Supplementary Information (SI) for ChemComm.


# Development of a molecular glue-based Lin28 degrader to regulate cellular proliferation and stemness

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## A. Materials and Methods

### Materials

All commercially available reagents for organic synthesis were purchased from Sigma-Aldrich, Tokyo Chemical Industry Co., Ltd, or ThermoFisher Scientific and used without further purification unless otherwise specified. Solvents were purchased from commercial vendors and used without further purification unless otherwise mentioned. Dry solvents were prepared using the ultimate solvent purification system CT-SPS-SA [Glass Contour]. The progress of the reaction was monitored using thin-layer chromatography (TLC) (silica gel 60, $F_{254}$ 0.25 mm) or liquid chromatography-mass spectrometry (LC-MS) LCMS-2020 [Shimadzu]. Components on TLC were visualized by UV light (254 nm) or by treating the TLC plates with $KMnO_4$ or phosphomolybdic acid followed by heating. Reverse-phase preparative high-performance liquid chromatography (HPLC) was performed with LC-6AD [Shimadzu] using YMC-Pack ODS-A AA20S05-2520WT (S-5 μm, 12 nm, 20 × 250 mm) [YMC]. Eluents used for purification are reported in parentheses.

### Compound characterization

$^{1}H$ and $^{13}C$ NMR spectra were obtained on Bruker AVANCE III HD 500 [Bruker]. Chemical shifts were reported in parts per million ($\delta$, ppm). As an internal standard, $^{1}H$ NMR spectra were calibrated using tetramethylsilane (TMS, 0.00 ppm). $^{13}C$ NMR spectra were calibrated using the residual solvent peak (DMSO-$d_6$, 39.52 ppm). Multiplicities were indicated as follows: s (singlet), d (doublet), m (multiplet), and so on. Coupling constants were reported in Hz. Low-resolution mass spectrometry (LRMS) was obtained by LCMS-2020 [Shimadzu] using electron spray ionization (ESI). High-resolution mass spectrometry (HRMS) was confirmed by Orbitrap Exploris 120 [ThermoFisher Scientific] using ESI from the Research Facilities Center at Seoul National University (Department of Chemistry).

#### Cell culture and compound treatment

Human choriocarcinoma cell line JAR was cultured in RPMI-1640 medium containing 10% (v/v) FBS and 1× antibiotic-antimycotic solution. Human embryonic kidney cell line HEK293T, mouse myoblast cell line C2C12, and mouse astrocyte cell line C8-D1A were cultured in DMEM supplemented with 10% (v/v) FBS and 1× antibiotic-antimycotic solution. Human ovary cell line PA-1 cells were cultured in MEM supplemented with 10% (v/v) FBS and 1× antibiotic-antimycotic solution. SH-SY5Y cells were cultured in Advanced DMEM/F12 [Gibco #12634-010] supplemented with 10% (v/v) FBS and 1× antibiotic-antimycotic solution. All cells were maintained in a humidified incubator with 5% $CO_2$ at 37 ℃. Cells were plated in a 12-well plate and incubated for 24 h to allow cell adhesion. Compounds were then treated to the cells at the indicated concentration and time. JAR and SH-SY5Y cells were obtained from the Korean Cell Line Bank (KCLB). HEK293T, PA-1, C2C12, and C8-D1A cells were obtained from American Type Culture Collection (ATCC).

**Cell lysis**

Cells were washed with cold PBS and lysed with RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM Sodium chloride, 1% (w/v) sodium deoxycholate [Sigma #30970], 1% IGEPAL CA-630 [Sigma #18896], 2 mM $Na_3VO_4$ [Sigma #450243], 5mM NaF [Sigma #S7920], 1× protease inhibitor cocktail [Roche #04693132001]). The lysate was centrifuged at 20000 g for 15 min at 4 ℃ to be clarified. Total protein concentration was measured by BCA assay [Thermo #23225] and diluted to equal concentration.

**Western blot**

Lysates were mixed with 5× Lammeli sampling buffer (0.315M Tris-HCl pH 6.8, 10% SDS, 8% DTT, 0.1% bromophenol blue, and 50% glycerol) and denatured at 95 ℃ for 5 min. Proteins were separated by SDS-PAGE and transferred to PVDF membranes [Bio-Rad #1620177] using Trans-Blot Turbo transfer system [Bio-Rad]. Membranes were blocked with TBS-T buffer with 2% bovine serum albumin (BSA) [MP Biomedicals #0216006980] at room temperature (r.t.) for 24 h, and then incubated overnight at 4 ℃ with primary antibody, diluted in recommended dilution. Membranes were washed with TBS-T and incubated with HRP-linked secondary antibodies (1:4000 dilution) at r.t. for 1 h. The membranes were washed three times with TBS-T and visualized by chemiluminescence [Dogenbio #DG-WPAL250]. Images were recorded with ChemiDoc MP and analyzed by Image Lab 4.0 [Bio-Rad]. Antibodies used here were Lin28A [Abcam #ab46020], Lin28B [CST #4196], α-tubulin [CST #3873], c-Myc [CST #5605], IMP1 [CST #8482], ubiquitin [Santa Cruz #sc-8017], RNF126 [Santa Cruz #sc-376005], Anti-mouse IgG, HRP-linked antibody [CST #7076], Anti-rabbit IgG, HRP-linked antibody [CST #7074].

**Quantitative PCR**

According to procedure guidelines, RNA was isolated from JAR cells using TRIzol [Invitrogen #15596018]. AccuPower CycleScript RT PreMix (dT20) kit [bioneer #K-2044] was used on a C1000 Touch thermal cycler [Bio-Rad #1841000] to obtain cDNA, following the manufacturer's protocol. qPCR was conducted on a StepOne Real-Time PCR Systems [Applied biosystems #4376357] with the KAPA SYBR FAST qPCR Master Mix (2×) [Kapa biosystems #KK4605]. Relative gene levels were determined by StepOne Software v2.3 [Applied biosystems], using GAPDH as a control. Detailed primer sequences are provided below.

List of qPCR primers

| | |
|---|---|
| *c-Myc* | F: 5'-AATGAAAAGGCCCCCAAGGTAGTTATCC-3'<br>R: 5'-GTCGTTTCCGCAACAAGTCCTCTTC-3' |
| *hGAPDH* | F: 5'-ACCAGCCCCAGCAAGAGCACAAG-3'<br>R: 5'-TTCAAGGGGTCTACATGGCAACTG-3' |
| *IMP1 (IGF2BP1)* | F: 5'-CTTTGTAGGGCGTCTCATTGGC-3'<br>R: 5'-CCTTCACAGTGATGGTCCTCTC-3' |
| *Lin28A* | F: 5'-GCCCCTTGGATATTCCAGTC-3'<br>R: 5'-TGACTCAAGGCCTTTGGAAG-3' |
| *Lin28B* | F: 5'-GCCCCTTGGATATTCCAGTC-3' |

| | |
|---|---|
| | R: 5'-TGACTCAAGGCCTTTGGAAG-3' |
| *RNF126* | AccuTarget Human Real-Time PCR Primer, Human, RNF126<br>[Bioneer PHS-P01 #P137806V] |
| *Synaptophysin* | F: 5'-CAACACCTCGGTGGTGTTCG-3'<br>R: 5'-CCTGAGGCCCGTAGGAATC-3' |
| *MAP2* | F: 5'-CATGGGTCACAGGGCACCTATTC-3'<br>R: 5'-GGTGGAGAAGGAGGCAGATTAGCTG-3' |
| *NSE* | F: 5'-CCCAGAACTTCCCTGATTGA-3'<br>R: 5'-AAGTGGAAGACACGTGGGAC-3' |
| *AChE* | F: 5'-CCTCCTTGGACGTGTACGAT-3'<br>R: 5'-AAACAGCGTCACTGATGTCG-3' |
| *SOX2* | F: 5'-AACCCCAAGATGCACAACTC-3'<br>R: 5'-CGGGGCCGGTATTTATAATC-3' |

**TaqMan assays**

Total RNA was extracted from JAR cells using TRIzol [Invitrogen #15596018]. Reverse transcription was conducted on a C1000 Touch thermal cycler [Bio-Rad #1841000] with TaqMan MicroRNA Reverse Transcription Kit [Applied biosystems #4366596]. TaqMan Universal Master Mix II, no UNG [Applied biosystems #4440040], was used to conduct qPCR with StepOne Real-Time PCR Systems [Applied biosystems #4376357]. Relative gene levels were determined by StepOne Software v2.3 [Applied biosystems], using U6 snRNA as a control. TaqMan microRNA Assays [Applied biosystems 4427975] used here were U6 snRNA [#001973], Let-7a [#000377], Let-7d [#002283], Let-7f [#000382], Let-7g [#002282], Let-7i [#002221], miR-98 [#000577], miR-20b [001014].

**siRNA transfection**

JAR cells were seeded in a 12-well plate at a density of 80,000 cells per well and incubated for 24 h. Cells were transfected with 20 nM siRNF126 [Bioneer SDH-1001 #55658-1] or AccuTarget Negative Control siRNA [Bioneer #SN-1002] using Lipofectamine RNAiMAX Transfection Reagent [Invitrogen #13778075] for 72 h. Cells were washed once with medium without siRNA. Cells were then treated with DMSO or SB1349 (5 μM) for 24 h.

**Cellular thermal shift assay (CETSA)**

Cells were incubated at 100 pi dish for 24 h. The cells were washed with DPBS, lifted using 0.25% trypsin, and harvested into a 50-mL tube. Cells were centrifuged at 1000 g for 3 min and resuspended to RPMI-1640 medium containing 60 μM SB1301, 60 μM SB1349 or DMSO as a control in 37 ℃ for 2 h. The cells were then harvested, aliquoted and treated at the indicated temperature for 3 min. Cells were centrifuged at 1000 g, 4 ℃ for 3 min and washed with PBS. The resulting cells were resuspended in 0.4% IGEPAL CA-630 in PBS and lysed through a three-time freeze-and-thaw cycle using liquid nitrogen. Lysates were cleared through centrifugation at 20000 g for 15 min at 4 ℃. The samples were mixed with 5× Lammeli sampling buffer and denatured at 95 ℃ for 5 min. Proteins were analyzed by western blot.

**Cell viability assay**

Cells were seeded in a 96-well plates (JAR: 15,000/well, HEK293T: 40,000/well, C2C12: 8,000/well, PA-1: 25,000/well, C8-D1A 20,000/well) and incubated for 24 h, followed by the treatment with SB1349 for 24 h. EZ-Cytoz [DoGenBio #EZ-BULK150] was added to each well and incubated for 1-2 h. Absorbance at 455 nm was measured with Synergy HT Microplate Reader [BioTeK]. The viability was calculated by the absorbance ratio between compound-treated wells and DMSO-treated wells.

**SH-SY5Y cell differentiation**

SH-SY5Y cells were seeded in a 96-well flat transparent bottom black plate [Corning #3904] at a density of 10,000 cells per well and incubated for 24 h. Following incubation, the cells were treated with designated concentrations of SB1349 or Retinoic acid [TCI #R0064] in Advanced DMEM/F12 supplemented with 1% (v/v) FBS and 1× antibiotic-antimycotic solution for 72 h. After the treatment, the media was replaced with Advanced DMEM/F12 supplemented with 10% (v/v) FBS and 1× antibiotic-antimycotic solution. The cells were then imaged using IN Cell Analyzer 2500 [GE Healthcare]. Neurite number and length were analyzed from brightfield images using NeuroQuantify software, which can be downloaded from GitHub https://github.com/StanleyZ0528/neural-image-segmentation.[1] More than two fields per technical replicate were analyzed, with fields containing 100 to 400 cells chosen for analysis. Total neurite length per cell was calculated as (Average axon length)×(Axons count)/(Estimated cell count), and total neurite number per cell was calculated as (Axons count) /(Estimated cell count).

[1] Dang, K. M. et al. NeuroQuantify--An Image Analysis Software for Detection and Quantification of Neurons and Neurites using Deep Learning. Preprint at https://arxiv.org/abs/2310.10978 (2023).

## B. Supplementary Figures

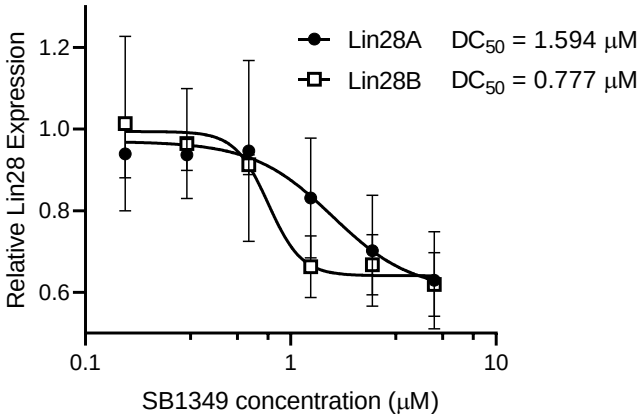



**Figure S1.** Quantification data for dose-dependent Lin28A/B degradation in Figure 2A.

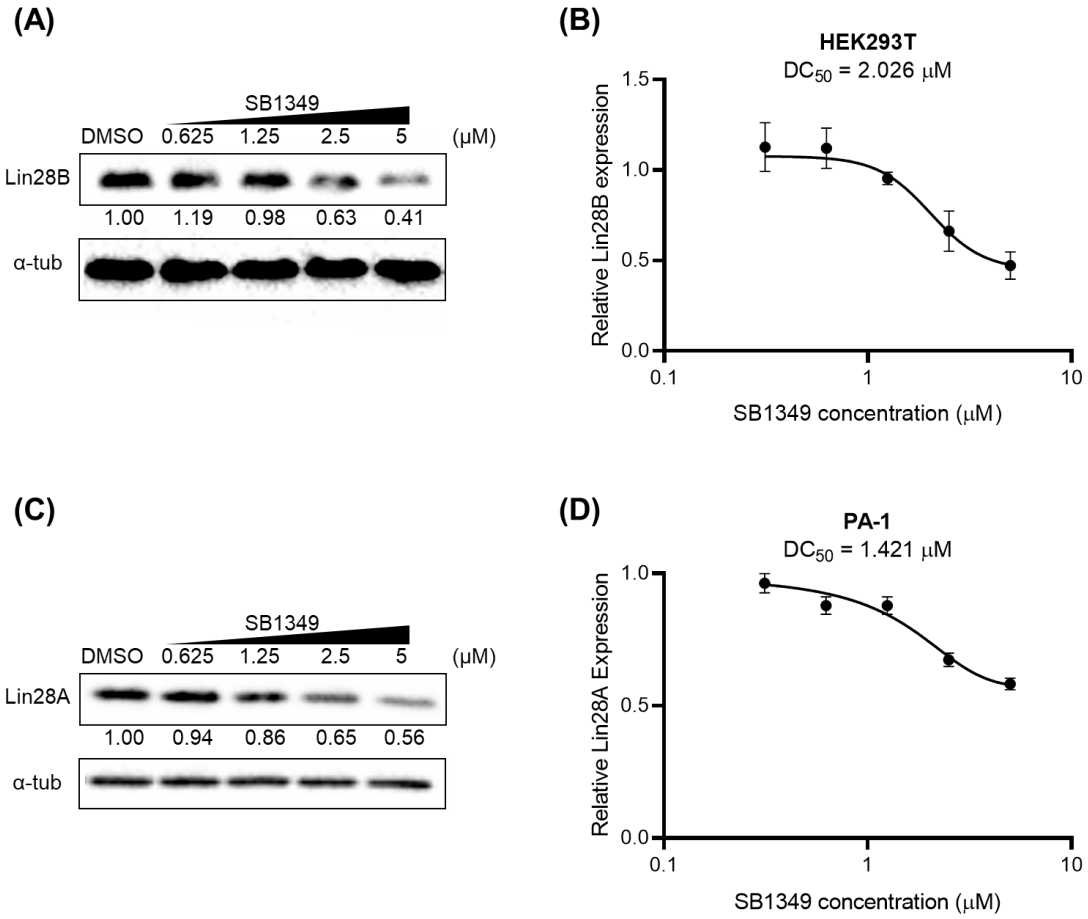



**Figure S2.** Dose-dependent degradation of Lin28 in HEK293T and PA-1 cells upon SB1349 treatment. (A, B) Representative western blot images and quantification of Lin28B degradation in HEK293T cell following SB1349 treatment. (C, D) Representative western blot images and quantification of Lin28A degradation in PA-1 cell following SB1349 treatment.

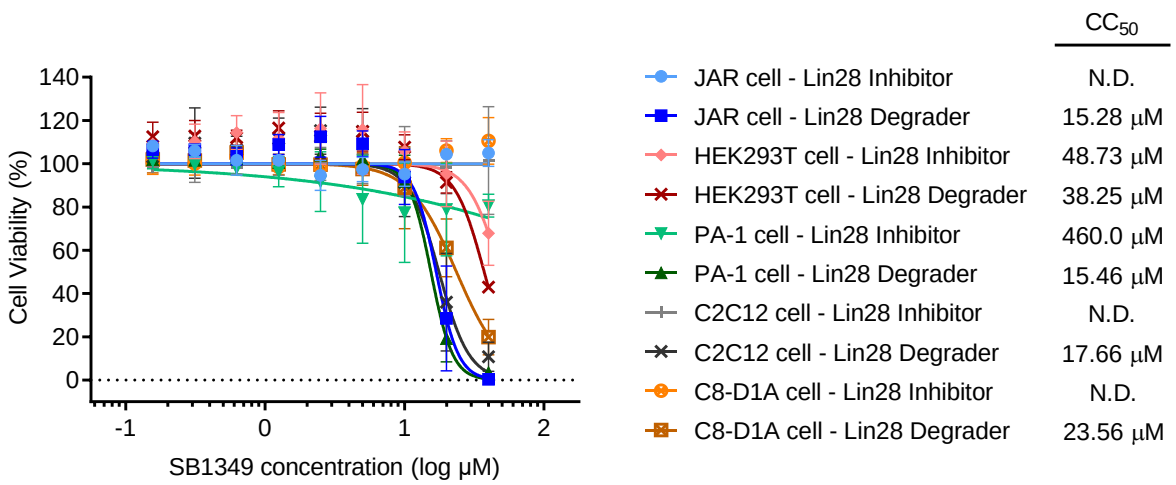



**Figure S3.** WST-based cell viability data of JAR, HEK293T, PA-1, C2C12, and C8-D1A cells after 24 h treatment with SB1349.

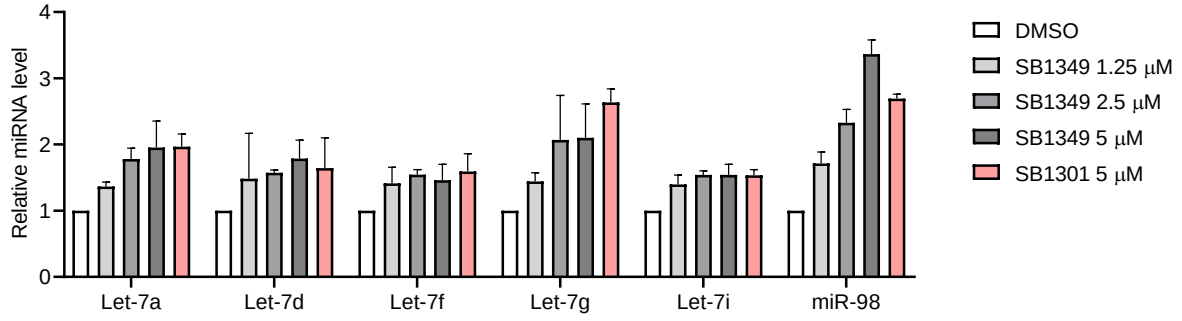



**Figure S4.** Quantification of mature let-7 miRNA by TaqMan assay in a dose-dependent manner. SB1349 and SB1301 increase the level of let-7 family genes in JAR cells.

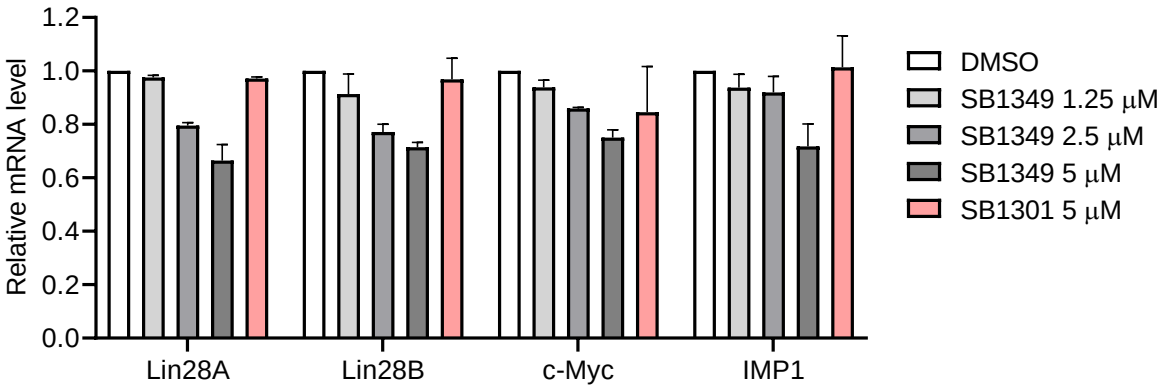



**Figure S5.** Quantification data of qRT-PCRs for let-7 target genes.

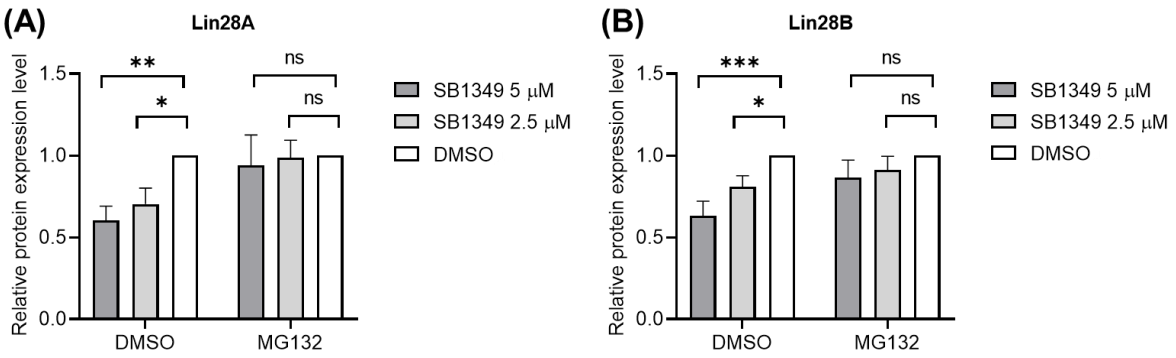



**Figure S6.** Quantification data of western blots for (A) Lin28A and (B) Lin28B in the presence or absence of SB1349 at two different concentrations.

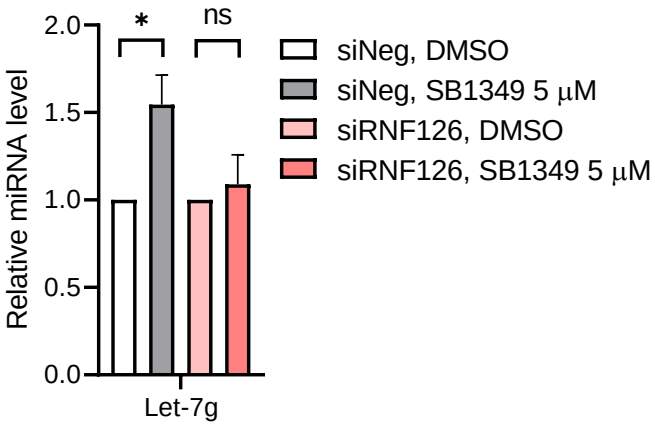



**Figure S7.** The enhanced cellular levels of mature let-7g were significantly decreased in RNF126-knocked down JAR cells. Each siRNA knockdown set was normalized to the DMSO treatment condition.

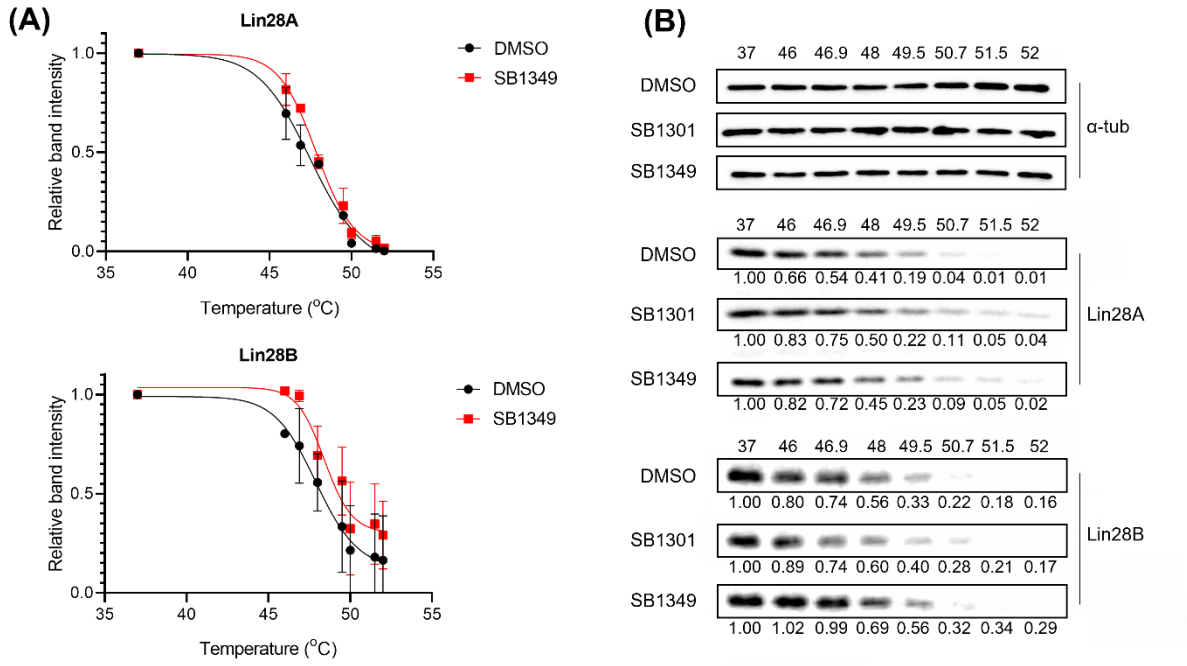



**Figure S8.** Cellular thermal stability shift assay (CETSA) of SB1301 and SB1349 in JAR cell. (A) Quantification data for CETSA of SB1349. Both Lin28A and Lin28B are thermally stabilized upon SB1349 treatment. (B) Western blot images of CETSA with SB1301 and SB1349.

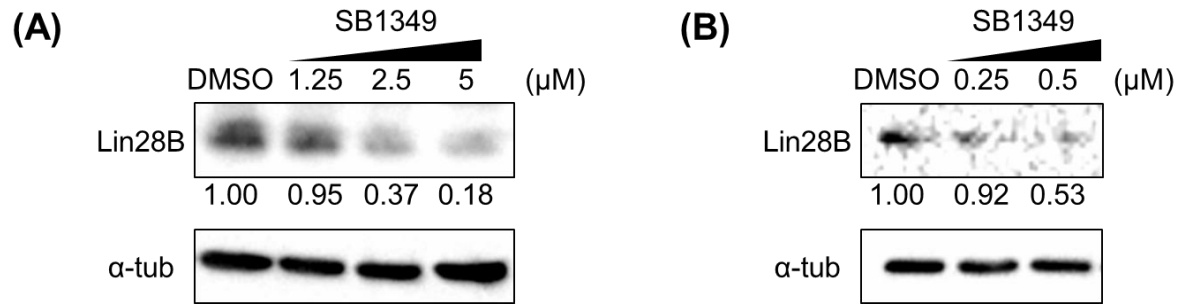



**Figure S9.** Dose-dependent degradation of Lin28B in SH-SY5Y cells. (A) After 24 h treatment with SB1349 in Advanced DMEM/F12 supplemented with 10% (v/v) FBS, and (B) after 72 h treatment with SB1349 in Advanced DMEM/F12 supplemented with 1% (v/v) FBS. SB1349 reduced Lin28B levels in a dose-dependent manner.

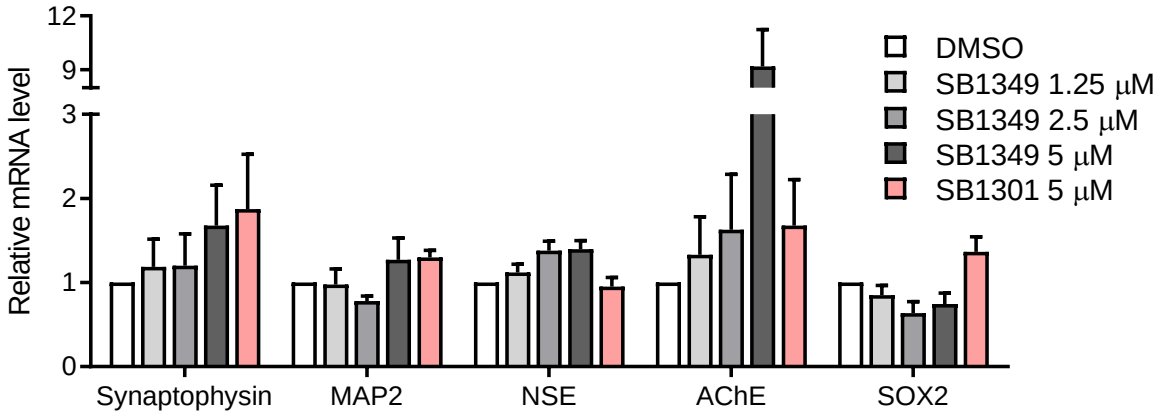



**Figure S10.** qRT-PCR measurement of mRNA markers for cell stemness and neuronal differentiation in SH-SY5Y cells after 24-h treatment with either SB1349 or SB1301 at two different concentrations.

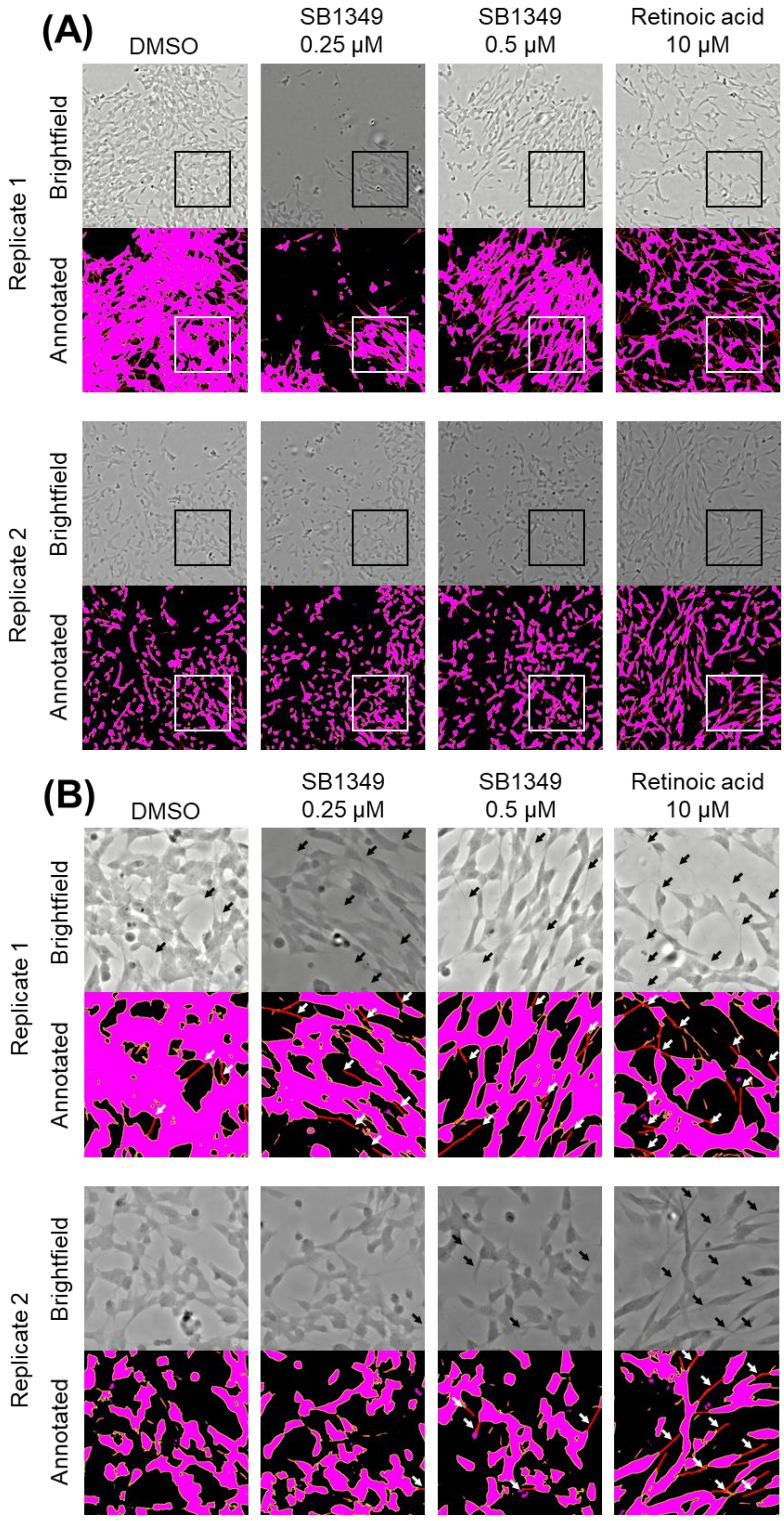



**Figure S11.** (A, B) Representative images from two independent biological replicates and their enlarged images. Fields with the median neurite length and number per cell were selected. Brightfield images were analyzed using NeuroQuantify software (segmented cells in pink; analyzed neurites in red with arrows).

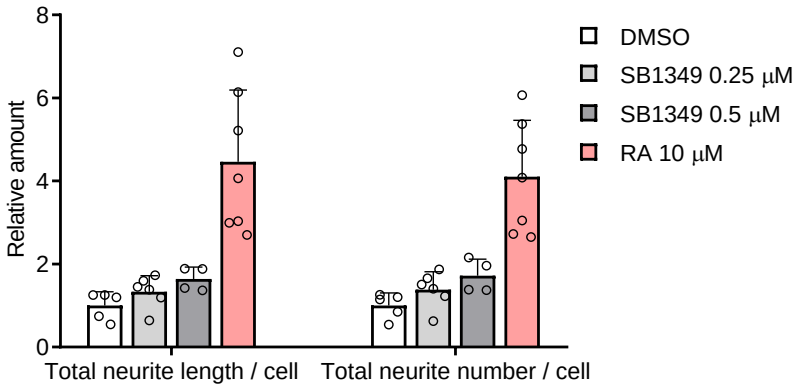



**Figure S12.** Neurite number and length of SB1349 0.5 μM, SB1349 0.25 μM, retinoic acid as a positive control, or DMSO as a vehicle. Total neurite length is calculated as (Average neurite length) × (Total neurite number). Brightfield images were analyzed using NeuroQuantify software.

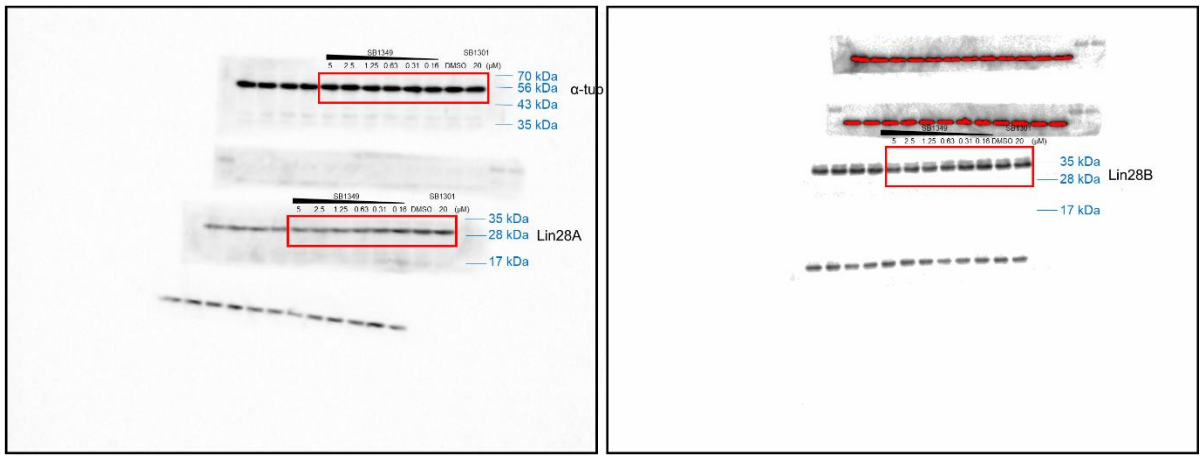



**Figure S13.** Uncropped images of Fig. 2A.

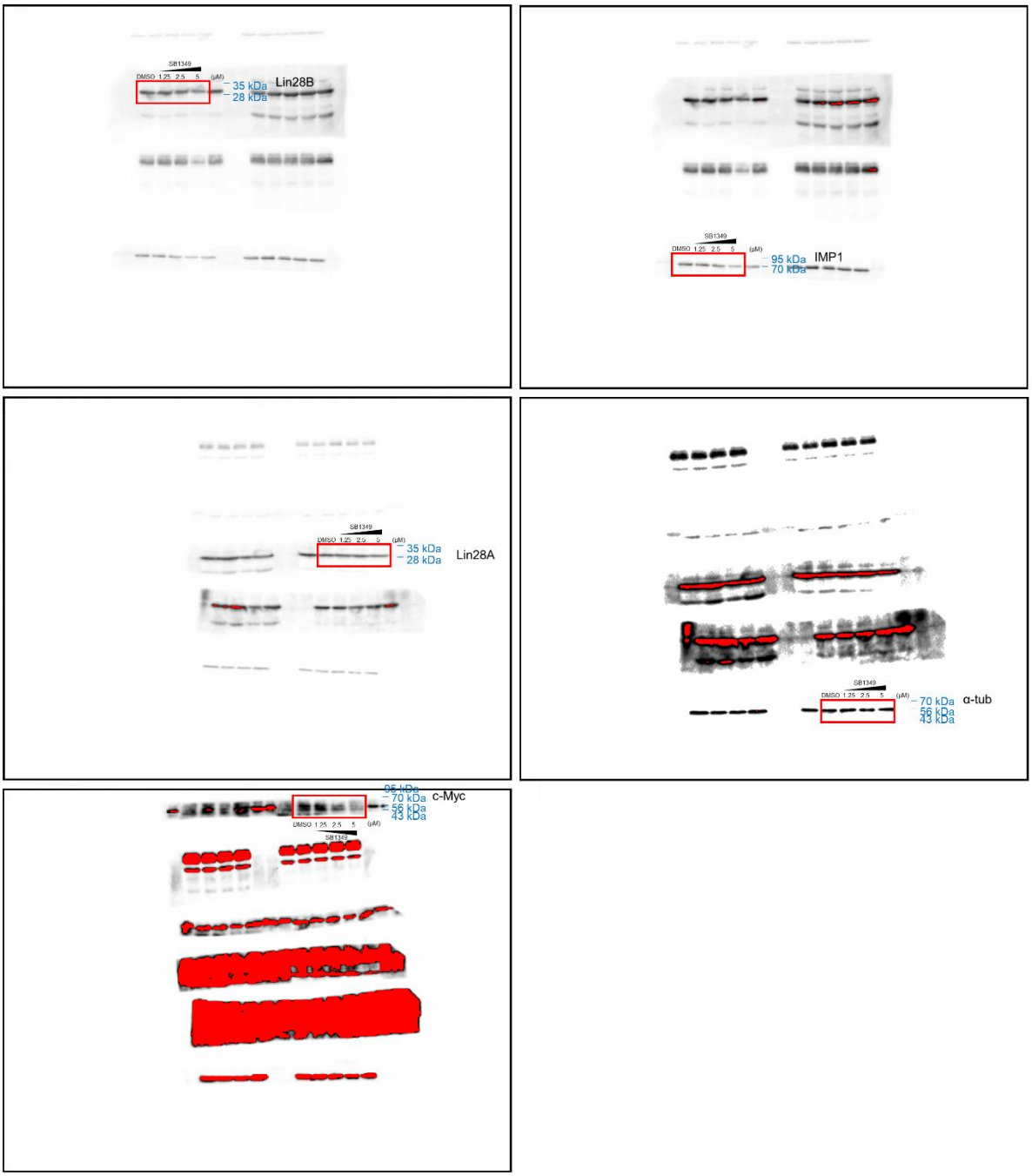



**Figure S14.** Uncropped images of Fig. 2E.

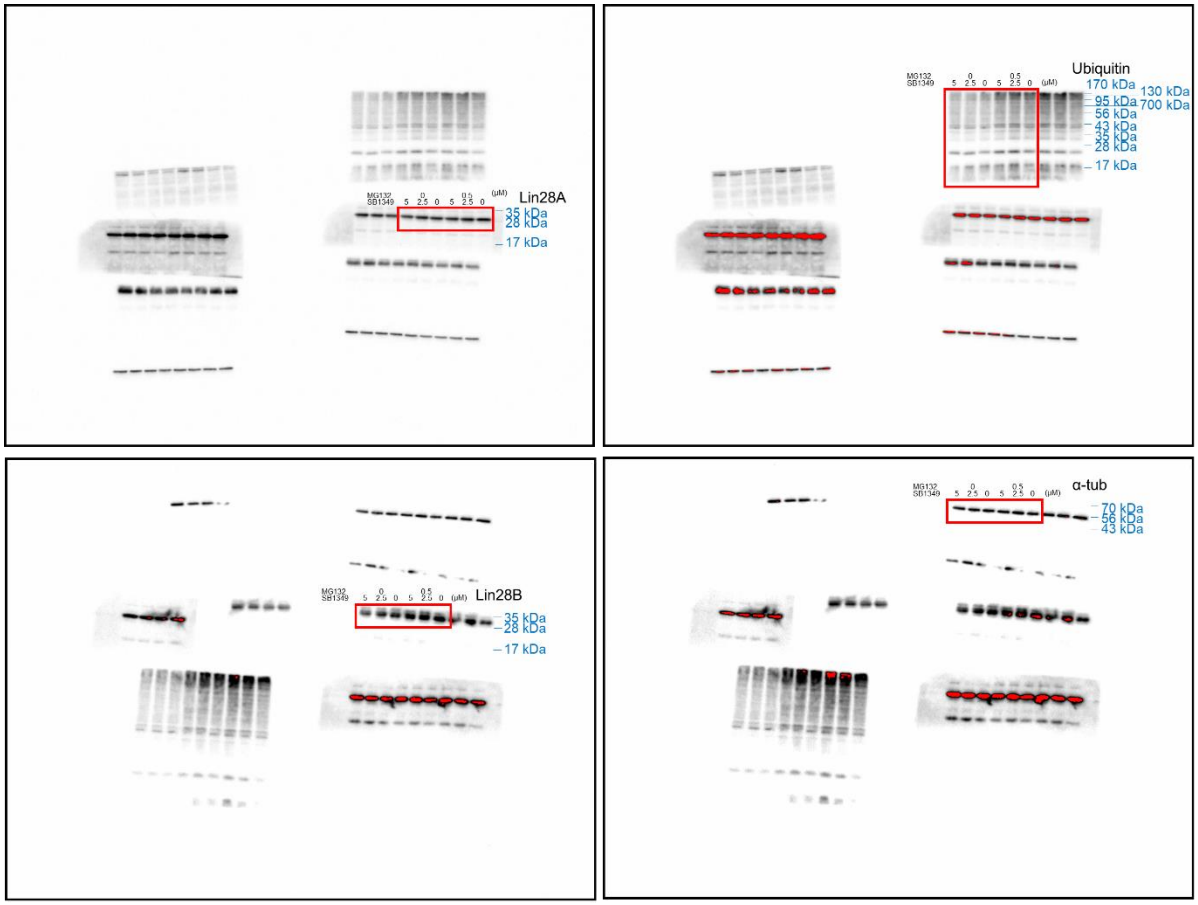



**Figure S15.** Uncropped images of Fig. 3A.

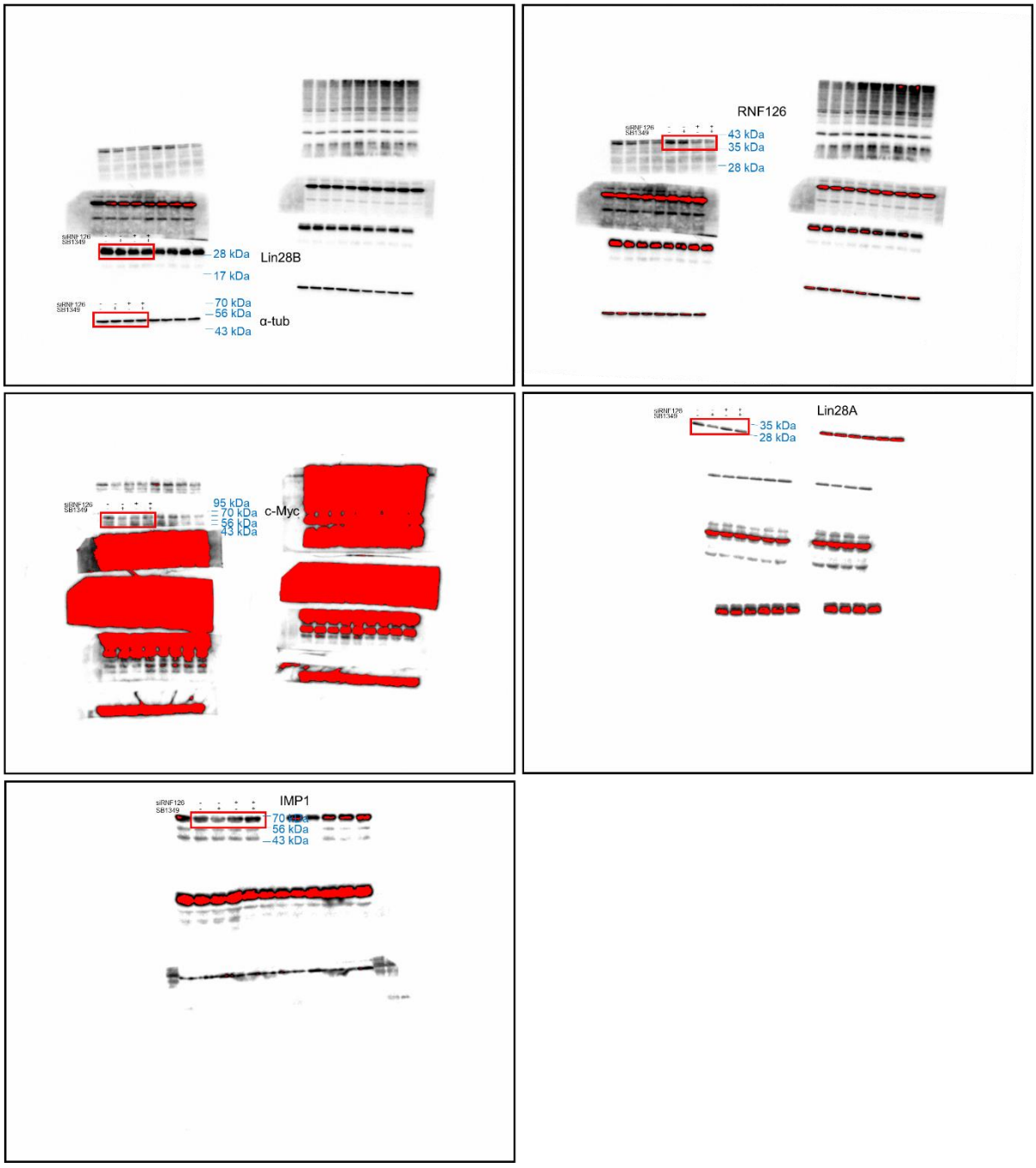



**Figure S16.** Uncropped images of Fig. 3B.

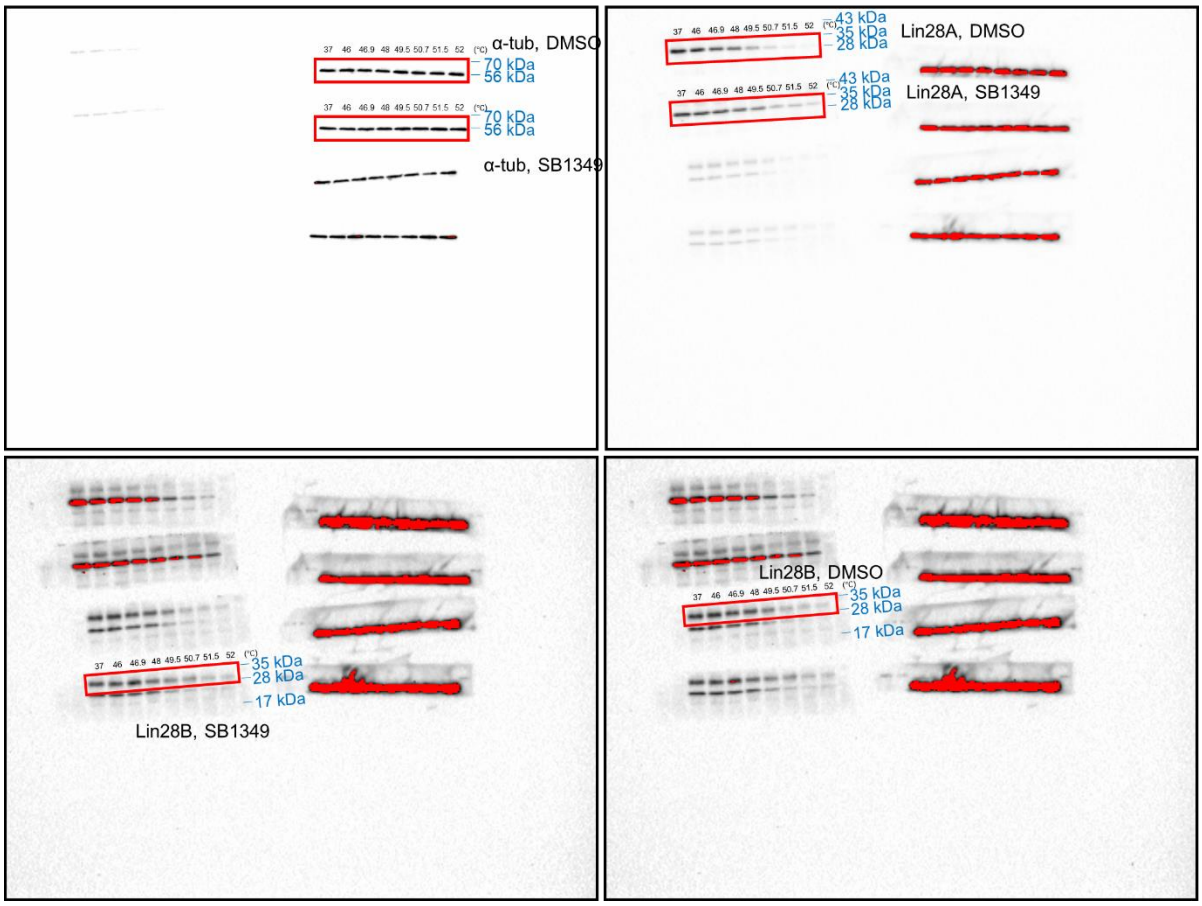



**Figure S17.** Uncropped images of Fig. 3E.

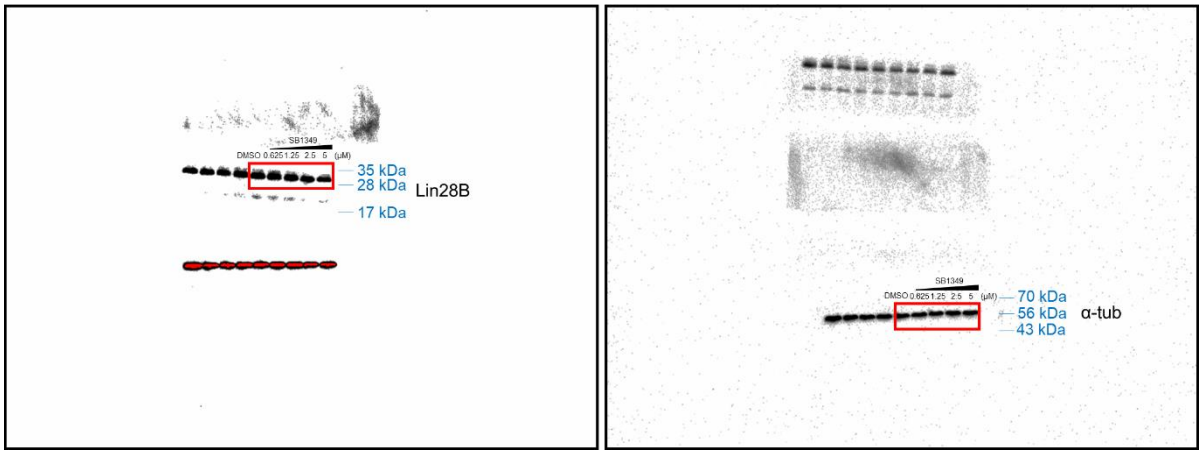



**Figure S18.** Uncropped images of Fig. S2A.

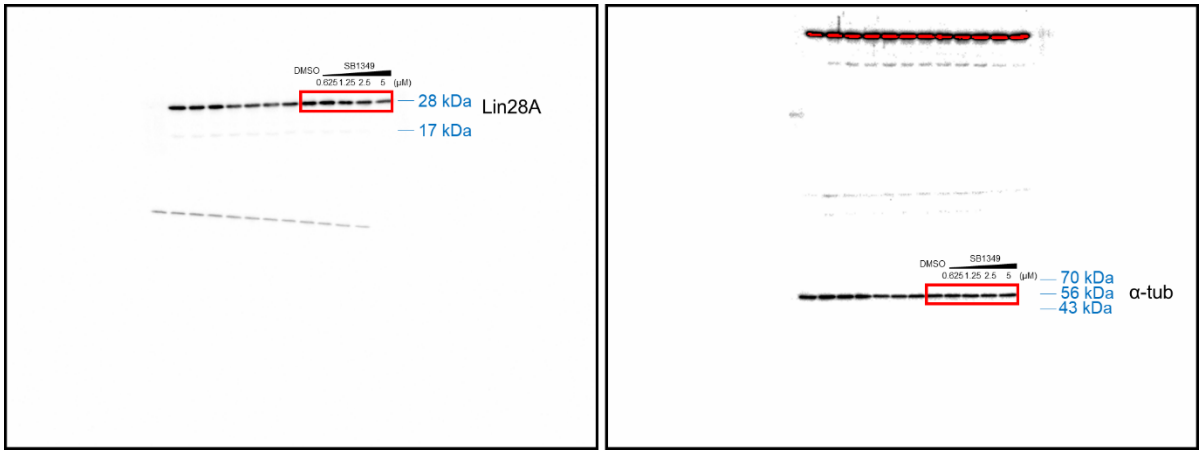



**Figure S19.** Uncropped images of Fig. S2C.

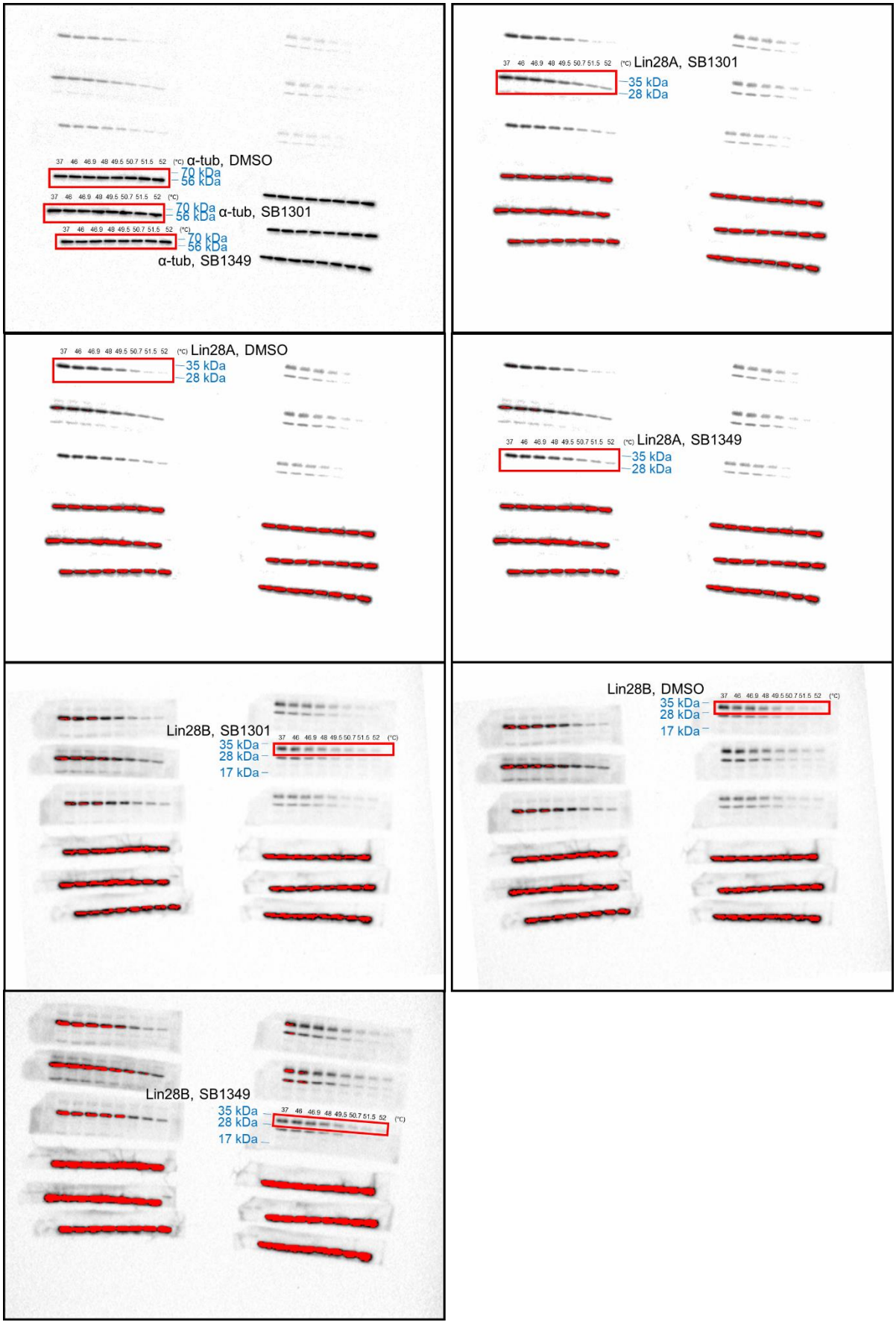



**Figure S20.** Uncropped images of Fig. S8B.

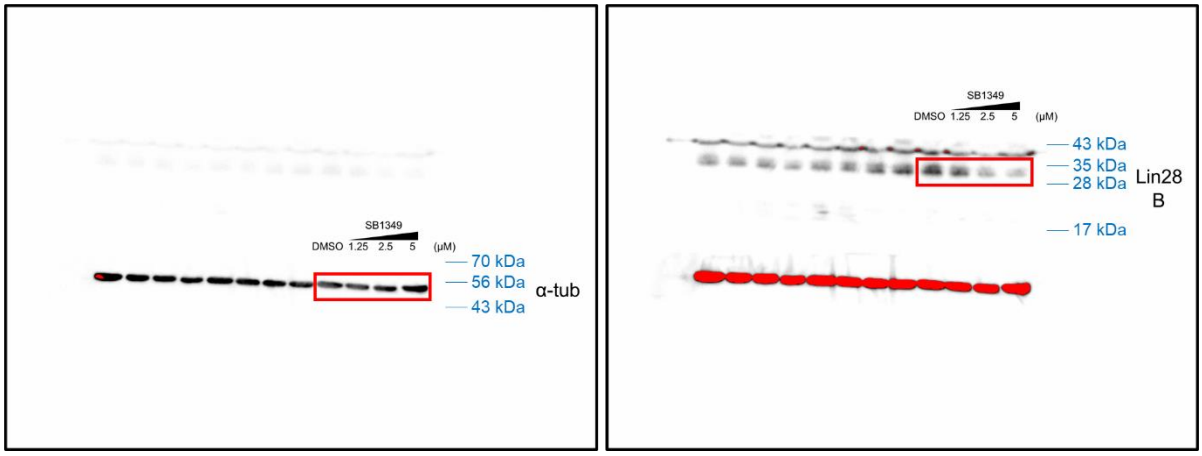



**Figure S21.** Uncropped images of Fig. S9A.

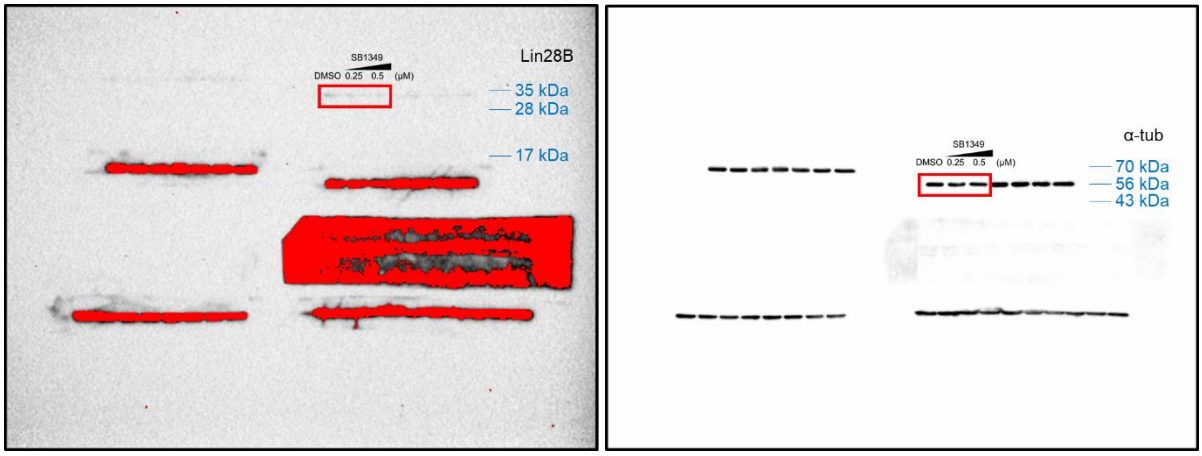
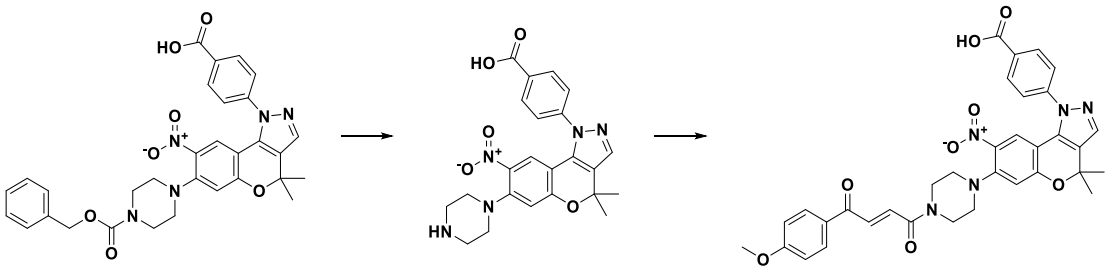



**Figure S22.** Uncropped images of Fig. S9B.

## C. Synthetic Procedures and Spectroscopic Data



**S1** **S2** **1**

The synthesis of the starting material **S1** was previously reported.[2] To a solution **S1** (19.30 mg, 0.033 mmol, 1.0 equiv.) in tetrahydrofuran (0.45 mL) and ethanol (0.45 mL) was added potassium hydroxide (18.69 mg, 0.333 mmol, 10 equiv.) 40% in water, then stirred at 100 °C for 8 h. After completion of the reaction checked by LC-MS, the reaction mixture was quenched with trifluoroacetic acid and the residue was removed by azeotropic evaporation with toluene three times. The resulting product **S2** was used directly for the following reaction without further purification. LRMS (ESI) *m/z* calcd for $C_{23}H_{24}N_5O_5$ $[M+H]^+$: 450.2, found: 450.1. To a solution of *trans*-3-(4-methoxybenzoyl)acrylic acid (10.30 mg, 0.050 mmol, 1.5 equiv.) and 1-hydroxybenzotriazole hydrate (7.65 mg, 0.050 mmol, 1.5 equiv.) in dry *N,N*-dimethylformamide (0.10 mL) was added *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (12.77 mg, 0.066 mmol, 2.0 equiv.). After being stirred at r.t. for 1 h, crude **S2** (<0.033 mmol) in dry *N,N*-dimethylformamide (0.10 mL) and 1,8-diazabicyclo[5.4.0]undec-7-ene (9.94 μL, 0.066 mmol, 2.0 equiv.) were added and left stirred at r.t. for 1 h. After completion of the reaction checked by TLC and LC-MS, the solvent was removed by azeotropic evaporation with toluene three times. The residue was purified by reverse-phase preparative HPLC (10% acetonitrile in water with 0.1% trifluoroacetic acid gradient to 75%) to afford **1** (6.54 mg, 10.3 μmol, 31% as 2-step yield) as a yellow solid. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 13.27 (s, 1H), 8.13 (d, $J$ = 8.4 Hz, 2H), 8.05 (d, $J$ = 8.6 Hz, 2H), 7.83 (s, 1H), 7.80 (d, $J$ = 15.2 Hz, 1H), 7.70 (d, $J$ = 8.2 Hz, 2H), 7.52 (s, 1H), 7.42 (d, $J$ = 15.2 Hz, 1H), 7.10 (d, $J$ = 8.8 Hz, 2H), 6.81 (s, 1H), 3.87 (s, 3H), 3.76–3.68 (m, 4H), 3.15–3.09 (m, 4H), 1.67 (s, 6H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 187.55, 166.51, 163.75, 157.30, 147.86, 142.79, 135.60, 133.51, 133.50, 132.62, 131.20, 130.69, 129.81, 129.45, 125.81, 122.78, 121.09, 114.32, 108.52, 107.24, 79.45, 55.67, 50.94, 50.20, 45.19, 41.39, 40.02, 39.85, 39.69, 39.52, 39.35, 39.19, 39.02, 28.86; HRMS (ESI) *m/z* calcd for $C_{34}H_{32}N_5O_8$ $[M+H]^+$: 638.2245, found: 638.2251, Δppm +0.94.

[2] Lim, D., Byun, W. G., Koo, J. Y., Park, H. & Park, S. B. Discovery of a Small-Molecule Inhibitor of Protein–MicroRNA Interaction Using Binding Assay with a Site-Specifically Labeled Lin28. *J. Am. Chem. Soc.* **138**, 13630–13638 (2016).

# $^{1}H$ and $^{13}C$ NMR Spectra