequencing by TSINGKE Biological Technology.

Cell lines: Human cancer lines SKOV3, NCI-N87, SKBR3, MDA-MB-231, A431, and HCC-1954 were obtained from American Type Culture Collection (ATCC, USA). SKOV3, NCI-N87, SKBR3, and HCC-1954 were grown in RPMI 1640 medium (Gibco, USA) supplemented with 10% FBS (Corning, USA) and 1% penicillin/streptomycin. MDA-MB-231 and A431 were grown in DMEM (Gibco, USA) with 10% FBS and 1% penicillin/streptomycin. Freestyle 293-F cell line was purchased from Thermo Fisher Scientific, and cultured in Freestyle 293 Expression Medium (Gibco, USA). All cell lines were cultured in a humidified incubator at 37 ^{o}C under 5% CO_2.

Antibodies and reagents: The primary antibodies for HER2 (#2165s, CST, USA), PD-L1 (#13684T, CST, USA), β-actin (HC201, Trans, China), Rab5 (66339-1-IG, Proteintech, China), anti-Rab7 antibody (9367T, CST, USA), anti-Rab11 antibody (15903-1-AP, proteintech, China), GAPDH (HC301, Trans, China), anti-mouse-HRP antibody (ZB2305, ZSGB-Bio, China), anti-rabbit-HRP antibody (ZB2301, ZSGB-Bio, China), anti-mouse-IgG AF488 antibody (L3036, SAB, China), and anti-rabbit-IgG AF488 antibody (A23220, Abbkine, China), chloroquine (C193834, Aladdin, China), MG132 (FB05934, FelixBio, China), chlorpromazine (T22292, TargetMol, China), nystatin (CSN11592, CNSpharm, USA), EIPA (CSN25462, CNSpharm, USA), Lysotracker green (40738ES50, Yeasen, China), Golgi-Tracker Green (C1045S, Beyotime, China), DAPI (D9542, Sigma, USA) and maleimide-PEG3-TAMRA (BDR-15, Confluore Biological Technology, China) were obtained from the indicated suppliers.

IgG expression and purification: The recombinant antibodies were prepared by transient expression in Freestyle 293-F mammalian cells. After transfection of the expression plasmids, cells were incubated for five days at 37 °C with 5% CO_2. The culture supernatant was then collected by centrifugation at 4000 rpm for 10 min, and the antibody was purified by Protein A affinity column. The eluted solution was buffer-exchanged into PBS (pH 7.4) by ultrafiltration centrifugal devices (Millipore). The purity and integrity of all antibodies were assessed by 10% SDS-PAGE under reducing and non-reducing conditions.

Flow cytometry for internalization assay: Cells were plated in 12-well plates and grown to ~70%, and were treated with 100 nM TAMRA-labeled antibody in a complete growth medium for 24 h. Following treatment, cells were washed with 0.2 M glycine buffer containing 0.15 M NaCl (pH 3.0) and then three times with PBS to remove unbound antibodies. Cells were trypsinized for 3 min and centrifuged at 1000 rpm for 5 min and then resuspended in PBS. Flow cytometry was performed using CytoFLEX S (Beckman Coulter). Data were analyzed by FlowJo 10.

Confocal microscopy for colocalization imaging: Cells were plated on chamber slides and grown to ~80% confluency, and then treated with 100 nM TAMRA-labeled antibody in a complete growth medium for 24 h at 37 °C. After the removal of the culture medium, cells were washed three times with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were washed three times with PBS followed by the addition of 100 nM Lysotracker Green for 30 min at 25 °C. Cells were then washed with PBS three times, and incubated with DAPI (1:1000) for 10 min. After washing with PBS three times, cells were imaged using an Olympus FV3000 confocal microscope at a 60×oil immersion objective.

Protein degradation analysis by western blot: Cells upon reaching 70% confluency were treated with 100 nM SignalTACs or isotype control for 48 h in a complete growth medium. Then, cells were washed three times with cold PBS and lysed with RIPA buffer supplemented with phenylmethanesulfonyl fluoride (PMSF) on ice for 30 min. Cells were then scraped and centrifuged at 12000 rpm for 30 min at 4 °C. The supernatant was collected and protein concentration was determined by BCA assay

(Thermo Fisher Scientific). Equal amounts of protein were loaded onto a 10% Bis-Tris gel and separated by SDS-PAGE. The gel was then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked with 5% (w/v) non-fat milk for 1 h at room temperature, and then incubated with primary antibodies (1:1000) overnight at 4 ºC. After washing three times with TBS with 0.1% Tween-20 (TBST), the membrane was incubated with secondary HRP-linked antibodies (1:5000) for 1 h at room temperature and then washed three times with TBST. Proteins were detected by enhanced chemiluminescence (GlarityTM Western ECL Substrate, Bio-Rad) and the signals were recorded and quantified with Tanon-Image Software (Tanon).

Cell surface protein degradation analysis by flow cytometry: Cells were plated (250,000 cells per well in a 12-well plate) one day before the experiment. Cells were incubated with 300 μL of complete growth medium with 100 nM of Tz or Tz-P3 for 48 h. Cells were washed three times with cold PBS and trypsinized for 3 min. After centrifugation, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, washed three times, and permeabilized with 0.1% Triton X-100 for 10 min. Cells were washed three times with PBS, blocked in 1% BSA in PBS for 1 h at room temperature, and incubated with primary antibody (1:300) overnight at 4 ºC. Cells were washed three times with PBS and incubated with secondary antibody (1:500) for 1 h at room temperature, followed by washing three times with PBS. The cells were prepared for analysis by flow cytometry system using CytoFLEX S (Beckman Coulter). Data was analyzed by FlowJo 10.

Confocal microscopy for membrane protein degradation: Cells were plated on chamber slides at ~80% confluence. Cells were incubated with 300 μL of complete growth medium with 100 nM antibodies for 48 h. After washing three times with cold PBS, cells were stained using a cell membranes staining kit according to the manufacturer's protocol (Beyotime Biotechnology). Cells were washed three times in PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and rinsed three times with PBS. Cells were blocked in 1% BSA in PBS for 1 h at room temperature and incubated with primary antibody (1:300) overnight at 4 ºC. Cells were then washed three times with PBS and incubated with secondary antibody (1:500) for 1 h at room temperature. Cells were washed three times with PBS and then

stained with DAPI (1:1000) for 10 min at room temperature. Samples were imaged using an Olympus FV3000 confocal microscope at a 60×oil immersion objective.

Confocal microscopy for subcellular location: Cells were plated on chamber slides at ~70% confluence. Cells were incubated with 300 μL of complete growth medium with 100 nM antibodies for 6 h, 12 h, or 24 h and then washed three times with cold PBS. For Golgi staining, cells were stained using a Golgi-Tracker Green staining kit according to the manufacturer's protocol (Beyotime Biotechnology). After washing three times with cold PBS, cells were fixed with 4% paraformaldehyde for 20 min at room temperature and then stained with DAPI (1:1000) for 10 min at room temperature. For Rab5, Rab7, or Rab11 staining, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were then blocked in 1% BSA in PBS for 1 h at room temperature and incubated with primary antibody Rab5, Rab7, or Rab11 overnight at 4 ºC. Cells were then washed three times with PBS and incubated with secondary antibody (1:500) for 1 h at room temperature. Cells were washed three times with PBS and then stained with DAPI (1:1000) for 10 min at room temperature. Samples were imaged using an Olympus FV3000 confocal microscope at a 60×oil immersion objective.

siRNA knockdown: SKOV3 cells were plated in a 6-well plate and grown to ~70% confluency. Cells were transfected with 20 pmol of siRNA (Rui Biotech) and Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. After incubating with the transfection medium for 6 h at 37 ºC, the cells were treated with a complete growth medium with 100 nM antibodies for another 48 h at 37 ºC before further experiments.

Cell proliferation assay: Cell proliferation was assessed using an EdU kit (Beyotime Biotechnology, China) following the manufacturer's instructions. Cells were plated on chamber slides at 70% confluence and then incubated with 100 nM antibodies or isotype for 48 h. Cells were treated with EdU working buffer for 2 h, washed three times with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After washing three times with PBS, cells were incubated with click reaction buffer for 30 min at room

temperature. Cells were washed three times with PBS and imaged with an Olympus FV3000 confocal microscope at a 60×oil immersion objective.

Cell colony formation assay: NCI-N87 cells were plated (1000 cells per well in a 12-well plate) 3 days before the experiment. Cells were then incubated with 1 mL of complete growth medium with various concentrations of Tz or Tz-P3 at 37 ºC for 15 days. Cells were washed three times with PBS, and then fixed with 4% paraformaldehyde for 15 min. After the cells were washed three times with PBS, 1 mL crystal violet reagent (Beyotime Biotechnology, China) was added and incubated for 20 min. After the cells were washed three times with ddH_2O, the photos were taken with a camera.

Tumor xenograft mouse models: NCI-N87 cells (2×10^6) were suspended in 100 μL complete growth medium and injected into the right flank of 5–6 week old female BALB/c mice (four mice/group, Guangdong Medical Laboratory Animal Center). After the tumor size reached 400–500 mm^3, the animals were randomly divided into three groups (n = 4). Tumor-bearing mice were intraperitoneally injected with PBS, Tz (10 mg/kg), and Tz-P3 (10 mg/kg) every four days for a total of four times. Tumor size and mouse weight were measured every 2 days. Tumor volume was calculated as follows: tumor volume = length × $width^2/2$. The mice were sacrificed 16 days after the first injection, and tumor samples were collected, weighed, and further analyzed. The animal experimental procedure used in this study was approved by the Animal Care and Use Committee of Sun Yat-sen University (SYSU-IACUC-2023-001033). All animals were maintained in pathogen-free conditions and cared for in accordance with policies and certification of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Immunohistochemistry of the tumor tissues: The collected tumors were fixed in 4% paraformaldehyde for 24 h at 4 °C and then embedded in paraffin. Serial sections (5 μm) were prepared for immunohistochemistry (IHC). The sections were dewaxed to water, and placed in xylene I for 10 min, xylene II for 10 min, and anhydrous ethanol for 5 min. The antigen retrieval was performed under high pressure and temperature in 0.01 M sodium citrate buffer (pH = 6.0). The slices were then incubated with 3% hydrogen peroxide in methanol for 10 min at room temperature to

quench the endogenous peroxidase activity. The sections were incubated with the primary antibody dilution buffer for 1 h at room temperature. After washing with PBS, HRP-conjugated secondary antibodies specific to the species of the primary antibodies were added.

Statistical analysis: All experiments were performed at least three times. The data are presented as mean ± s.e.m (standard error). All statistical analyses were performed using GraphPad Prism 7. Student's t-test was used to analyze differences between 2 groups, while one-way ANOVA followed by Tukey's post hoc test or Dunnett's multiple comparisons was applied for multiple comparisons.

2. Characterization of antibodies

1) Tz-P3

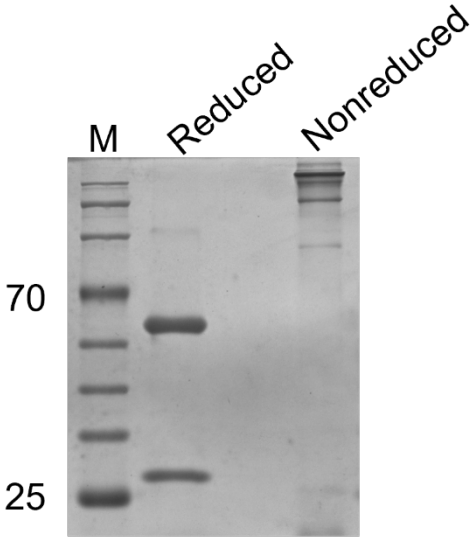

Figure S1. SDS-PAGE analysis of Tz-P3 under reducing and nonreducing conditions.

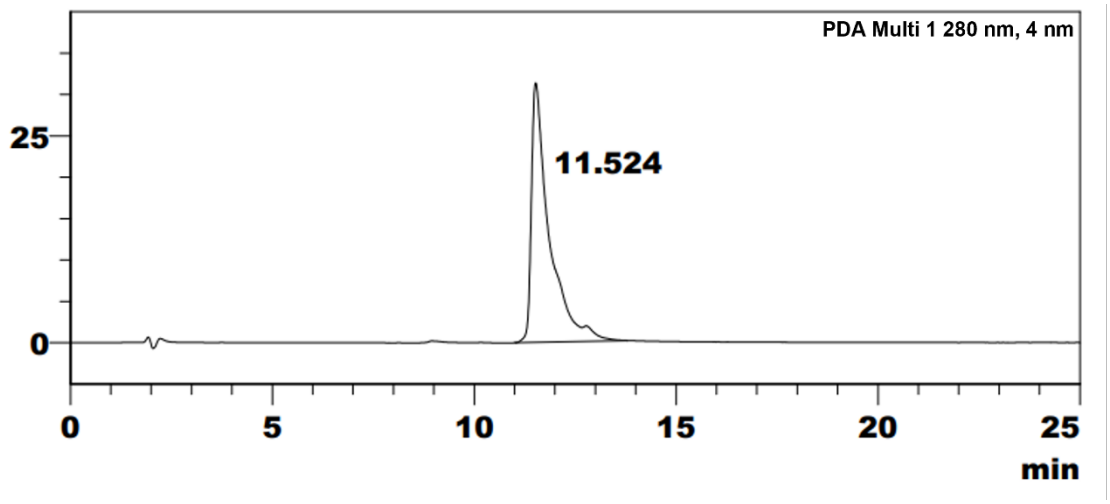

Figure S2. RP-HPLC analysis of Tz-P3.

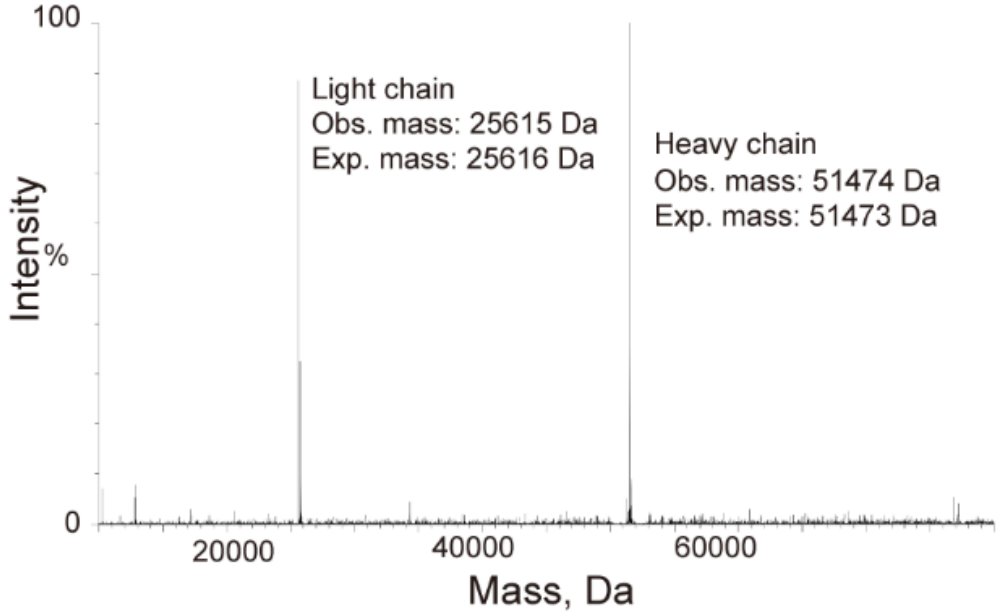

Figure S3. ESI-MS spectrum of Tz-P3.

2) Tz-P2-P3

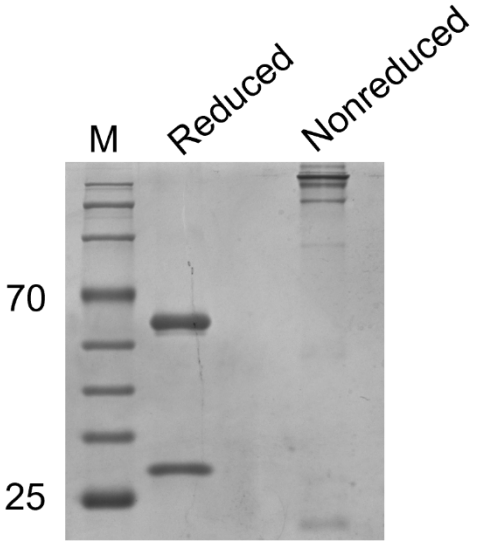

Figure S4. SDS-PAGE analysis of Tz-P2-P3 under reducing and nonreducing conditions.

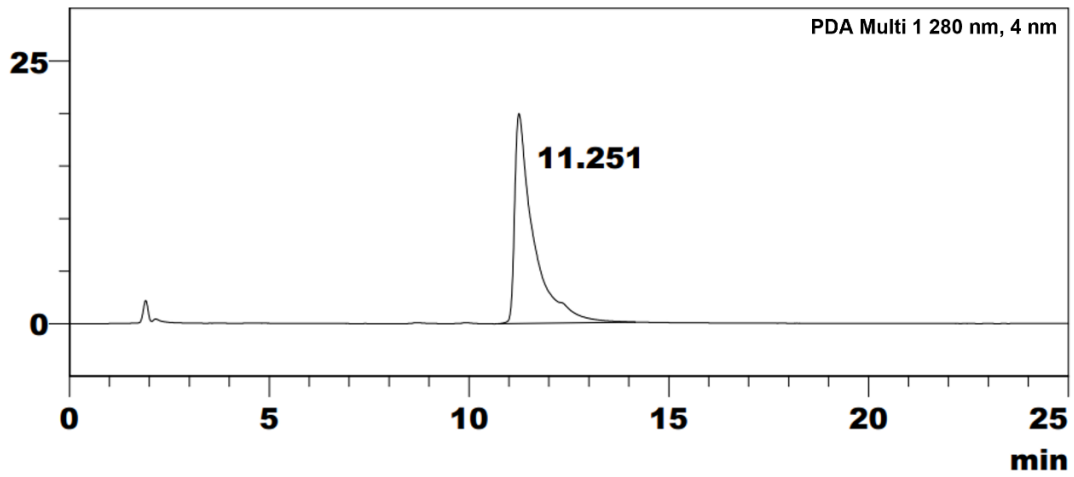

Figure S5. RP-HPLC analysis of Tz-P2-P3.

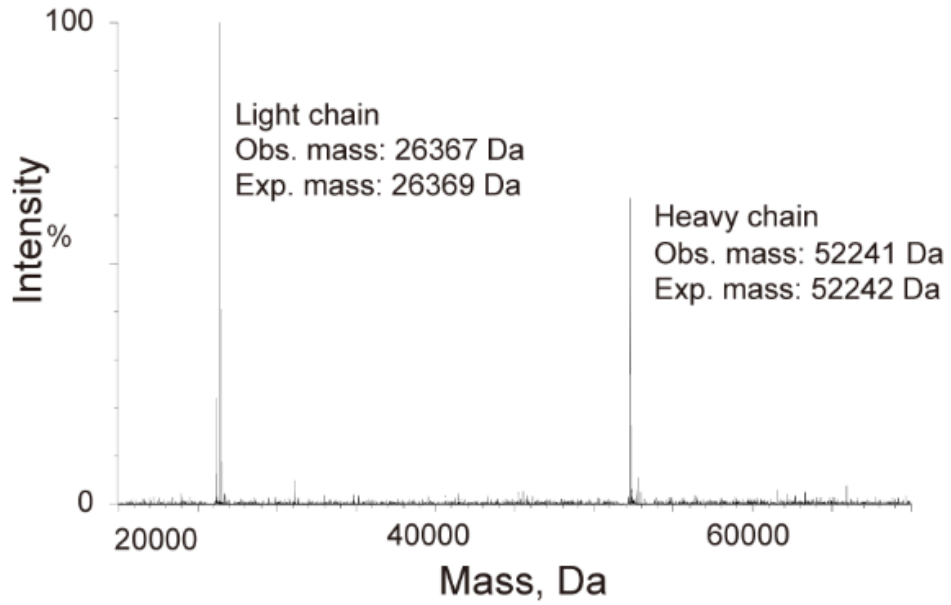

Figure S6. ESI-MS spectrum of Tz-P2-P3.

3) Az-P3

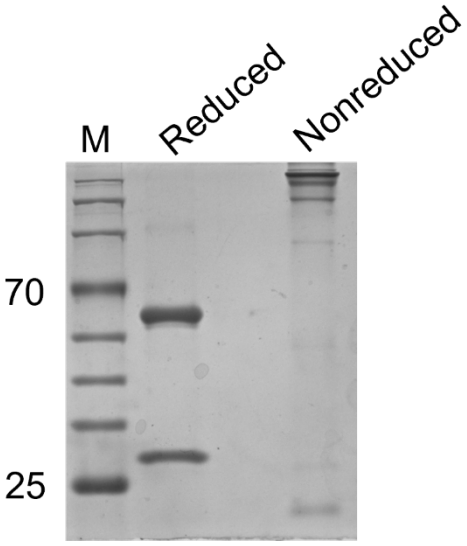

Figure S7. SDS-PAGE analysis of Az-P3 under reducing and nonreducing conditions.

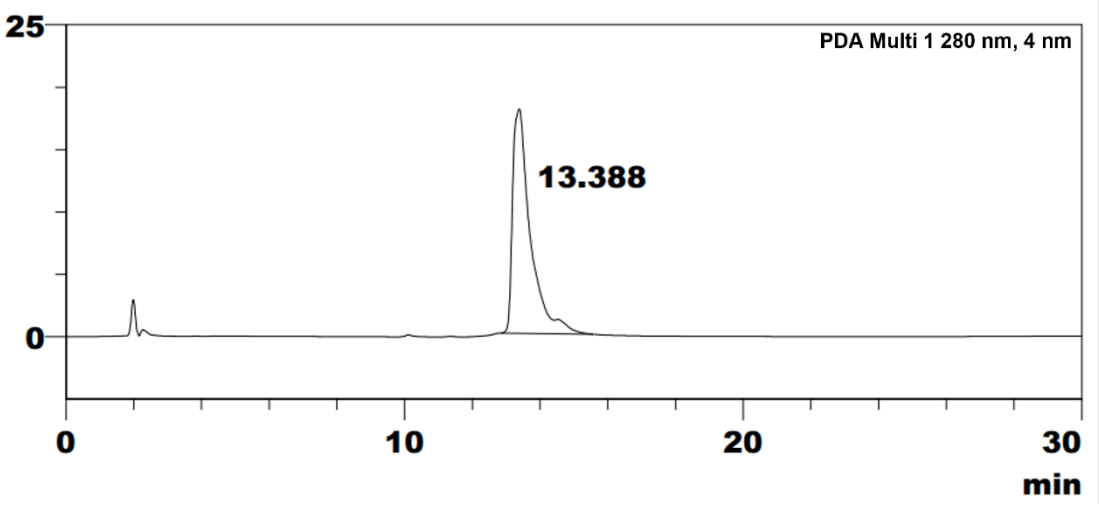

Figure S8. RP-HPLC analysis of Az-P3.

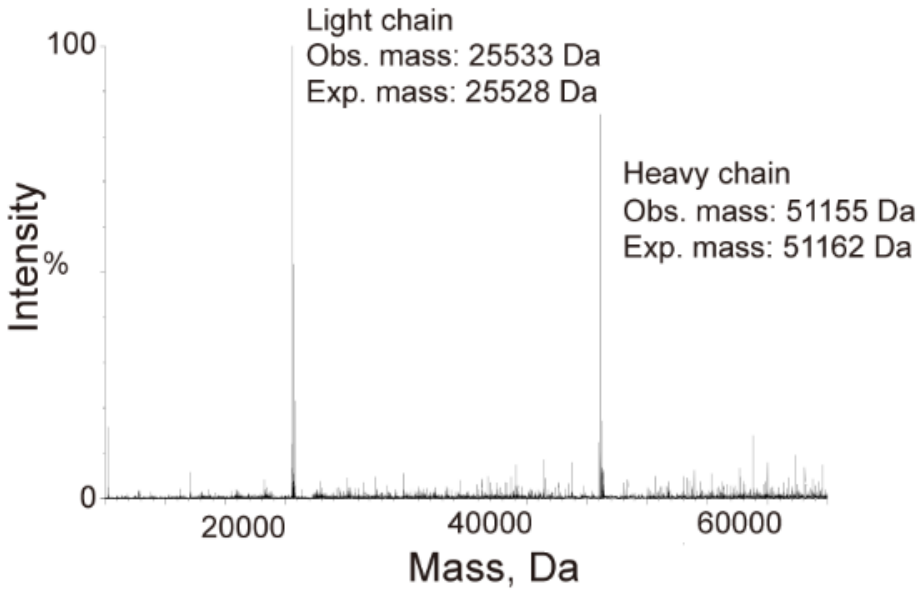

Figure S9. ESI-MS spectrum of Az-P3.

4) Az-P2-P3

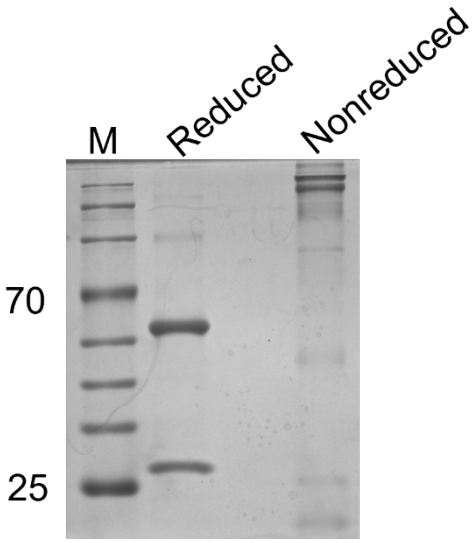

Figure S10. SDS-PAGE analysis of Az-P2-P3 under reducing and nonreducing conditions.

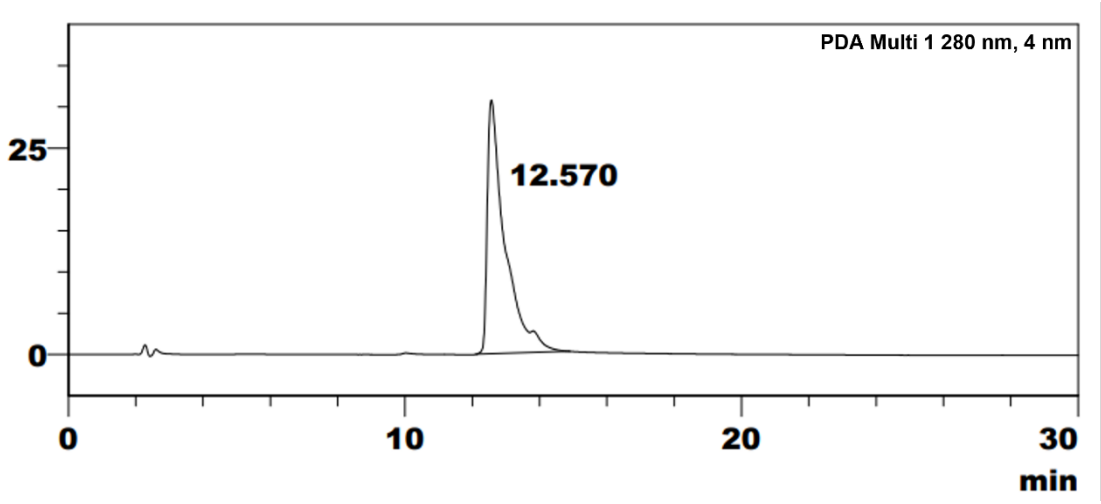

Figure S11. RP-HPLC analysis of Az-P2-P3.

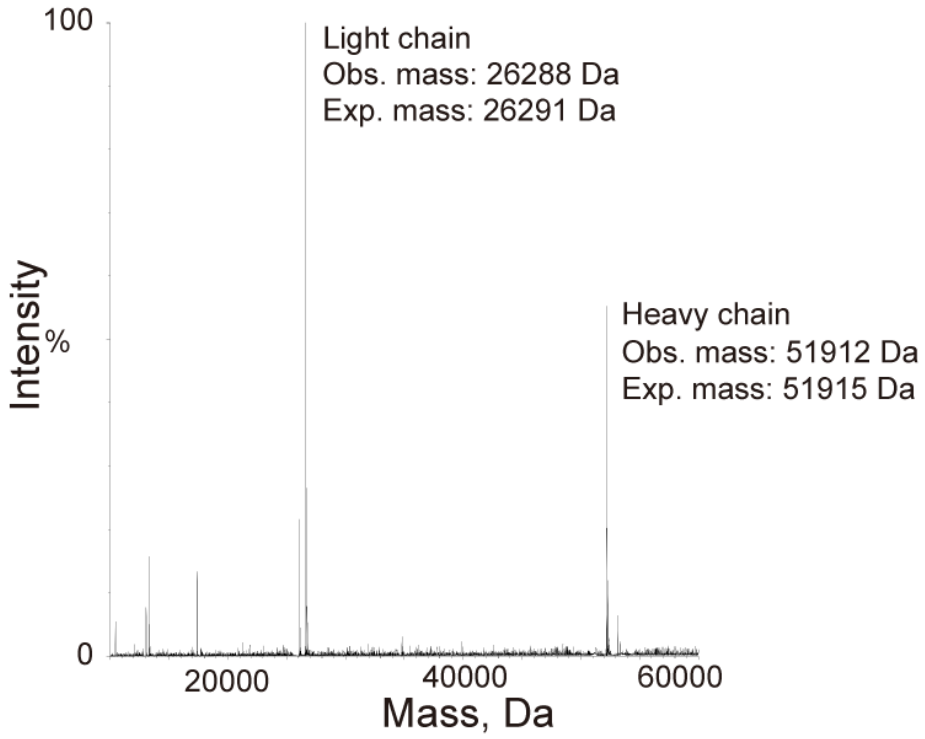

Figure S12. ESI-MS spectrum of Az-P2-P3.

3. Stability test examination of Tz and Tz-P3

Table S1. The chemical stability study of Tz and Tz-P3.

Sample	Detection Items	0 h	25℃		40℃	
			12 h	60 h	12 h	60 h
Tz	Concentration (mg/mL)	0.92	0.93	0.97	1.09	1.02
	Purity (SDS-PAGE Reducing)	> 95%	> 95%	> 95%	> 95%	> 95%
	Purity (SDS-PAGE Non-Reducing)	> 95%	> 95%	> 95%	> 95%	> 95%
	Purity (SEC-HPLC)	96%	97%	97%	96%	94%
Tz-P3	Concentration (mg/mL)	0.82	0.84	0.88	0.97	0.93
	Purity (SDS-PAGE Reducing)	> 95%	> 95%	> 95%	> 95%	> 95%
	Purity (SDS-PAGE Non-Reducing)	> 90%	> 90%	> 90%	> 90%	> 90%
	Purity (SEC)	95%	99%	91%	98%	90%

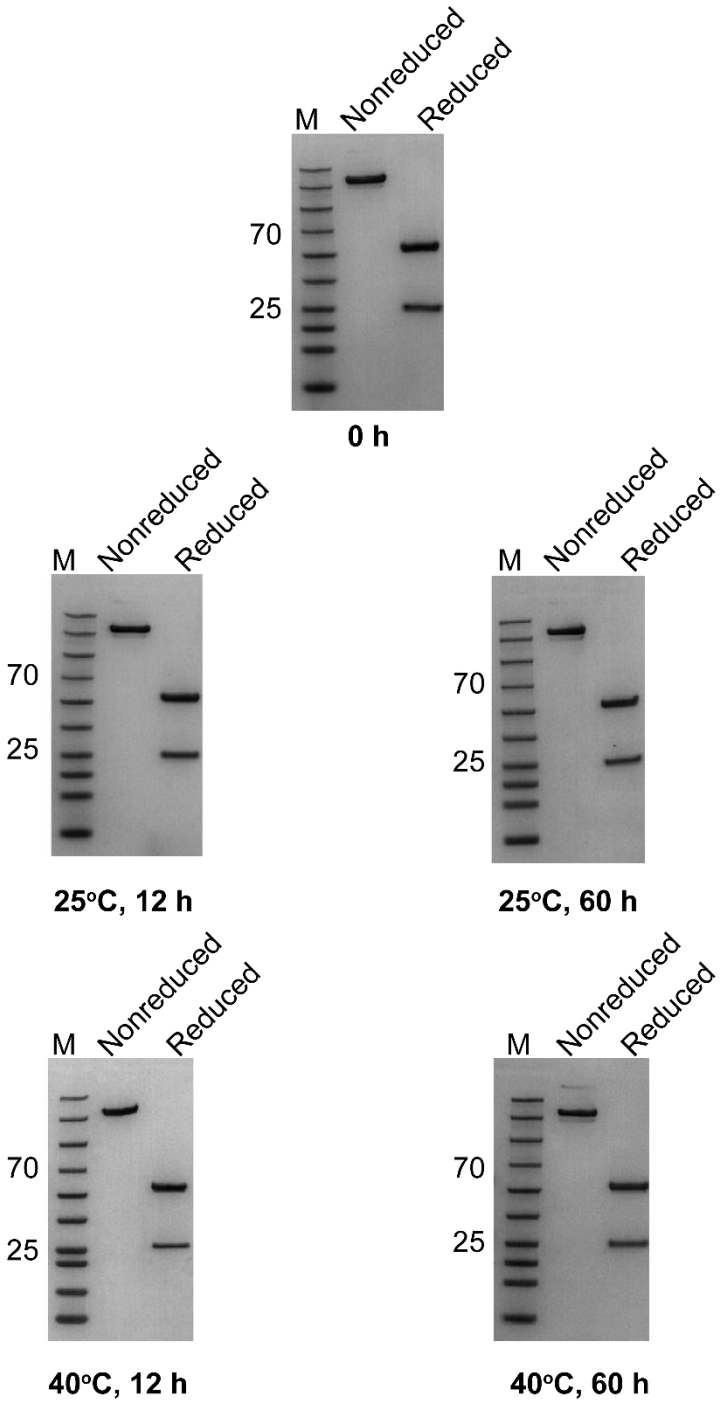

Figure S13. SDS-PAGE analysis of Tz after incubation at 12h and 60 h at 25 °C and 40 °C.

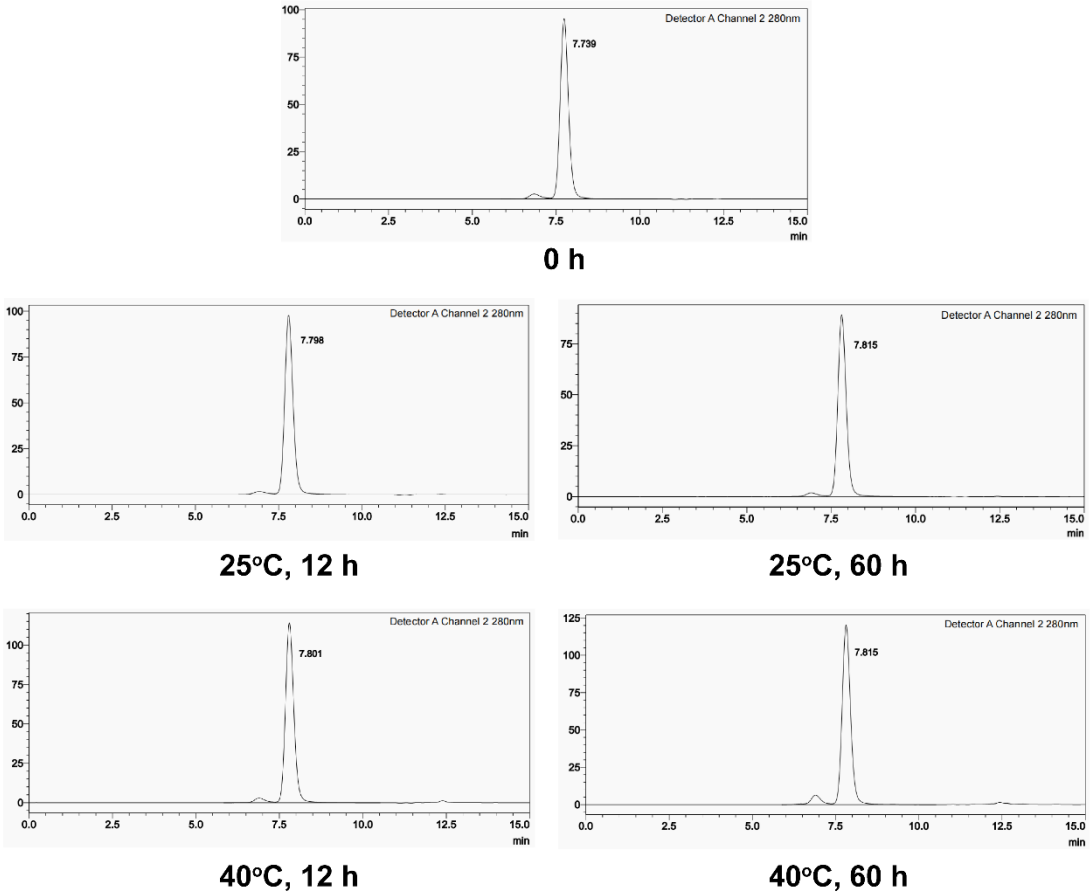

Figure S14. SEC-HPLC analysis of Tz after incubation at 12h and 60 h at 25 ºC and 40 ºC.

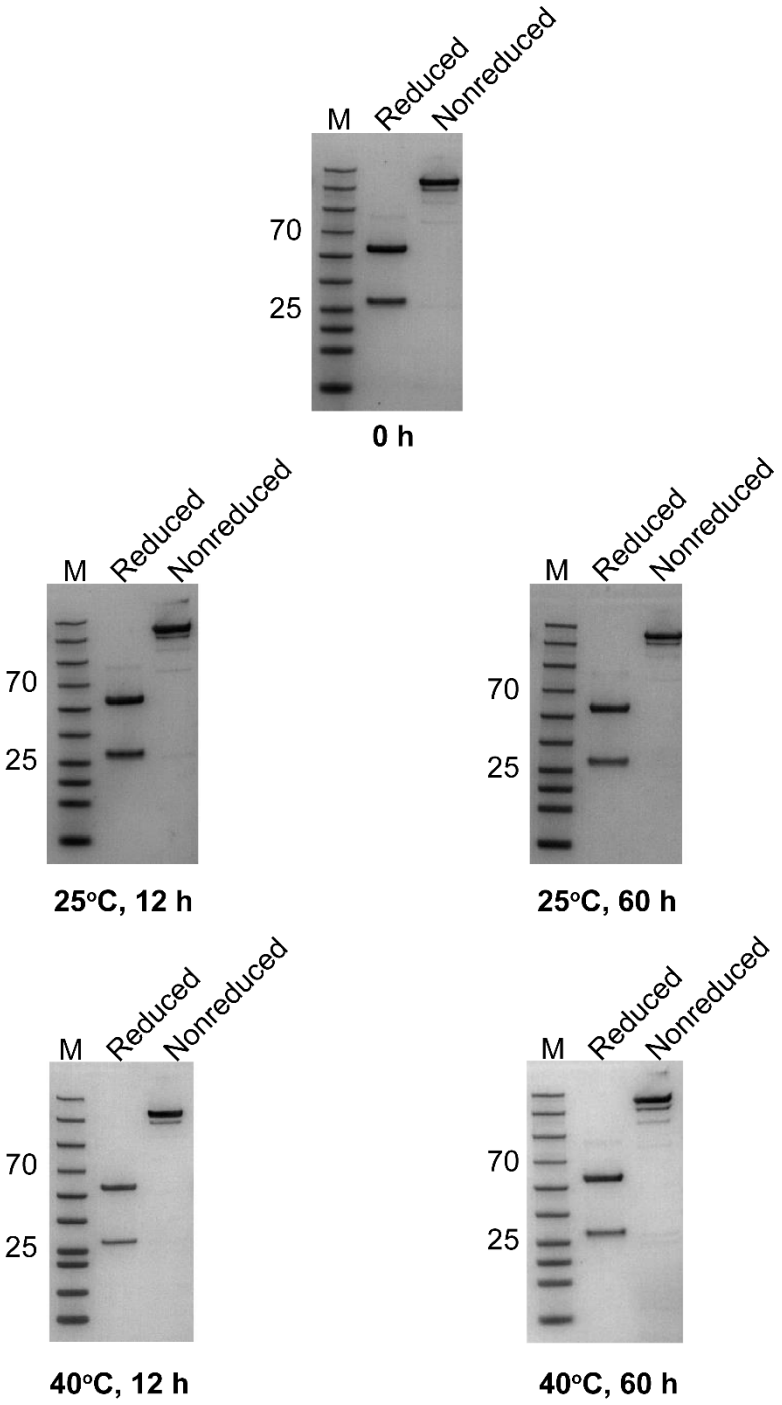

Figure S15. SDS-PAGE analysis of Tz-P3 after incubation at 12h and 60 h at 25 °C and 40 °C.

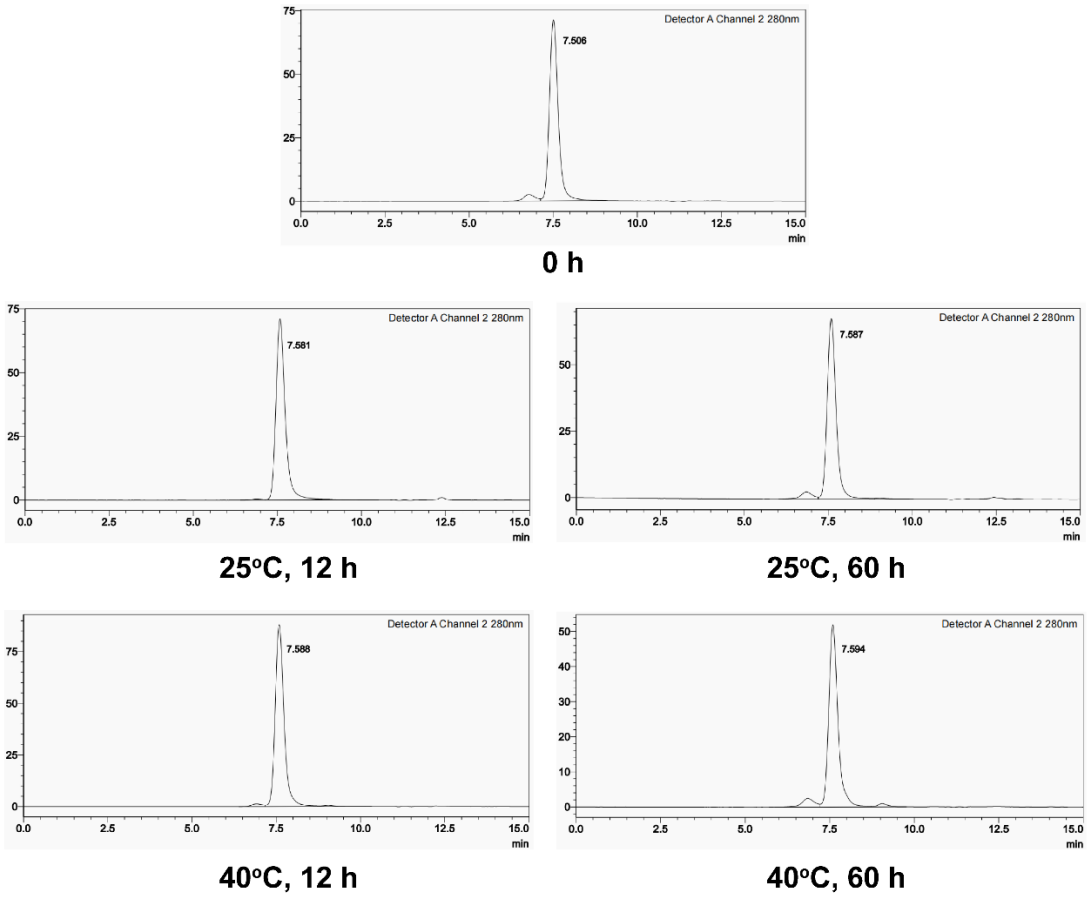

Figure S16. SEC-HPLC analysis of Tz-P3 after incubation at 12h and 60 h at 25 °C and 40 °C.

4. Comparative study of SignalTACs with different signal peptides

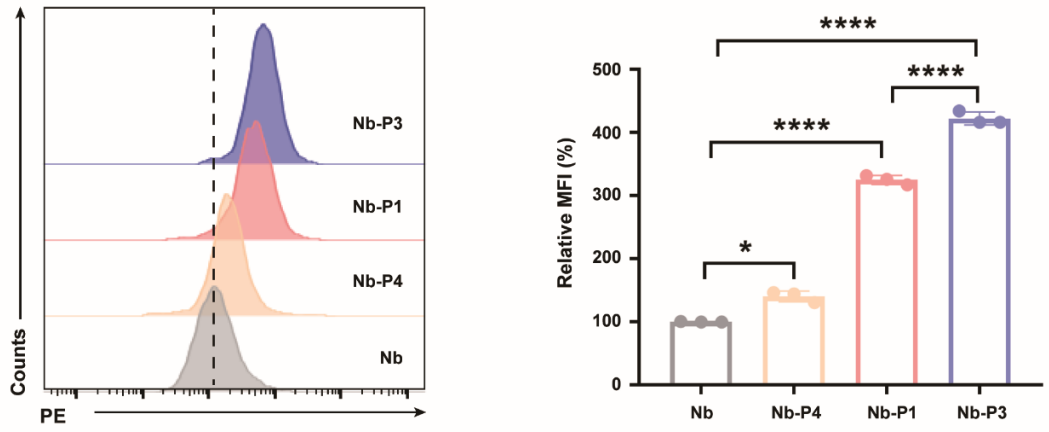

Figure S17. Mean fluorescence intensity for SKOV3 incubated with 2 μM Nb, Nb-P4, Nb-P1, or Nb-P3 for 6 h as determined by flow cytometry; P4: MTKEYQDLQHL. Statistical analysis was determined by one-way ANOVA with Tukey's post hoc test. *P<0.05, ****P<0.0001.

5. Confocal microscopic study

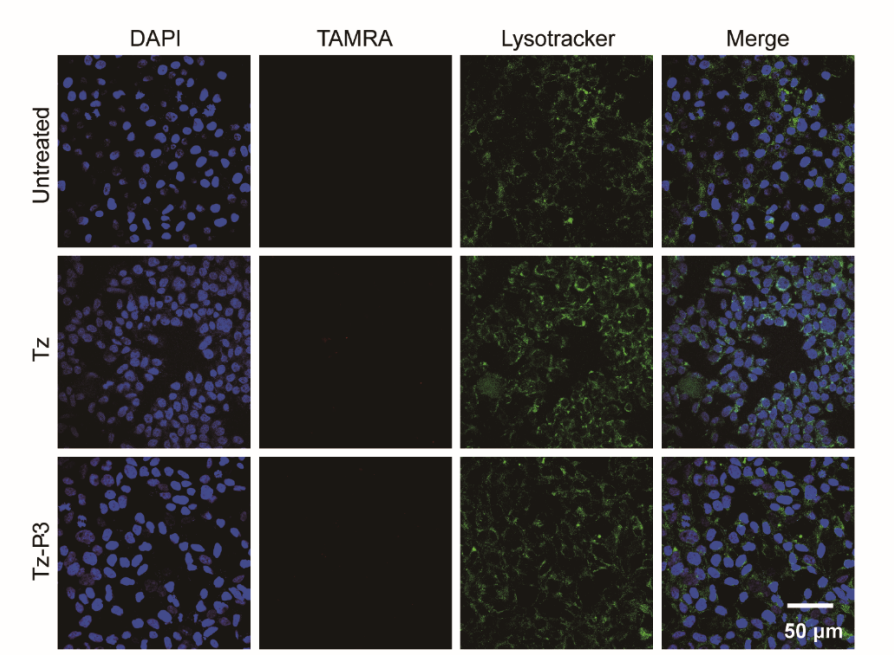

Figure S18. Confocal microscopy images of 293T cells incubated with 100 nM TAMRA-labeled Tz or Tz-P3 for 24 h.

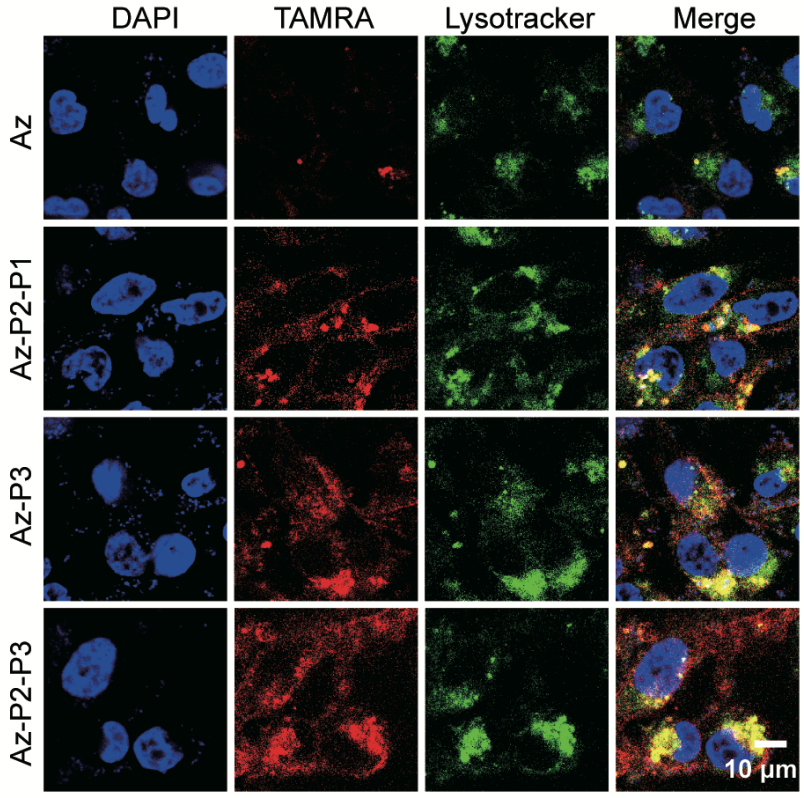

Figure S19. Confocal microscopy images of MDA-MB-231 cells incubated with 100 nM TAMRA-labeled Az, Az-P2-P1, Az-P3, or Az-P2-P3 for 24 h.

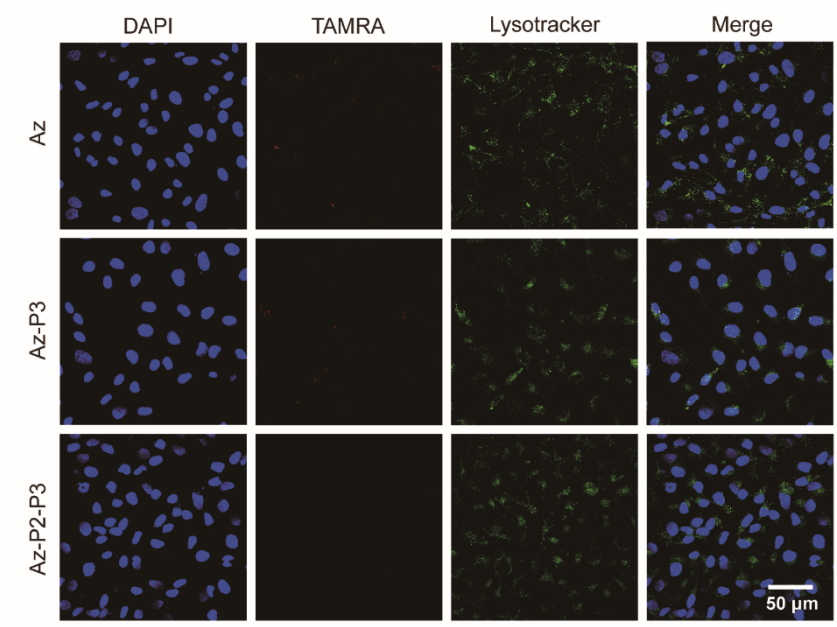

Figure S20. The confocal images of PD-L1-negative SKOV3 cells treated with 100 nM TAMRA labeled Az, Az-P3, and Az-P2-P3 for 24 h.

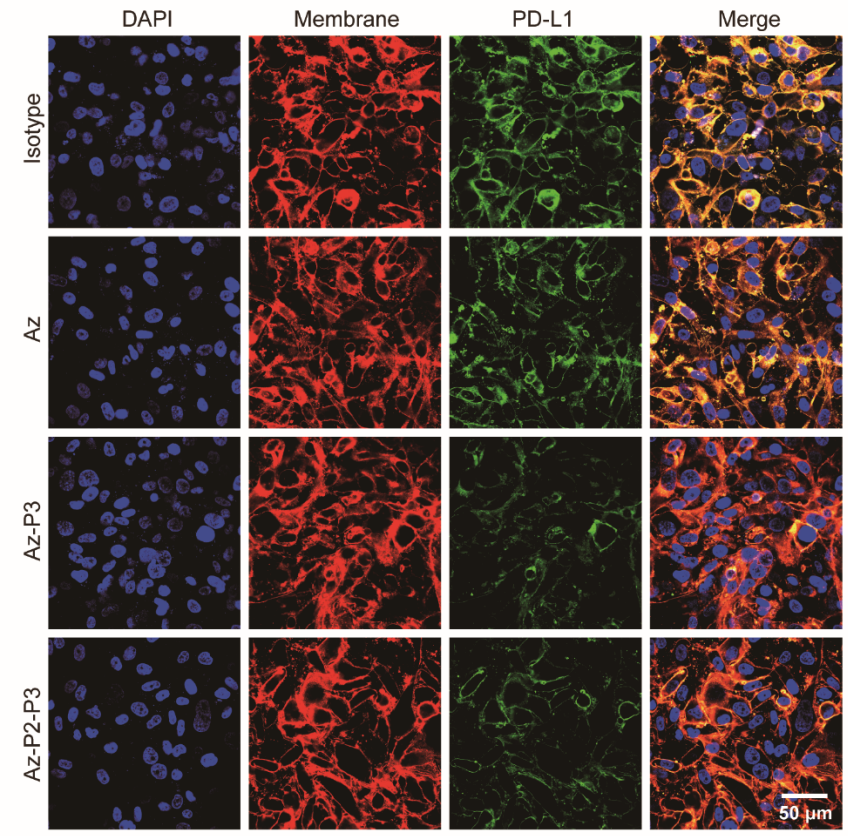

Figure S21. Visualization of PD-L1 degradation in MDA-MB-231 cells by confocal microscopy after treatment with 100 nM Az, Az-P3, or Az-P2-P3 for 48 h.

6. Western blotting analysis

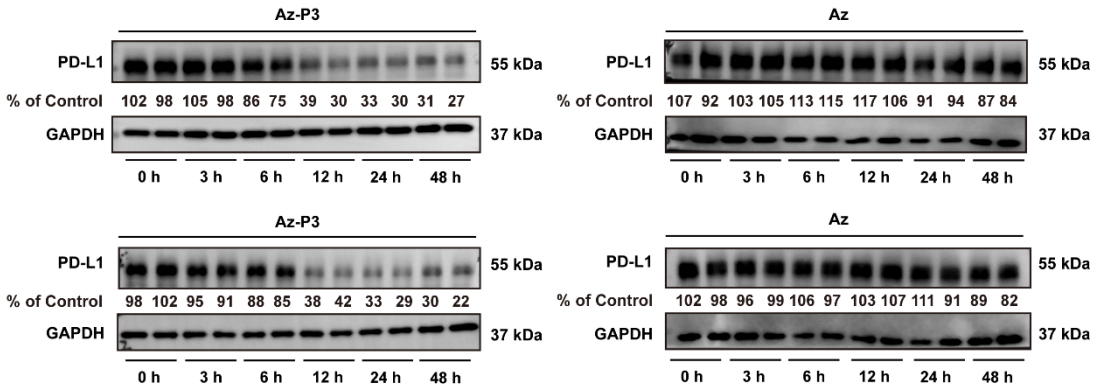

Figure S22. Western blotting analysis of PD-L1 degradation in MDA-MB-231 cells after treatment with 100 nM Az-P3 or Az for 3, 6, 12, 24, and 48 h.

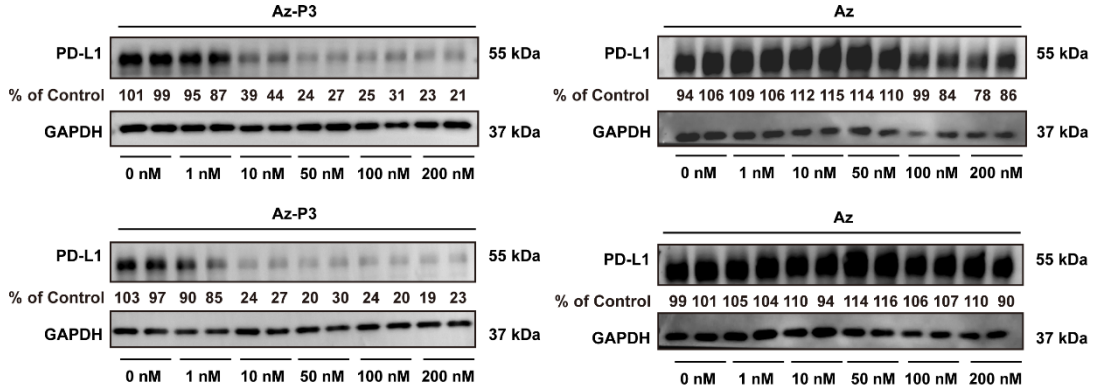

Figure S23. Western blotting analysis of PD-L1 degradation in MDA-MB-231 cells after treatment with 1 nM, 10 nM, 50 nM, 100 nM, and 200 nM Az-P3 or Az for 48 h.

7. In vitro antitumor effect of Tz-P3

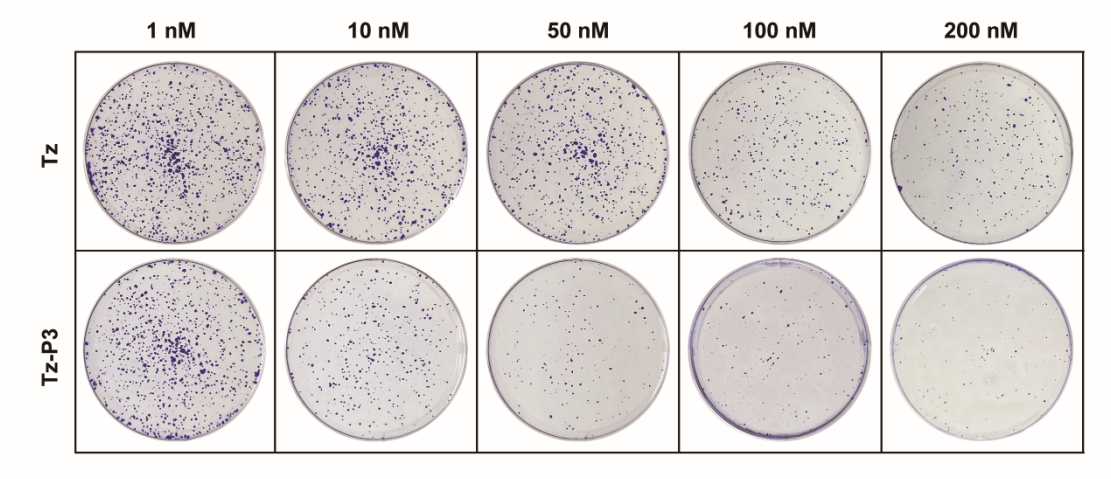

Figure S24. Images of colony formation assay of NCI-N87 cells after treatment after treatment of Tz or Tz-P3 at 1 nM, 10 nM, 50 nM, 100 nM, or 200 nM for 48 h.

8. In vivo antitumor effect of Tz-P3

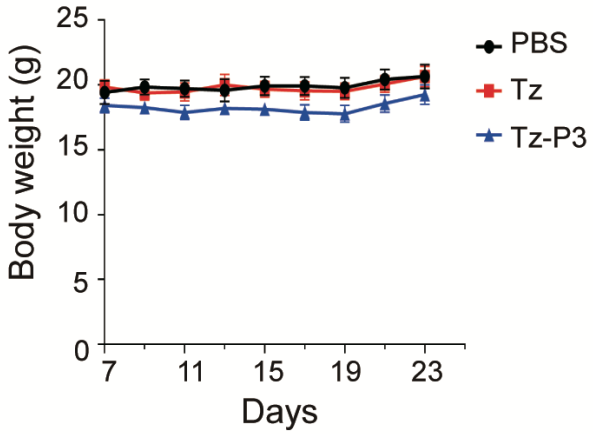

Figure S25. Evaluation of body weight of each group during the treatment (n = 4). Values are mean ± s.e.m using one-way ANOVA with Turkey's post hoc test. No significant body weight loss was observed for each group over the course of study.

9. siRNA-mediated protein knockdown study

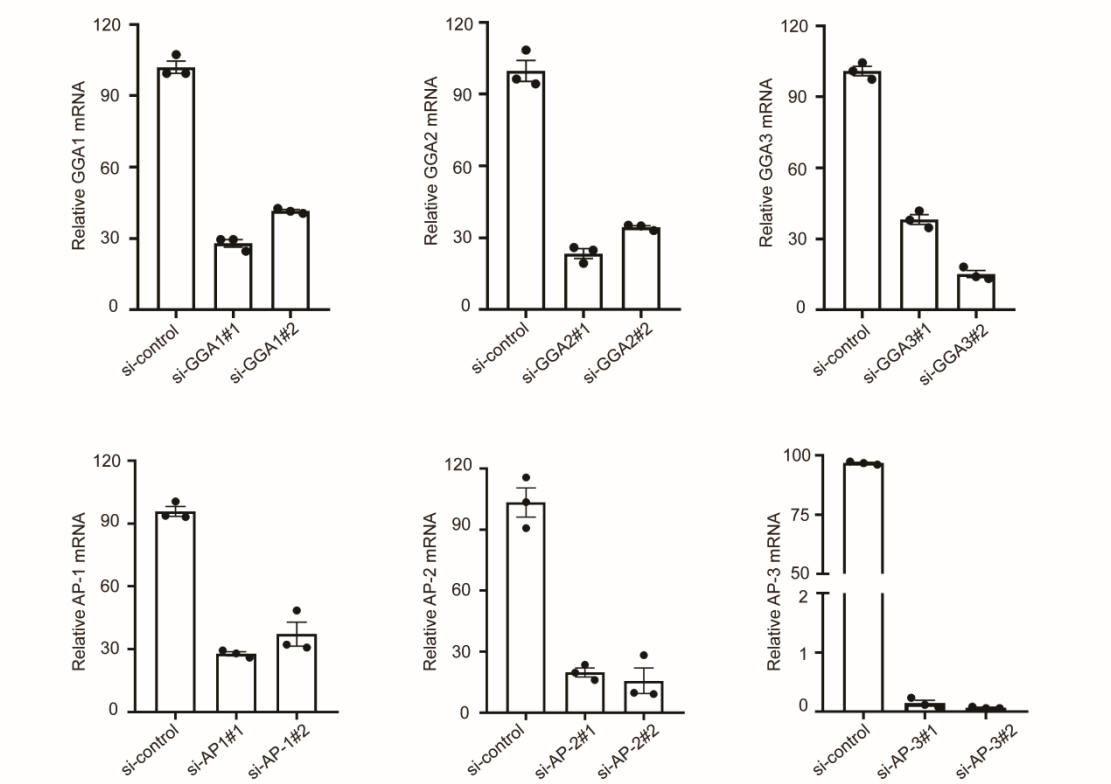

Figure S26. RT-qPCR analysis of mRNA of APs and GGAs mRNA in SKOV3 cells after siRNA treatment for 24 h.

Primer sequences for experiments

Table S2. Primer sequence for siRNA knockdown.

Gene	Forward	Reverse
AP1B1#1	GCUGCUGACAGCCCUCAAUTT	AUUGAGGGCUGUCAGCAGCTT
AP1B1#2	GCACGCUGCUCGACCUCAUTT	AUGAGGUCGAGCAGCGUGCTT
AP2S1#1	CCGGAACUUUAAGAUCAUUTT	AAUGAUCUUAAAGUUCCGGTT
AP2S1#2	GGAGGUCUUAAACGAAUAUTT	AUAUUCGUUUAAGACCUCCTT
AP3S1#1	GCGGCUCUCCAAGUUCUACTT	GUAGAACUUGGAGAGCCGCTT
AP3S1#2	UCUGUGAGCUGGAUUUGAUTT	AUCAAAUCCAGCUCACAGATT
GGA1#1	GAGGUGAACAACAAUGUGAAATT	UUUCACAUUGUUGUUCACCUCTT
GGA1#2	CCGAAGAAUGUGAUCUUUGAATT	UUCAAAGAUCACAUUCUUCGGTT
GGA2#1	CCGUGCCCUCUGAAUUAUGUUTT	AACAUAAUUCAGAGGGCACGGTT
GGA2#2	CUUACGGUACAAGCUGACAUUTT	AAUGUCAGCUUGUACCGUAAGTT
GGA3#1	GCACACGUUAGAGGAAGUUAATT	UUAACUUCCUCUAACGUGUGCTT
GGA3#2	CCAGAAGAAGCAAAGAUCAAATT	UUUGAUCUUUGCUUCUUCUGGTT

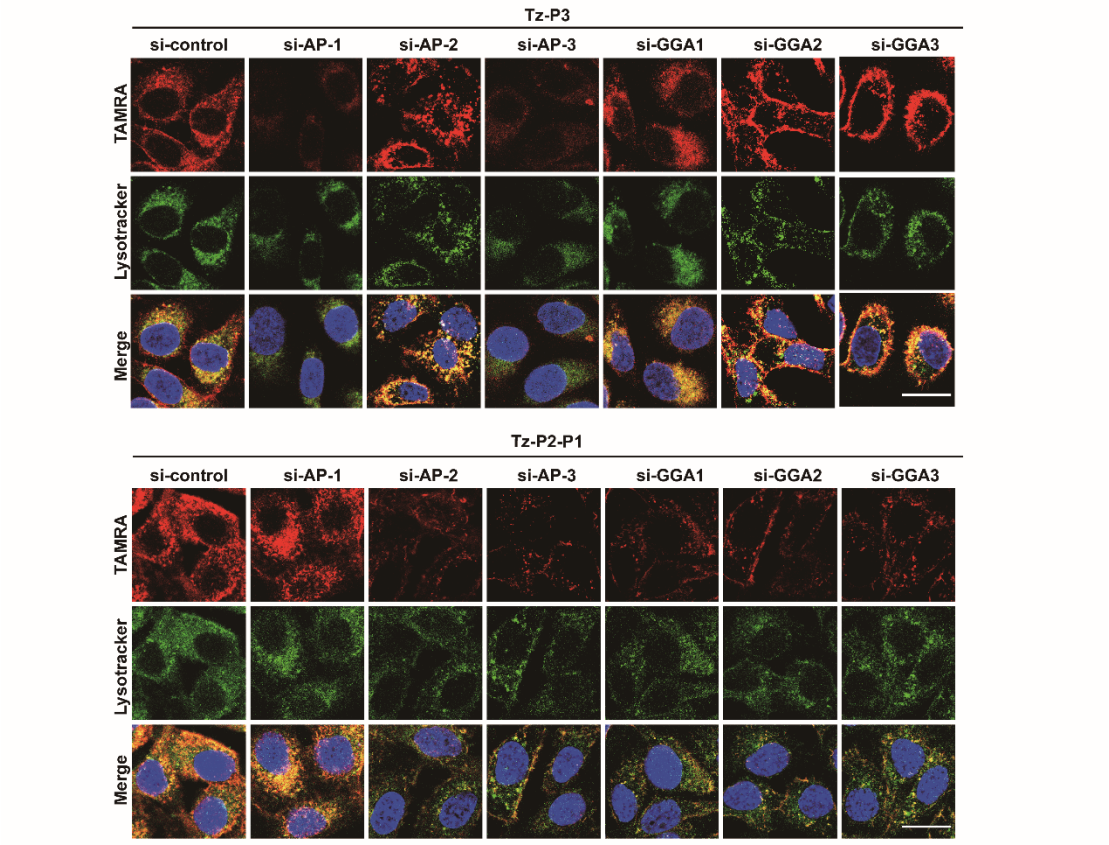

Figure S27. Confocal microscopy images of SKOV3 cell incubated with 100 nM Tz-P3 or Tz-P2-P1 for 24 h after siRNA knockdown of AP-1, AP-2, AP-3, GGA1, GGA2, or GGA3. Scale bars indicate 20 μm.

10. The intracellular trafficking study of SignalTACs

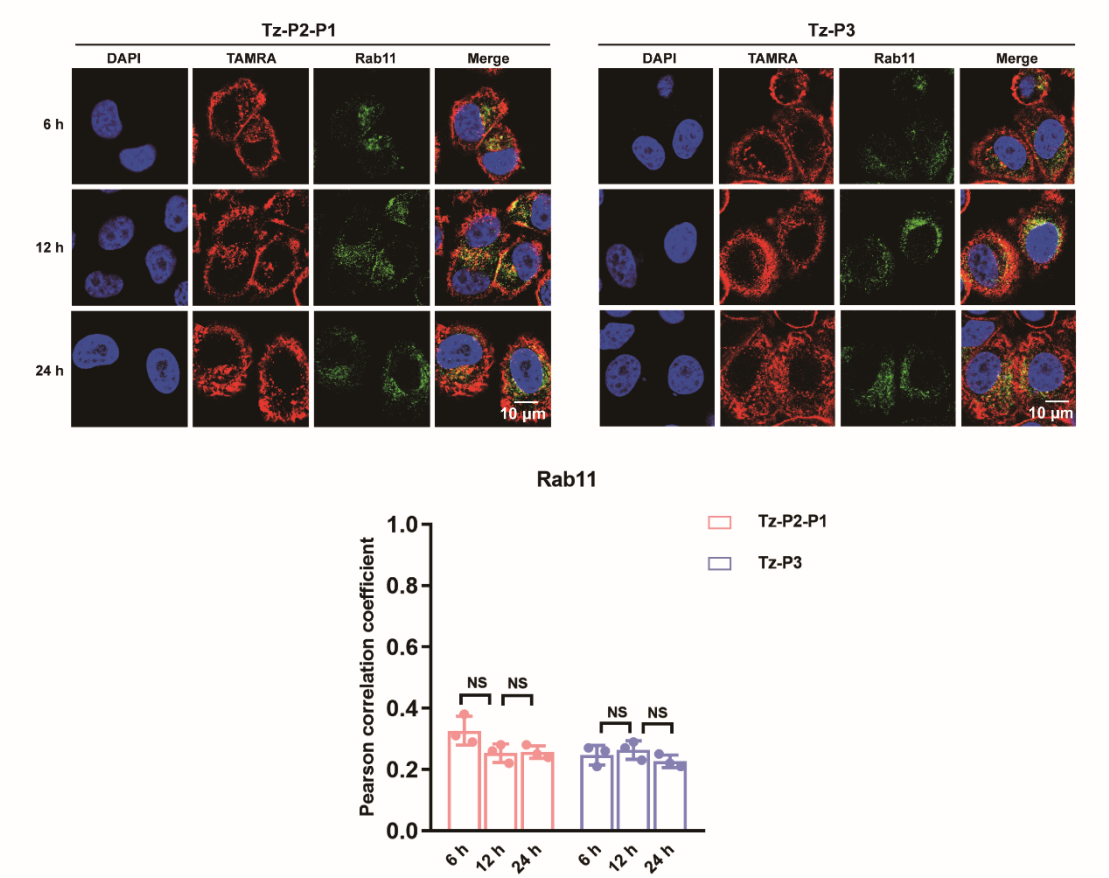

Figure S28. Visualization of Rab11 in SKOV3 cells by confocal microscopy after treatment with 100 nM Tz-P2-P1, or Tz-P3 for 6 h, 12 h or 24 h. The statistical result of the colocalization factor (Pearson's R value) are shown on the right panel. Values are means ± s.e.m. from three independent expriments. Statistical differences were determined by one-way ANOVA test. NS, no significance.

11. Unprocessed western blot images

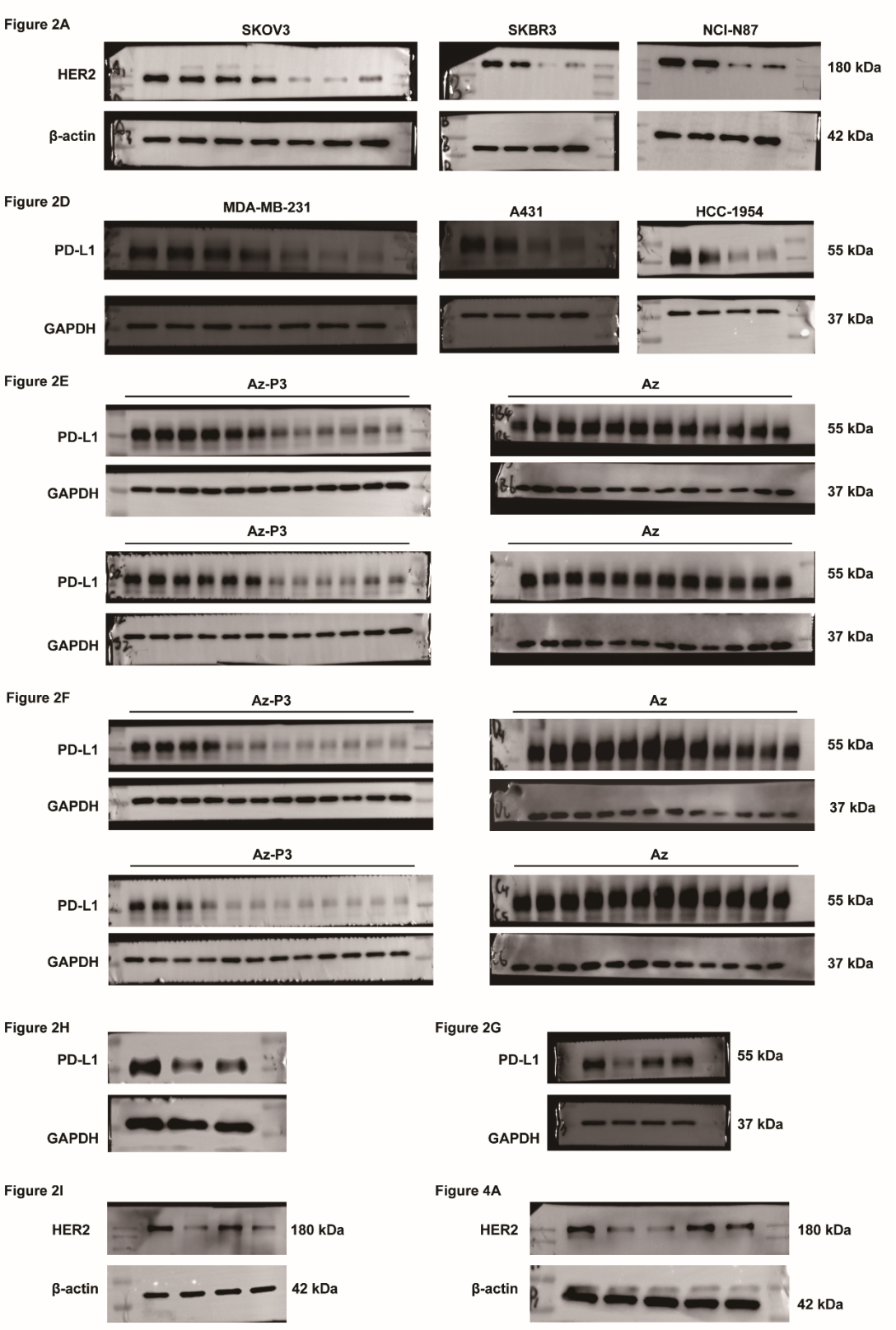

Figure S29. Full length gels for Figures 2 and 4.

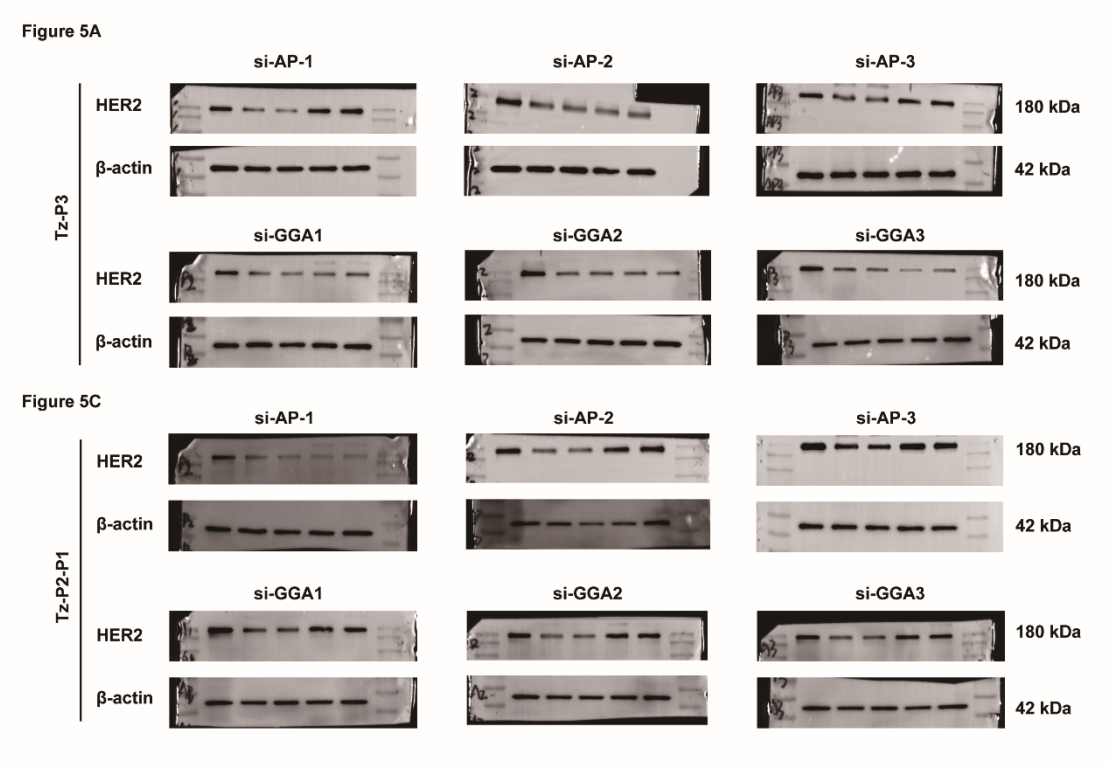

Figure S30. Full length gels for Figures 5.