Covalent PROTAC design method based on sulfonyl pyridone probe

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Supplementary Figures.

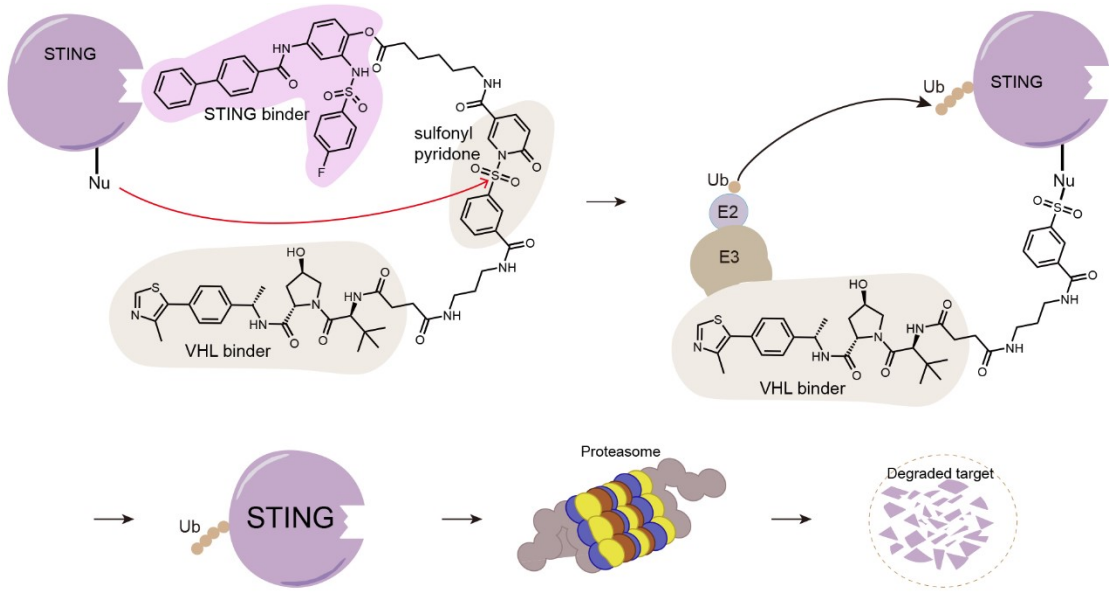


Fig. S1. Proposed model of SD02.

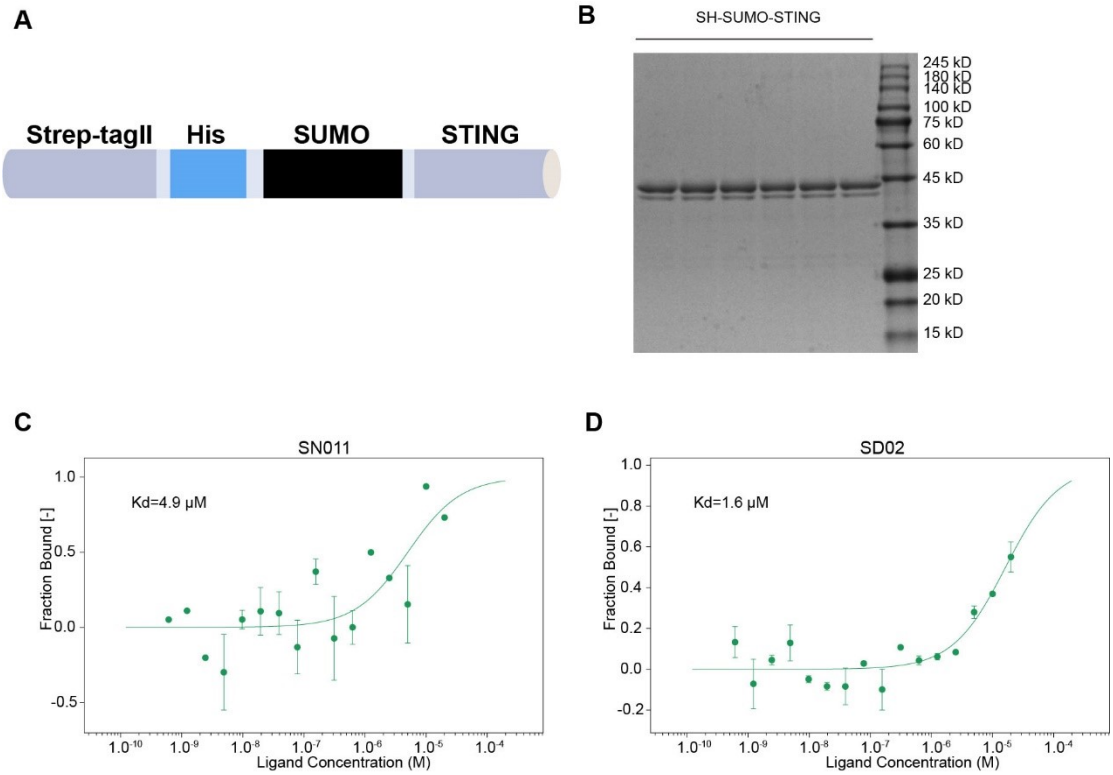


Fig. S2. (A) Domain structure of the SH-SUMO-STING fusion protein with structural features labeled: Strep-tagII, His tag, SUMO tag, and STING. (B) SDS-PAGE analysis for purified SH-SUMO-STING protein. (C-D) Individual MST Fitted binding curves obtained from MST data, describing the interaction between STING and (C) SN011, (D) SD02.

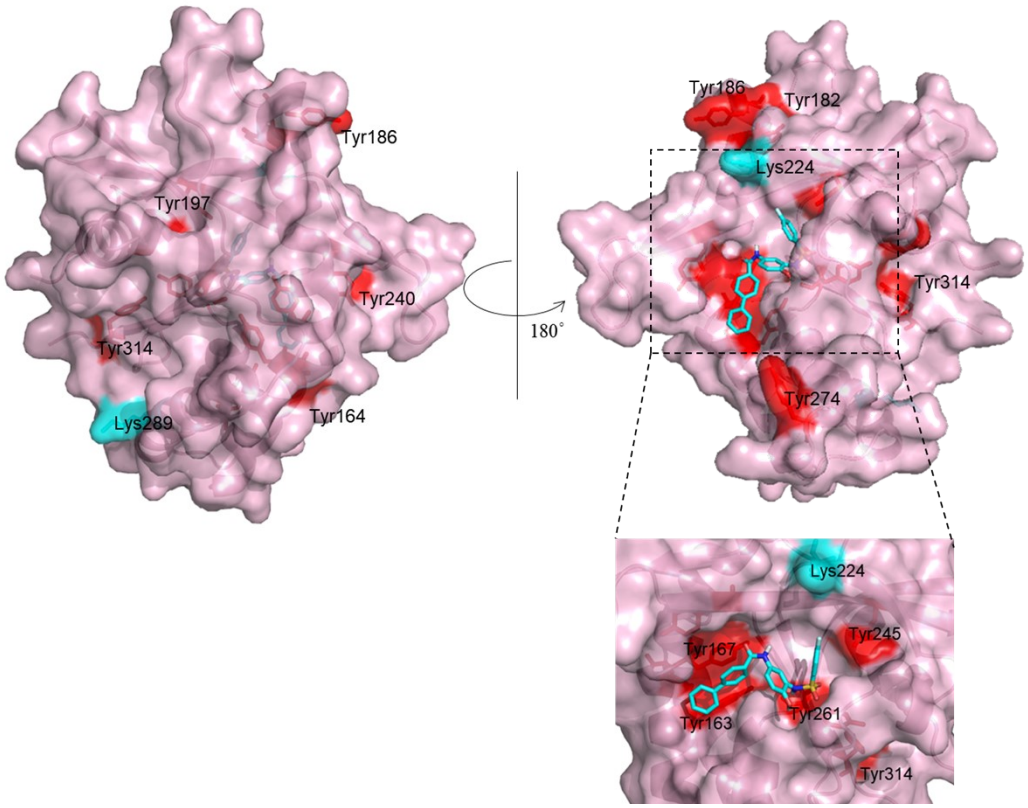


Fig. S3. STING structure and nucleophilic amino acid exposed on the surface. Exposed lysine (Lys 224 and Lys 289) were labeled as cyan sticks, and exposed tyrosine (Tyr 163, Tyr 164, Tyr 167, Tyr 182, Tyr 186, Tyr 197, Tyr 240, Tyr 245, Tyr 261, Tyr 274, Tyr 314) were labeled as red sticks.

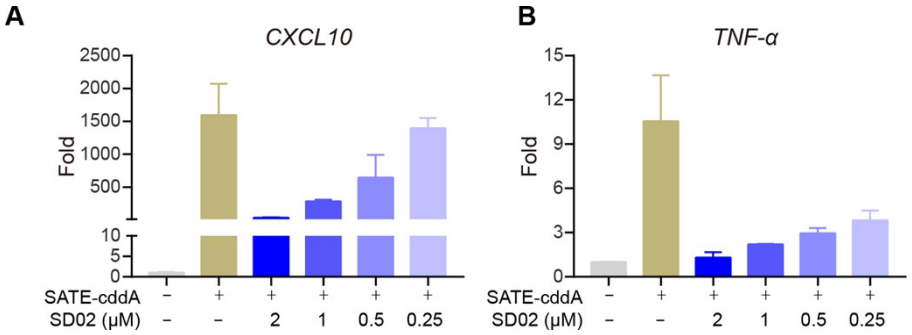


Fig. S4. (A-B) Real-time PCR was used to measure the mRNA expression levels of CXCL10 and TNF-α, which were then normalized to actin expression. THP1 cells were exposed to increasing doses of SD02 for 24 hours followed by treatment with 25 nM SATE-3',3'-c-di-dAMP for 4 hours.

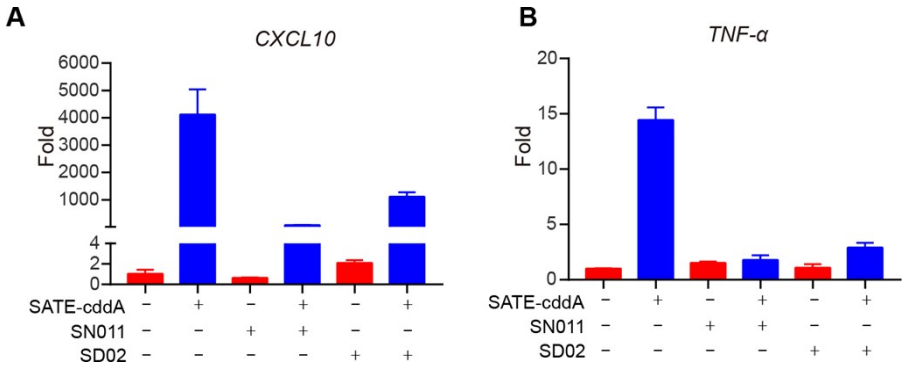


Fig. S5. (A-B) mRNA expression levels of CXCL10, and TNF-α were analyzed by real-time PCR in THP1 cells. Cells were treated with 2 μM SN011 or 2 μM SD02 for 24 hours, followed by stimulation with 25 nM SATE-3',3'-c-di-dAMP for 4 hours. The mRNA levels were quantified and normalized to actin expression.

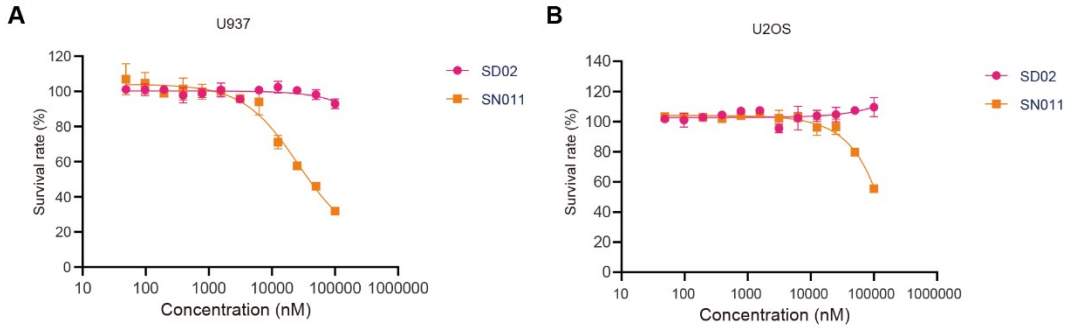


Fig. S6. (A-B) Cell growth inhibition effects were determined using a CCK8 assay in U937 and U2OS cells treated with increasing doses of SD02 or SN011 for 24 hours.

Reagents, cells, and antibodies

The VHL ligand 2 compound (CAS: 1948273-03-7) was obtained from Jiangsu Aikon Biopharmaceutical, while the SATE-3',3'-c-di-dAMP was generously provided by Dr. Junmin Quan from the School of Chemical Biology and Biotechnology at Peking University in Shenzhen, China. The Quanti-Luc reagent (cat. rep-qlc1) was purchased from InvivoGen (San Diego, CA, USA). THP1 cells were obtained from ATCC and THP1-Lucia™ cells were purchased from InvivoGen (cat. #thpl-isg). The cells were cultured in RPMI1640 supplemented with 10% FBS and 0.1% Normocin (InvivoGen, cat. #ant-ar-1) under standard conditions of 37 °C with 5% CO2.

For immunoblotting, the following antibodies were used: anti-STING antibody (Cell Signaling Technology, cat. 13647S), anti-DDDDK-tag antibody (Proteintech, cat. 20543-I-AP), anti-HA-tag antibody (Abcam, cat. ab9110), anti-Tubulin antibody (ProteinTech, cat. HC101), anti-Protein A-HRP (Cell Signaling Technology, cat. 12991), anti-phosphorylated IRF3 (Ser396) (Cell Signaling Technology, cat. 29047), anti-phosphorylated TBK1 (Ser172) (Cell Signaling Technology, cat. 5483), anti-IRF3 (Cell Signaling Technology, cat. 11904), anti-TBK1 (Cell Signaling Technology, cat. 3504), anti-rabbit IgG (HRP-linked) antibody (Cell Signaling Technology, cat. 7074), and anti-mouse IgG (HRP-linked) antibody (Cell Signaling Technology, cat. 7076). Marker(10-245kD) (Cat:DB245, MIKX Co., Ltd); MG132 (Cat. No.: HY-13259, MedChemExpress (Monmouth Junction, NJ, USA)); Cellsaving (Cat. No.: C40100, New Cell & Molecular Biotech)

Reagents for real-time PCR were acquired from Accurate Biotechnology (Hunan) Co., Ltd, Changsha, China, including AG RNAex Pro Reagent (Catalogue # AG21102), SYBR® Green Premix Pro Taq HS qPCR Kit (Catalogue # AG11701), and Evo M-MLV RT Premix for qPCR (Catalogue #AG11706).

Primer and gene were obtained from GenScript Biotech Corporation. Amino acids were obtained from Nanjing Peptide Biotech.

Chemistry

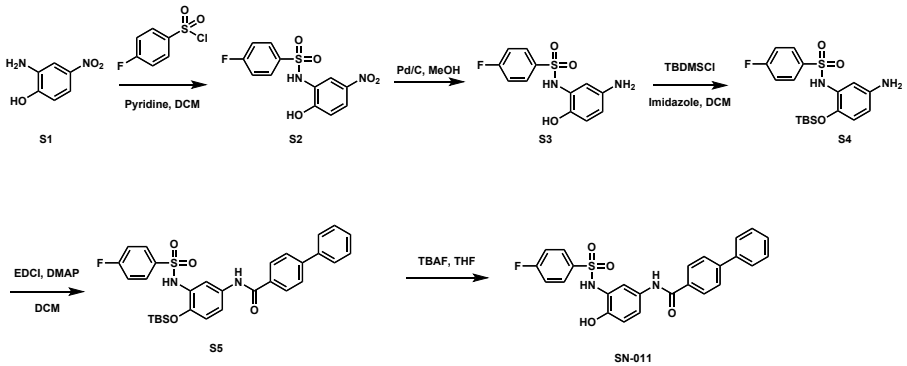
The solution of 4-Fluorobenzenesulfonyl chloride (3.04 g, 15.68 mmol) in DCM (20 mL) was added into the solution of 2-Amino-4-nitrophenol (2.00 g, 13.0 mmol) and pyridine (1.54 g, 19.5 mmol, 1.57 mL) in DCM (40 mL) dropwise at 0 °C. The reaction mixture was stirred for 12 h at room temperature. After the removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (PE：EA = 4：1) to give **S2** (2.7 g, 67%) as a yellow solid.

The solution of compound **S2** (1.5 g, 4.8 mmol) in MeOH (15 mL) was added with 10% Pd/C (0.1 g). The reaction mixture was stirred for 12 h at room temperature under H_2. After filling the solvent with Celite. After the removal of the solvent by evaporation, the residue gives **S3** (1.4 g) as a yellow solid.

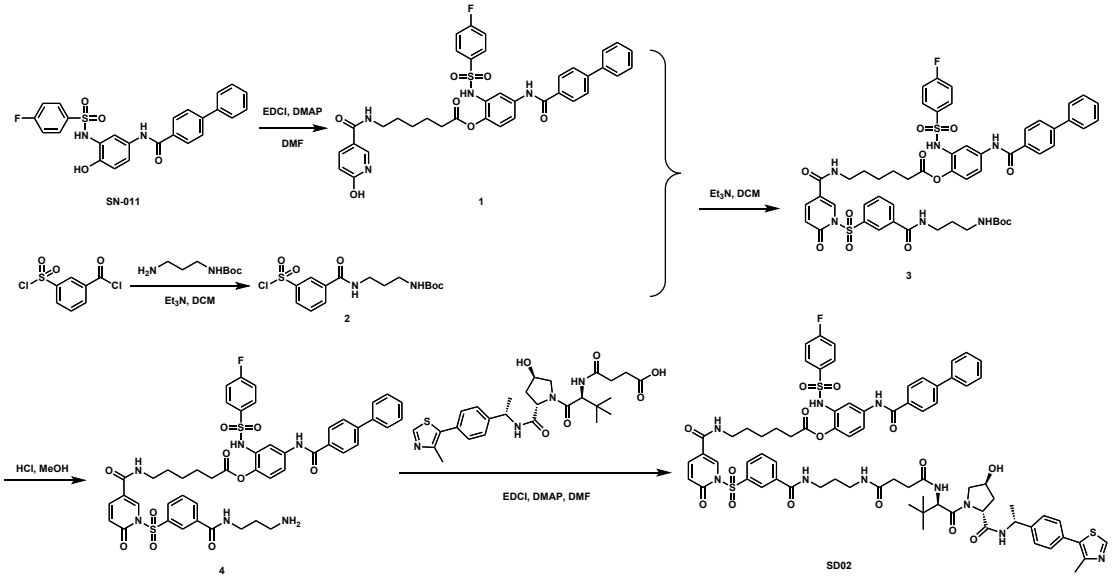
The solution of compound **S3** (1.98 g) and imidazole (458 mg) in DCM (20 mL) was added dropwise with the solution of TBSCl (1.0 g) in DCM (2 mL) at 0 °C. The reaction mixture was stirred for 12 h at room temperature. After the removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (DCM:MeOH = 10:1) to give **S4** (2.3 g, 83%) as a red solid.

Compound **S4** (740 mg) was added to the solution of 4-biphenylcarboxylic acid (370 mg) at 0 °C. The reaction mixture was stirred for 12 h at room temperature. After the removal of the solvent by evaporation, the crude product **S5** appeared as a red solid.

To the solution of compound **S5** (630 mg) in DCM (15 mL) was added dropwise the solution of TBAF (1 mol/L) in THF (1.2 mL) at 0 °C. The reaction mixture was stirred for 4 h at room temperature. After the removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (PE：EA = 5：1) to give **SN-011** (250 mg, 50%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.13 (s, 1H), 9.39 (d, J = 32.7 Hz, 2H), 8.08 – 7.99 (m, 2H), 7.87 – 7.79 (m, 4H), 7.78 – 7.69 (m, 4H), 7.51 (dd, J = 8.3, 6.8 Hz, 2H), 7.47 – 7.31 (m, 4H), 6.70 (d, J = 7.7 Hz, 1H).



Scheme 1.



Scheme 2.

To the solution of 6-[(6-oxo1H-pyridine-3-carbonyl)amino]hexanoic acid (500 mg), EDCI (460 mg), and DAMP (40 mg) in DMF (10 mL) was added compound **SN-011** (925 mg) at 0 °C. The reaction mixture was stirred for 12 h at room temperature. After the removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (DCM：MeOH = 50：1) to collect **1** (1.2 g, 86%) as a white solid.

To the solution of 3-(chlorosulfonyl)benzoyl chloride (574 mg) and TEA (560 μL) mixed in DCM (10 mL) was added dropwise the solution of tert-Butyl (3-aminopropyl)carbamate (418 mg) in DCM (10 mL) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C. After the removal of the solvent by evaporation, the residue was purified by flash column chromatography on SiO_2 (DCM：EA = 8：1) to collect **2** (868 mg, 96%) as a colorless oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.60 (s, 1H), 8.27 (dt, *J* = 7.8, 1.3 Hz, 1H), 8.19 – 8.08 (m, 1H), 7.72 (t, *J* = 7.9 Hz, 1H), 4.89 (s, 1H), 3.54 (q, *J* = 6.0 Hz, 2H), 3.28 (q, *J* = 6.1 Hz, 2H), 1.82 – 1.65 (m, 2H), 1.46 (s, 9H).

To the solution of compound **7** (50 mg) and TEA (200 μL, 1.44 mmol) in DCM (2 mL) and DMF (0.5 mL) was added the solution of compound **2** (108 mg, 0.29 mmol) in DCM (2 mL). The reaction mixture was stirred for 2 h at room temperature. After the removal of the solvent by evaporation, the residue was purified by HPLC to collect **3** (26 mg, 35%) as a brown solid.

The solution of compound **3** (26 mg) in MeOH (1 mL) was added dropwise to the solution of HCl (4 mol/L) in MeOH (5 mL) at 0 °C. The reaction mixture was stirred for 6 h at room temperature. After the removal of the solvent by evaporation, the residue was purified by HPLC to collect **4** (25 mg) as a brown solid.

To the solution of compound **4** (15 mg), EDCI (3.7 mg), and DAMP (0.4 mg) in DMF (1 mL) was added VHL-Ligand (15 mg) at 0 °C. The reaction mixture was stirred for 12 h at room temperature. After the removal of the solvent by evaporation, the residue was purified by HPLC to collect **SD02** (5.4 mg, 23%) as a brown solid. HRMS (ESI-TOF) Calcd. for [M+H] $C_{74}H_{79}FN_{10}O_{15}S_3$, 1463.49453 found 1463.49243.

SD02 was obtained after HPLC purification and freeze drying.^{1}H NMR (400 MHz, DMSO-d6) δ 10.67 (d, J = 7.3 Hz, 1H), 8.98 (s, 1H), 8.87 – 8.73 (m, 1H), 8.43 (d, J = 2.0 Hz, 1H), 8.39 – 8.23 (m, 2H), 8.18 (t, J = 5.6 Hz, 1H), 8.12 – 7.93 (m, 9H), 7.90 – 7.72 (m, 10H), 7.51 (td, J = 7.8, 2.7 Hz, 3H), 7.47 – 7.31 (m, 8H), 6.33 (d, J = 9.6 Hz, 1H), 4.90 (dd, J = 7.4, 2.5 Hz, 1H), 4.50 – 4.31 (m, 2H), 4.24 (d, J = 4.5 Hz, 1H), 3.34 - 3.31 (m, 3H), 3.23 – 3.05 (m, 5H), 2.45 (s, 3H), 2.06 – 1.93 (m, 3H), 1.70 (ddt, J = 18.9, 12.2, 5.9 Hz, 3H), 1.56 – 1.43 (m, 5H), 1.35 (dd, J = 6.9, 3.8 Hz, 3H), 1.31 – 1.18 (m, 3H), 0.87 (s, 9H).

Protein expression and purification

The SUMO-STING protein was expressed using *E. coli* BL21(DE3)pLyS strains. Plasmid-transfected E. coli strains were coated on K^+ and C^+ LB agar plates and incubated at 37°C overnight. Monoclones were selected and transferred to K^+ and C^+ LB liquid medium and grown in a shaker. When the OD_{600} of medium reached 0.4~0.6, the shaker was cooled to 18°C. Then 0.3 mM IPTG was added and the bacterial solution continued shaking at 18°C and 160 rpm for 24 h. Thalli was obtained by centrifugation at 6,500 rcf for 10 minutes at 4°C and stored at -20°C for subsequent use.

To extract the protein, the harvested cells were resuspended in lysis buffer (25 mM HEPES, 250 mM NaCl, 5% glycerol, pH 8.0) supplemented with 1 mg/mL DNAase, 5 mM $MgCl_2$, and 1 mM PMSF. The solution was then sonicated for 30 minutes, followed by centrifugation at 40,000 rcf for 30 minutes at 4°C. The supernatant was collected after filtration through a 0.45 μm membrane. The protein was purified using a Ni-affinity column, followed by desalting. The desalted protein was then subjected to a second round of purification using a STREP-TACTIN XT packed column. The eluate contained the purified SUMO-STING proteins, which were stored at -80°C for subsequent experiments. All purification steps were carried out at 4°C, and the eluted proteins were analyzed by SDS-PAGE.

The amino acid sequence of the purified SUMO-STING protein is as follows:

MNWSHPQFEKSSGSSGGGTHHHHHHGGSGGSGLQMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKI
KKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGENLYFQGAPAEISAVCE
KGNFNVAHGLAWSYYIGYLRLILPELQARIRTYNQHYNNLLRGAVSQRLYILLPLDCGVPDNLSMADPNIRFLD
KLPQQTGDHAGIKDRVYSNSIYELLENGQRAGTCVLEYATPLQTLFAMSQYSQAGFSREDRLEQAKLFCRTLED
ILADAPESQNNCRLIAYQEPADDSSFSLSQEVLRHLRQEEKEEVTVGSLKTSAVPSTSTMSQEPELLISGMEKPLP
LRTDFS.

Western Blotting and Immunoprecipitation

For western blotting, cell lysates were separated in 10% SDS-PAGE gels and subsequently transferred onto the nitrocellulose membranes. The membranes were then blocked with 5% BSA in TBST (Tris-buffered saline with 0.1% Tween-20) before being incubated overnight at 4°C with the indicated primary antibodies. The membranes were washed with TBST buffer three times before being incubated with the appropriate HRP-conjugated secondary antibodies at room temperature for 1 hour. After incubation and washing, the protein bands were exposed by the developer and detected by e-BLOT.

For immunoprecipitation, HEK293T cells were seeded at 4×10^5 cells per well overnight in a 6-well plate. The following day, the cells were transfected with 2 μg of plasmid(s) per well using lipofectamine 3000 (ThermoFisher). After 48 hours, cells were treated with DMSO or 5 μM SD02 for 24 hours, and 5 μM MG132 was added to block the SD02-induced degradation of STING. The cells were then lysed in IP lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 5% glycerol) containing protease and phosphatase inhibitor cocktail and rotated at 4°C for 30 minutes. Lysates were cleared by centrifugation at 12000 rpm for 10 minutes at 4°C to obtain the supernatants. Total protein concentrations were measured using the BCA Protein Colorimetric Assay Kit (Elabscience). A 50-μg supernatant was removed as an input sample. 1 μg of anti-DDDDK-tag antibody (ProteinTech) was added to the supernatants (100 μg) and incubated overnight at 4 °C with rotation. Protein A Sepharose beads (ThermoFisher) were added to the protein complexes for 2 hours with rotation, followed by washing with ice-cold PBS three times. Proteins were eluted in 40 μL SDS loading buffer by boiling for 10 minutes, and the samples were analyzed on SDS-PAGE gels. Gels were subjected to western blotting as described above.

Luciferase assay

THP1 Lucia cells (5-10 × 10^4 cells/well) were seeded into a 96-well cell culture plate. Different concentrations of SN011 or SD02 were prepared in the fresh medium. After 24 hours of incubation with the compound, the cells were treated with 25 nM SATE-3',3'-c-di-dAMP for 12 hours. Then, 10 μL from each well of the 96-well plate was transferred to a 96-well white plate. The luciferase activity of the cells was measured by a microplate reader with autosampler after adding QUANTI-Luc™ reagent to each well.

Tandem Mass Spectrometry (MS/MS)

The buffer of SUMO-STING was replaced with PBS with a desalting column (PD SpinTrap G-25). The protein was then measured by nanodrop to calculate the molarity of the substance. The ratio of SD02 to protein in the experiment was 2:1. The reaction was shaken at room temperature for 24 h. After the reaction, the buffer of the reaction liquid was replaced with H_2O by a desalting column. Then, the sample was separated in 10% SDS-PAGE gels and the gels were dyed with Coomassie Blue R-250 Stain Solution, and decolorized with decolorizing solution (10% acetic acid, 5% ethanol). After that, the target strips on the gels were cut into small pieces and decolorized by decolorization solution (25 mM NH_4HCO_3, 30% ACN) for 4-8 h until the glue blocks were transparent. Then, the glue blocks were incubated with 10 mM DTT/25 mM NH_4HCO_3 at 56℃ for 1 h and 60 mM IAA/25 mM NH_4HCO_3 in the dark at room temperature for 30min. After the reaction, 25 mM NH_4HCO_3 solution and 2 μg Trypsin were added overnight at 37℃. The next day, 0.1% FA water and 0.1% FA acetonitrile were used to collect the supernatant. Then, the supernatant was rotated dry in a centrifugal concentrator at room temperature and used for MS/MS analysis.

Real-time PCR

THP1 Lucia cells were treated with drugs for 24 hours and then incubated with SATE-3',3'-c-di-dAMP for 4 hours. For contrast assays, cells were treated with SN011 (2 μM) and SD02 (2 μM). For dose-dependent assays, cells were treated with varying concentrations of SD02 (0.25 nM, 0.5 nM, 1 μM, 2 μM). After harvesting the cells, RNA was extracted from each sample using RNAex Pro, following the manufacturer's instructions. The RNA was then reversely transcribed into DNA using Evo M-MLV reverse transcription reagent premix. SYBR® Green Pro Taq HS premixed qPCR kit and gene-specific primers were used to amplify DNA. Fluorescence real-time PCR analysis was performed in triplicate using a Q5 fluorescence quantitative PCR instrument. Relative expression was normalized to the levels of Actin. The sequences of qPCR primers required for the assay are as follows: 5′-AGCACTGGCTGGAATGAGAC-3′ and 5′-TTTCGGAGGTAACCTGTAAG-3′ for IFN-β, 5′-TGGCATTCAAGGAGTACCTC-3′ and 5′-TTGTAGCAATGATCTCAACACG-3′ for CXCL10, 5′-CCTCTCTCTAATCAGCCCTCTG-3′ and 5′-GAGGACCTGGGAGTAGATGAG-3′ for TNF-α, 5′-CTGGAACGGTGAAGGTGACA-3′ and 5′-AAGGGACTTCCTGTAACAACGCA -3′ for β-actin.

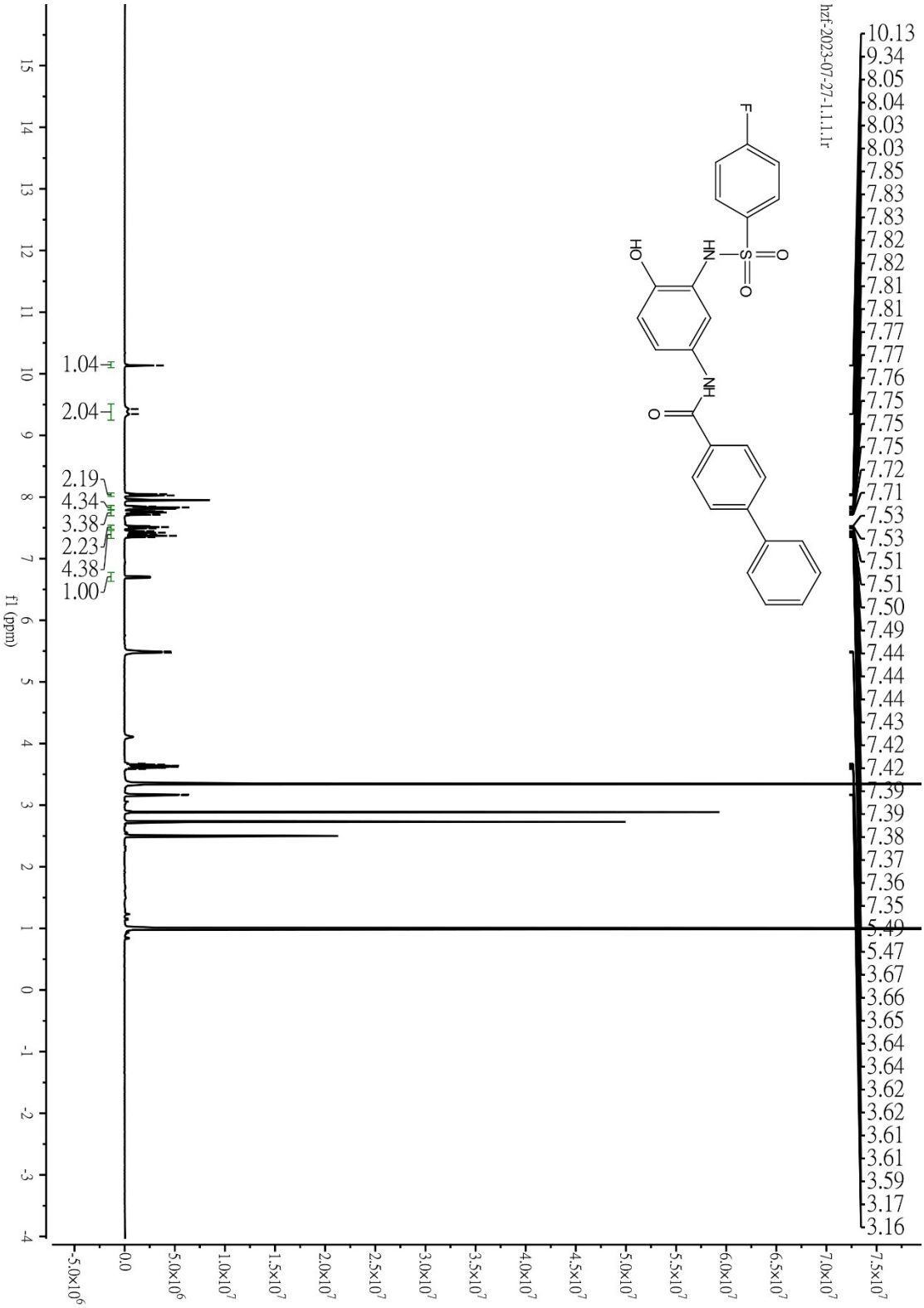
Cell viability assay

THP1 Lucia cells (5-10 × 10^4 cells per well) were seeded into a 96-well cell culture plate. Stock solutions of SN011 or SD02 (10 mM) were diluted in fresh medium to obtain various concentrations, which were added to the cells for a 24-hour incubation period. Following the incubation, CCK-8 reagent (10 μL per well) was added and incubated for another hour. The absorbance of each well was measured at 450 nm using a microplate reader. Similarly, 293T cells (5-10 × 10^3 cells per well) were seeded into a 96-well cell culture plate and subjected to the same procedure.

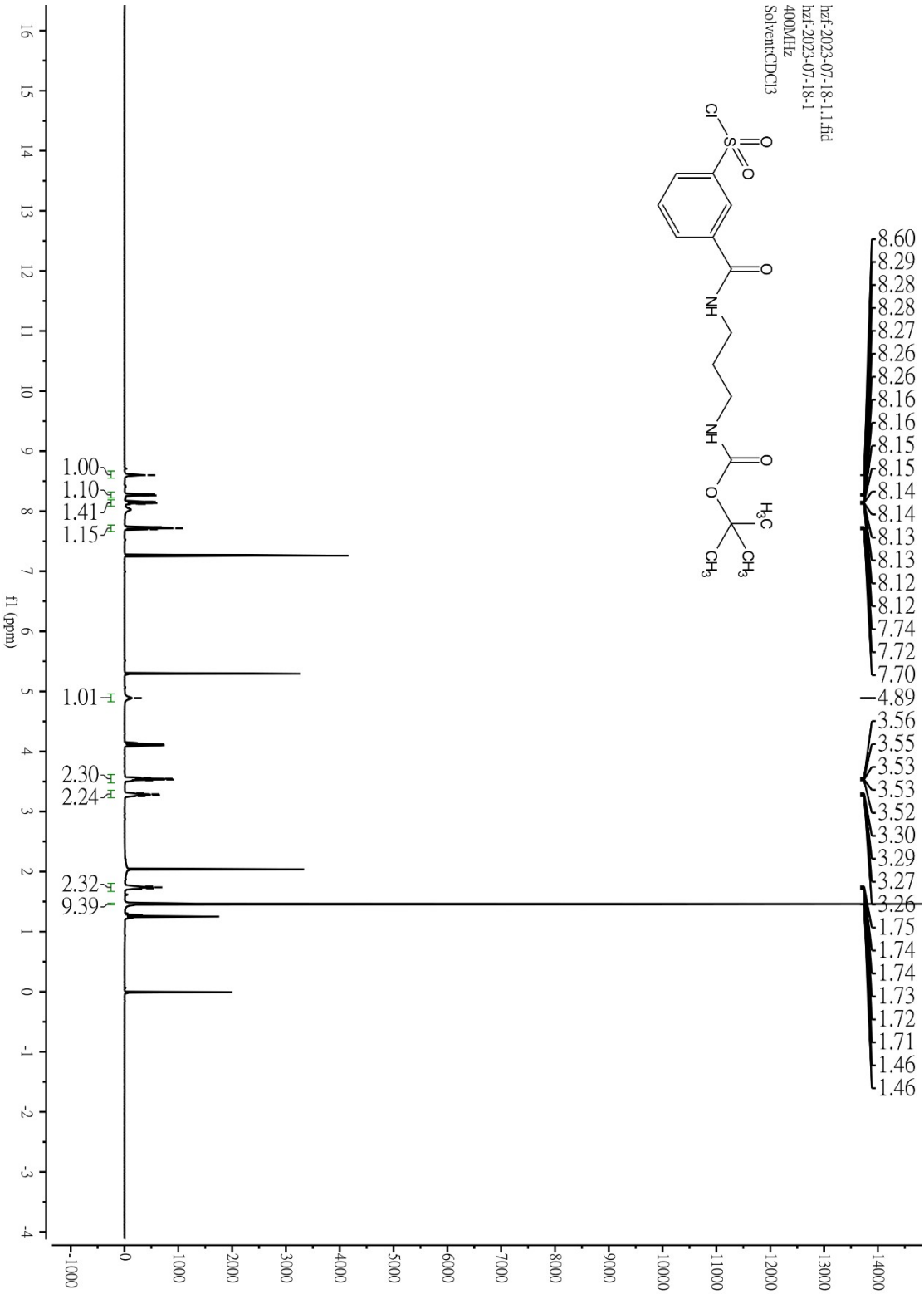
MicroScale Thermophoresis (MST)

The binding affinity of small molecules (SN011 and SD02) with purified SUMO-STING protein was determined using Monolith NT 115. The experiment was performed in triplicate. The SUMO-STING protein sample was labeled with NT-647-NHS dye in PBS and the mixed solution was incubated in the dark at room temperature for 30 minutes. Subsequently, the labeled protein was purified using column B and the excitation was optimized by adjusting the concentration to obtain fluorescence intensities above 200 counts. The labeled sample, at a final concentration of 40 nM, was used in PBS buffer containing 0.05% Tween 20. The MST settings were as follows: drug concentration (20 μM), max MST power (100%), laser on time (20 s), laser off time (1 s), and temperature control (25 °C). Finally, the data were analyzed using the MO. Affinity Analysis software package.

Appendix. NMR, HPLC trace and MS data of SD02 and key intermediates



NMR data of SN-011



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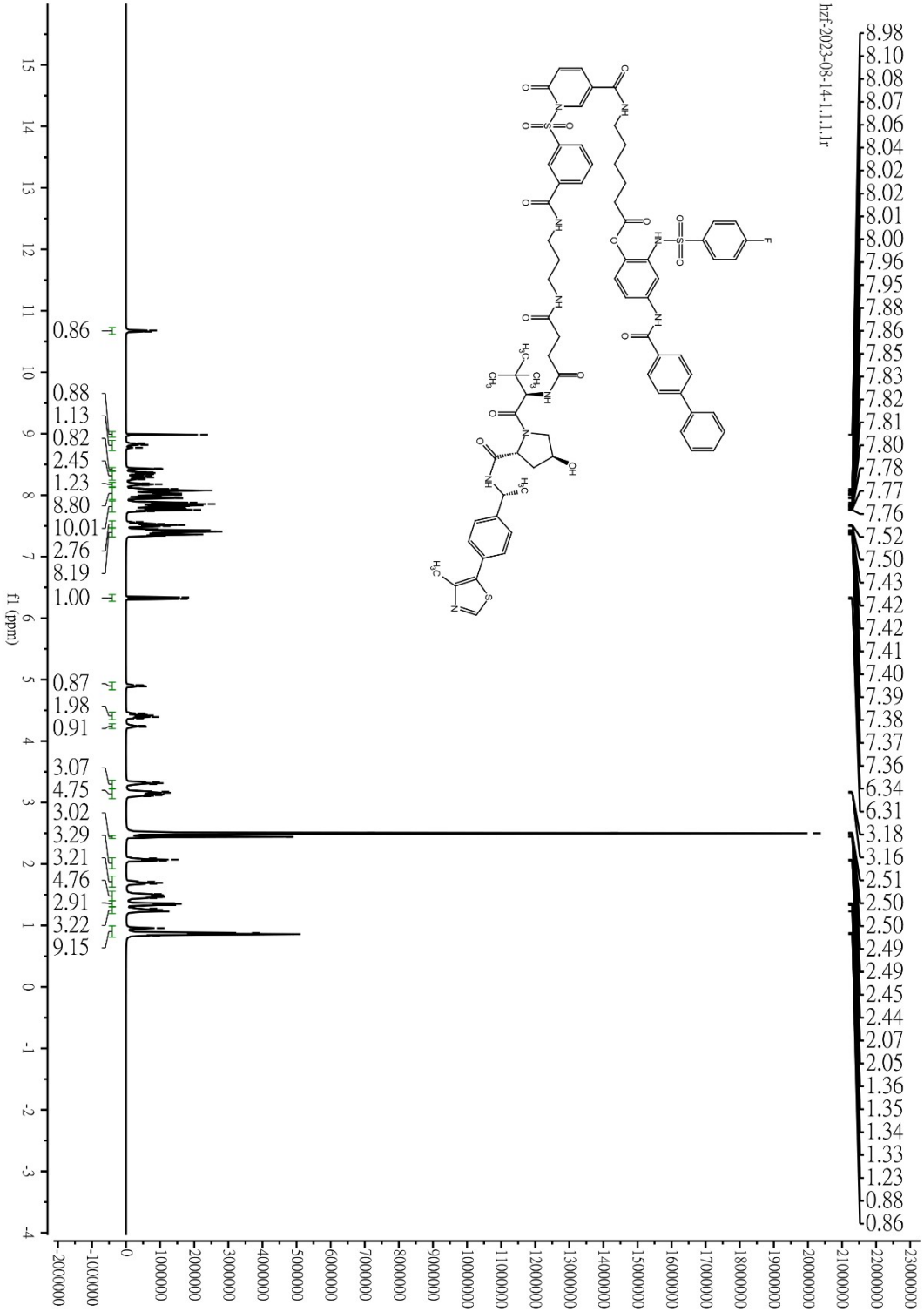
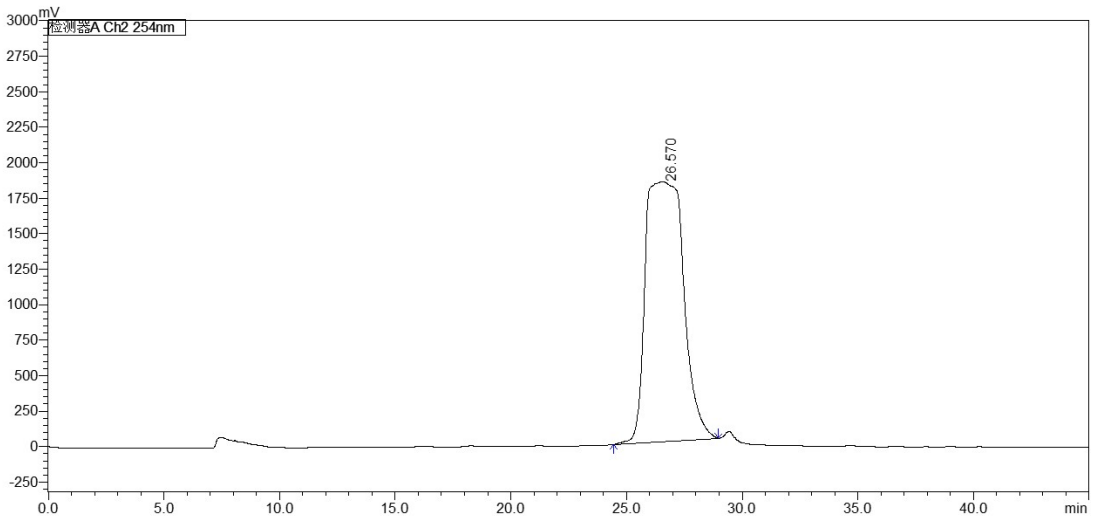
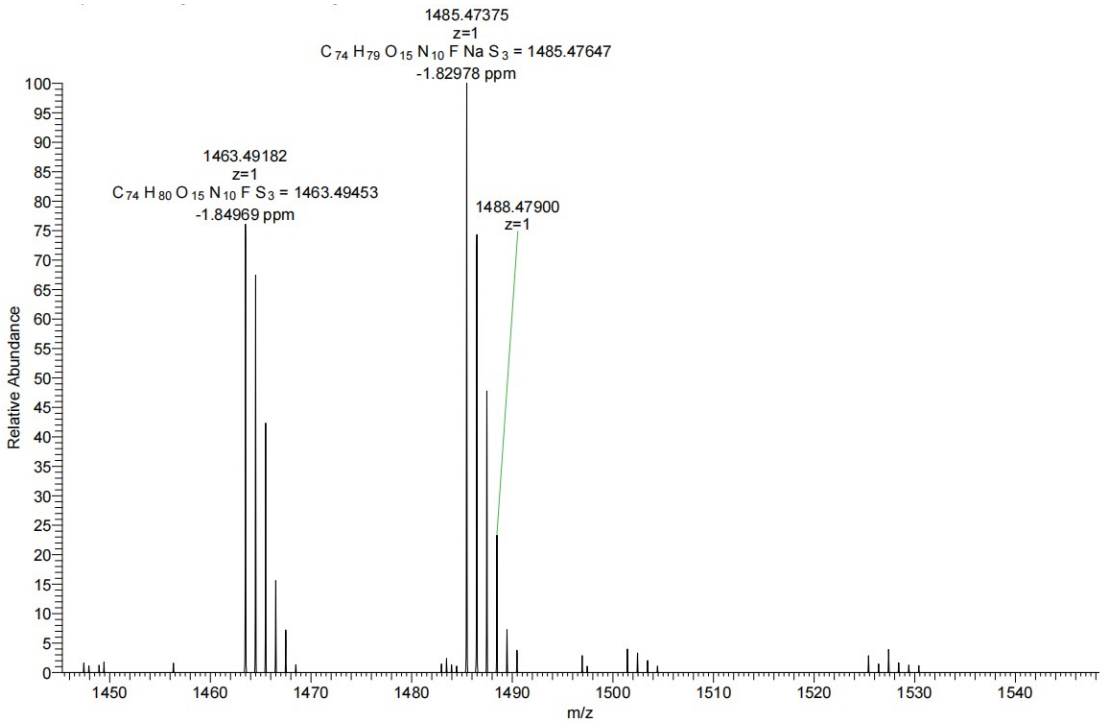


NMR data of SD02



HPLC chromatogram of SD02



mass spectrometry identification spectrum of the SD02

Original gel

Figure 2A

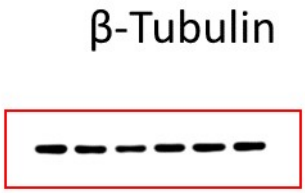


Figure 2B

Figure 2B

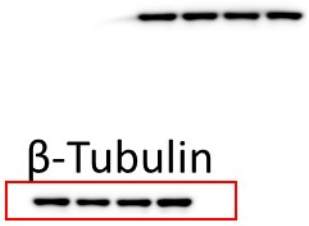


Figure 2C

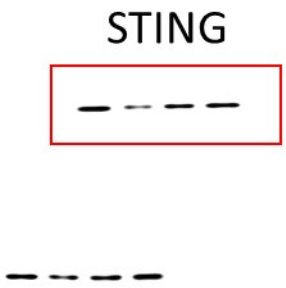


Figure 2C

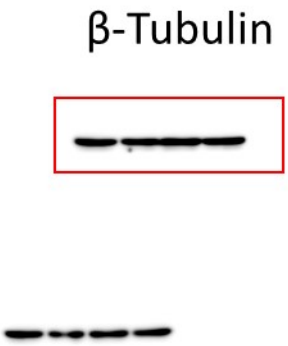


Figure 2D

Figure 2D

IP: HA

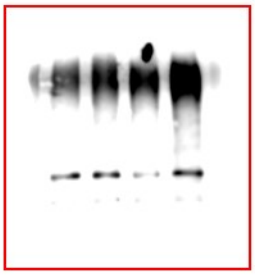
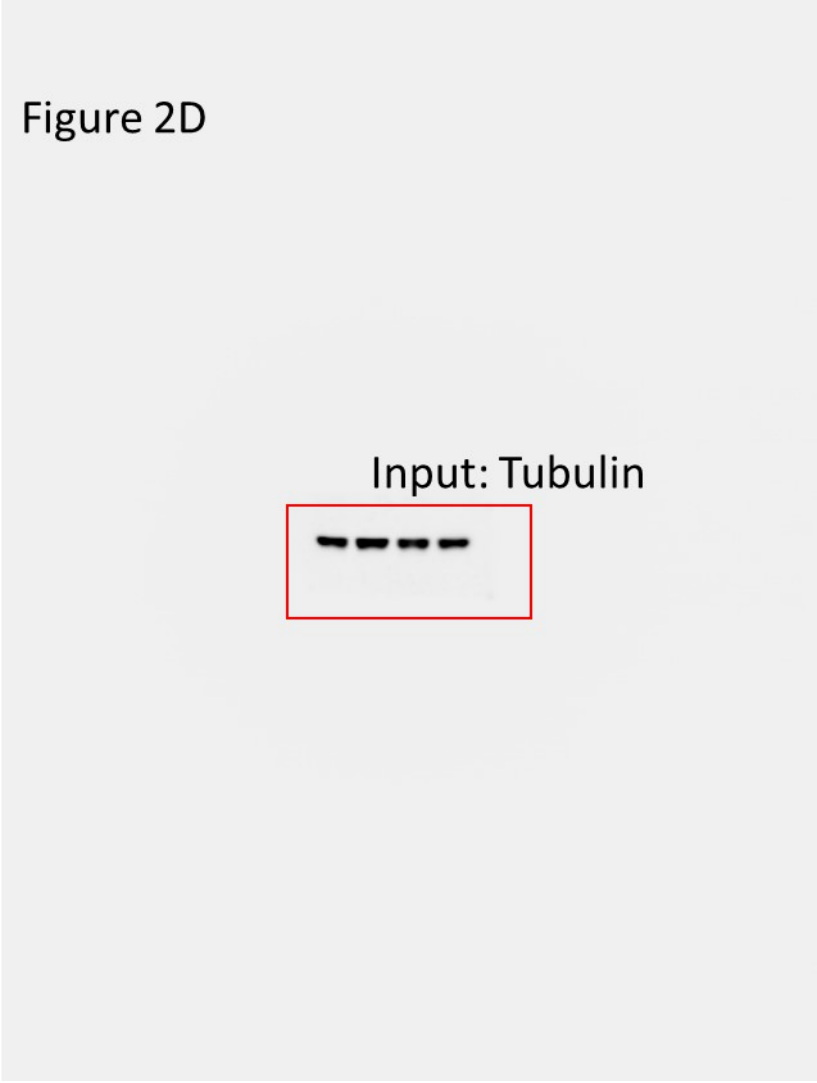


Figure 2D



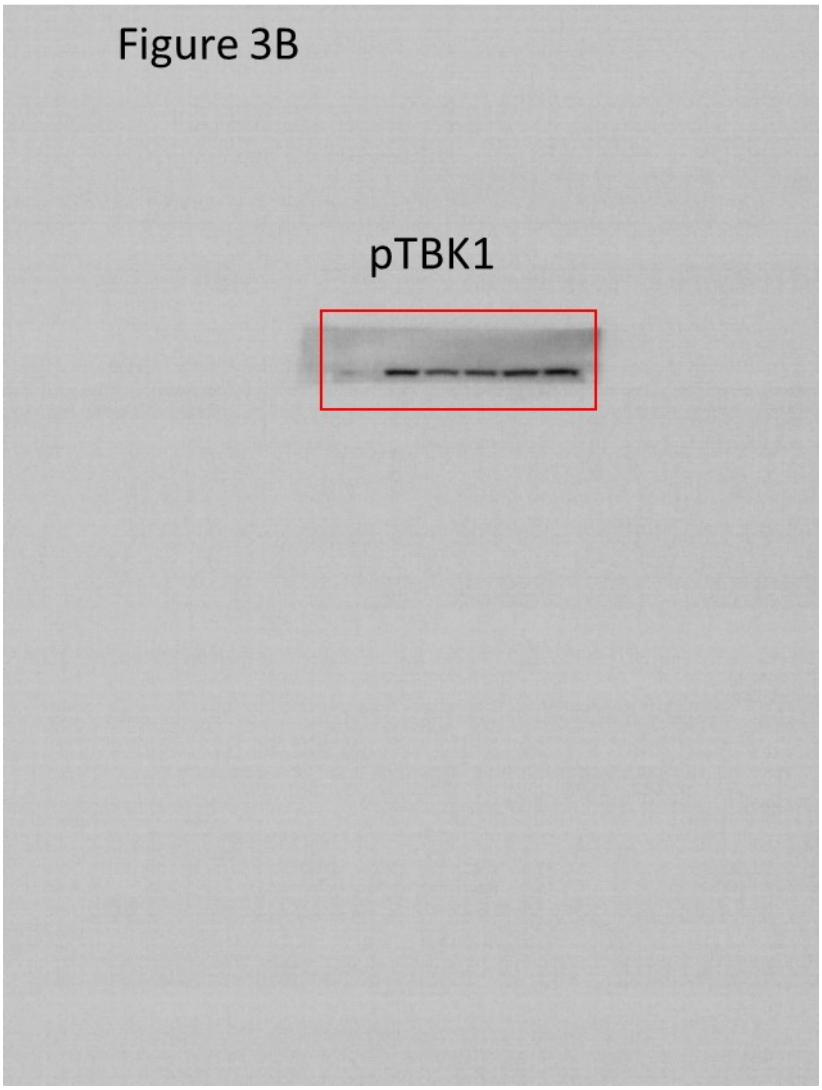
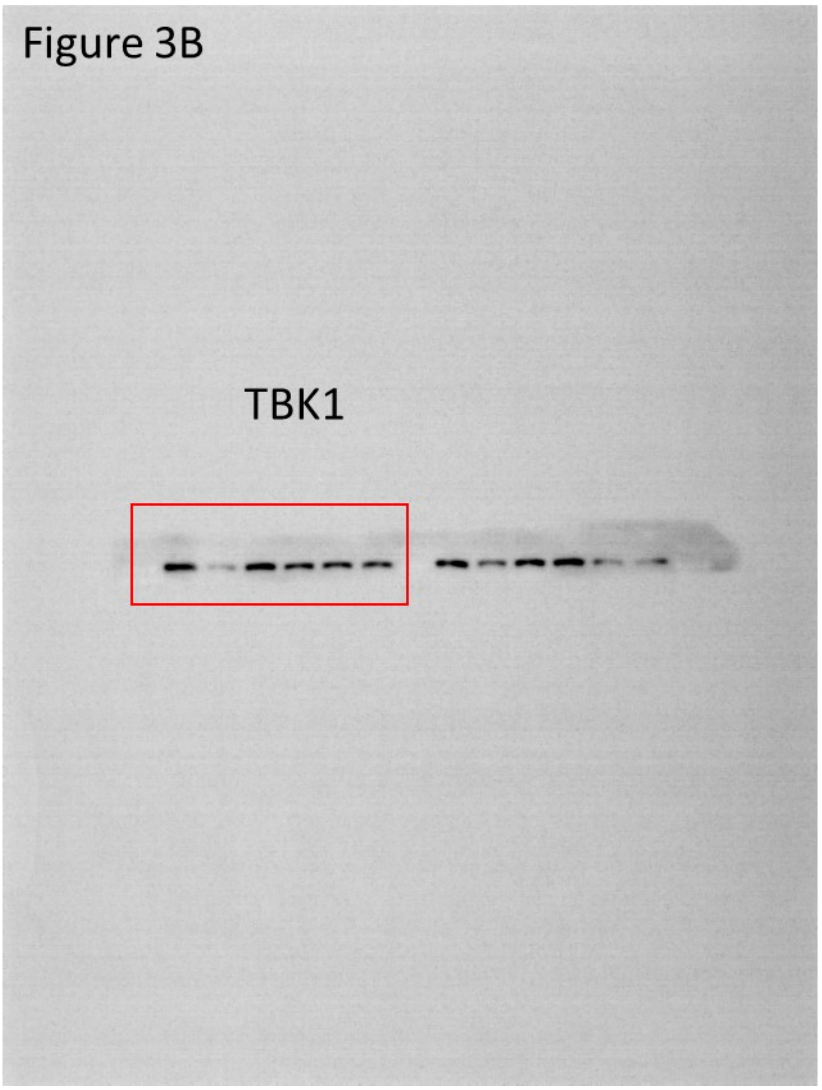


Figure 3B
pTBK1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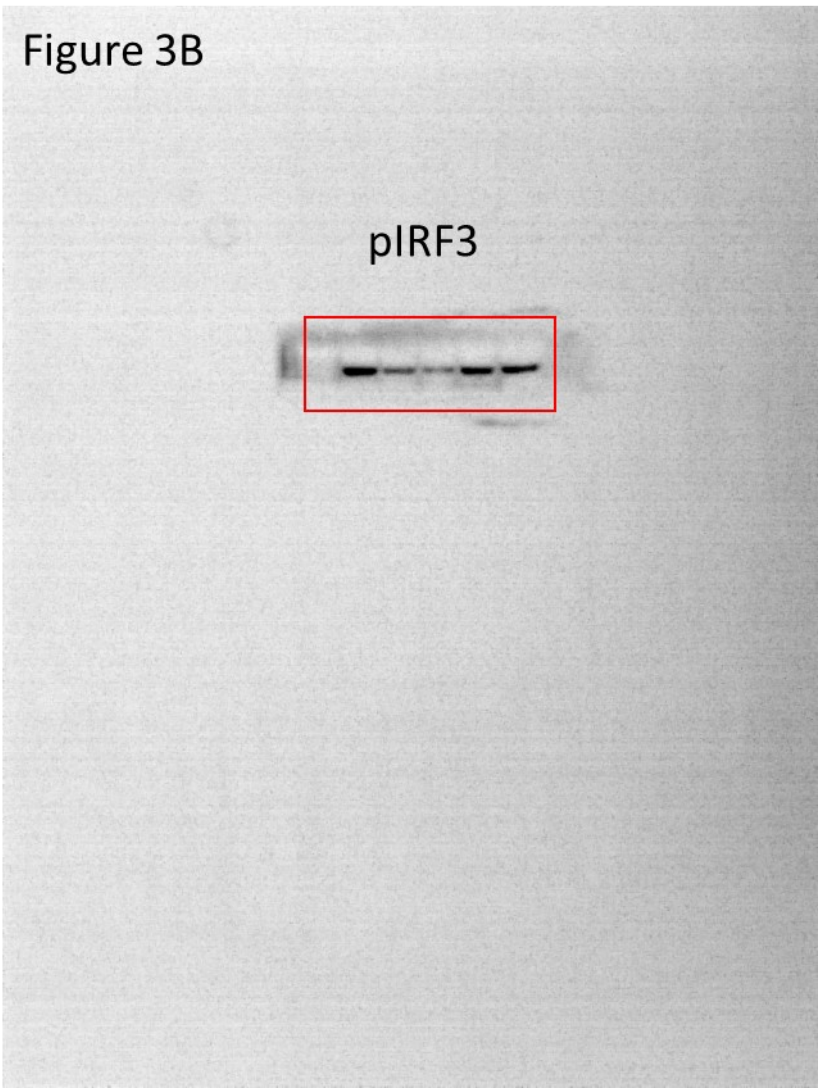
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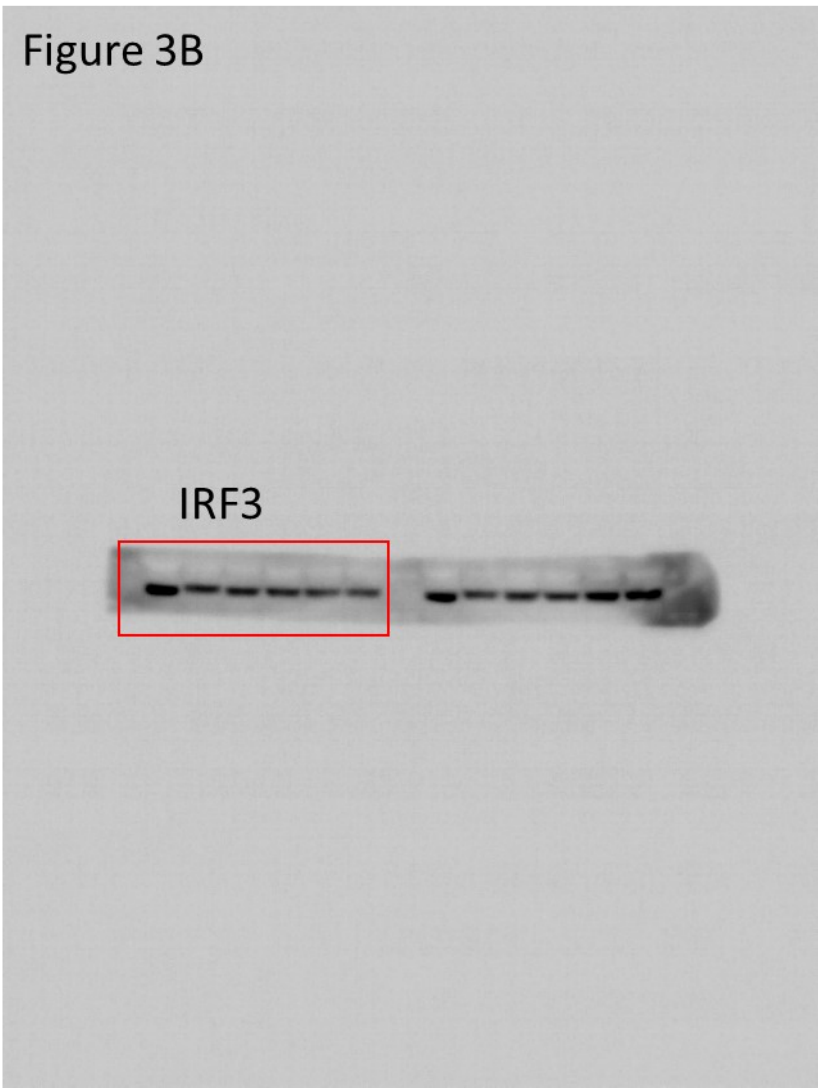
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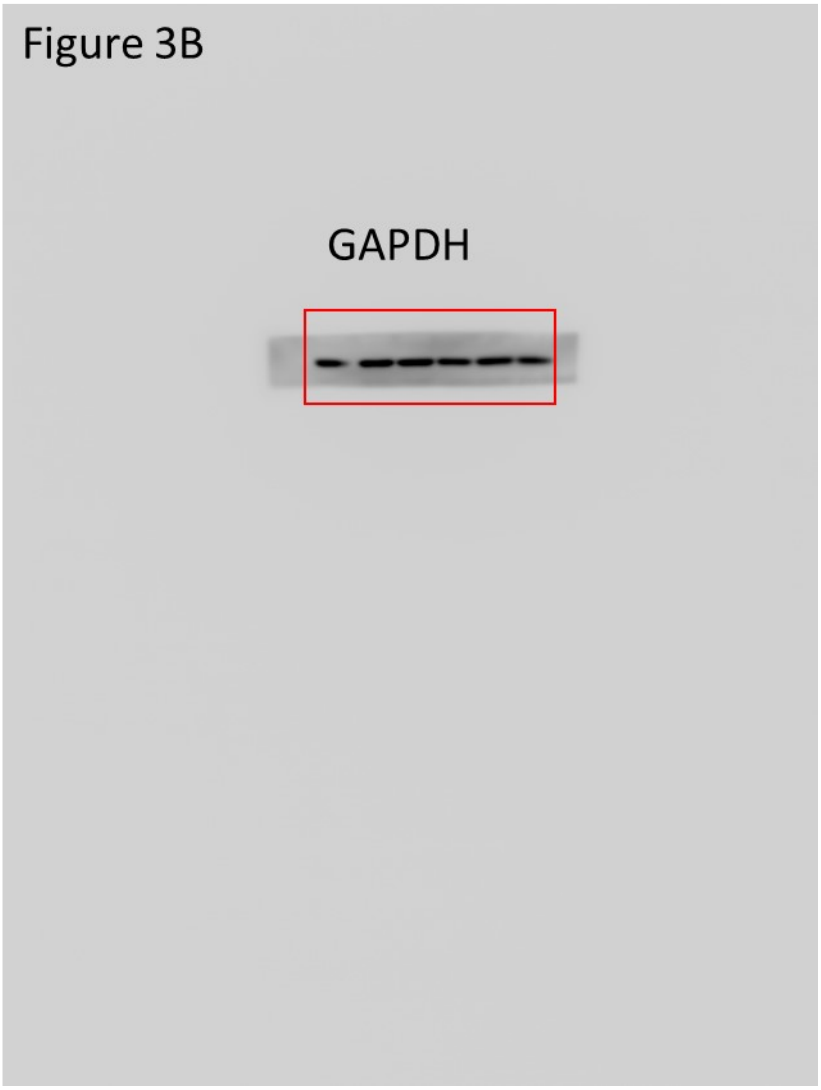
Figure 3B
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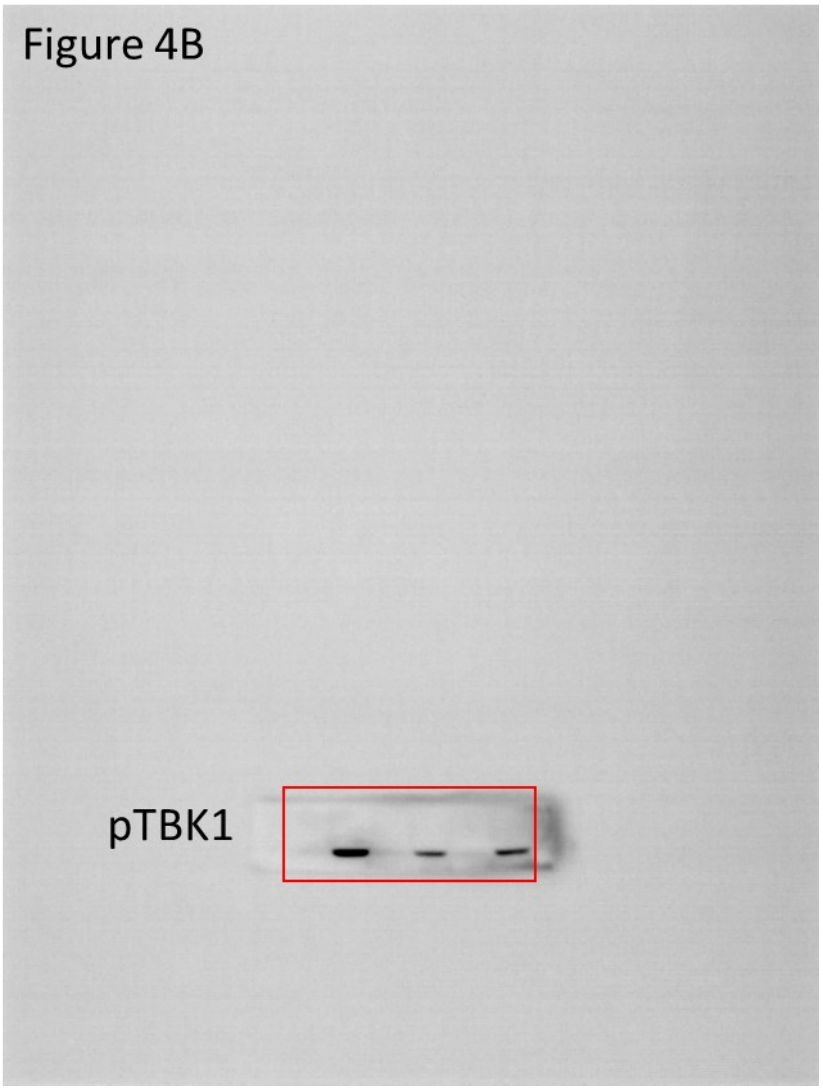
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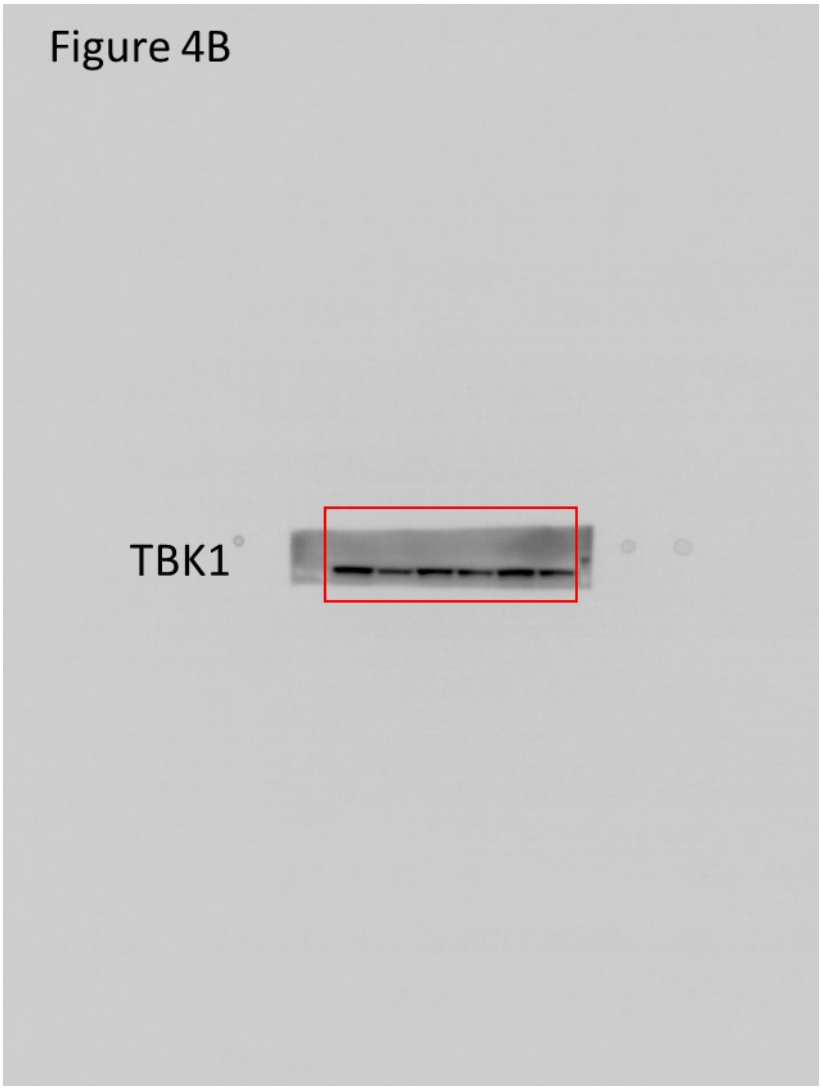
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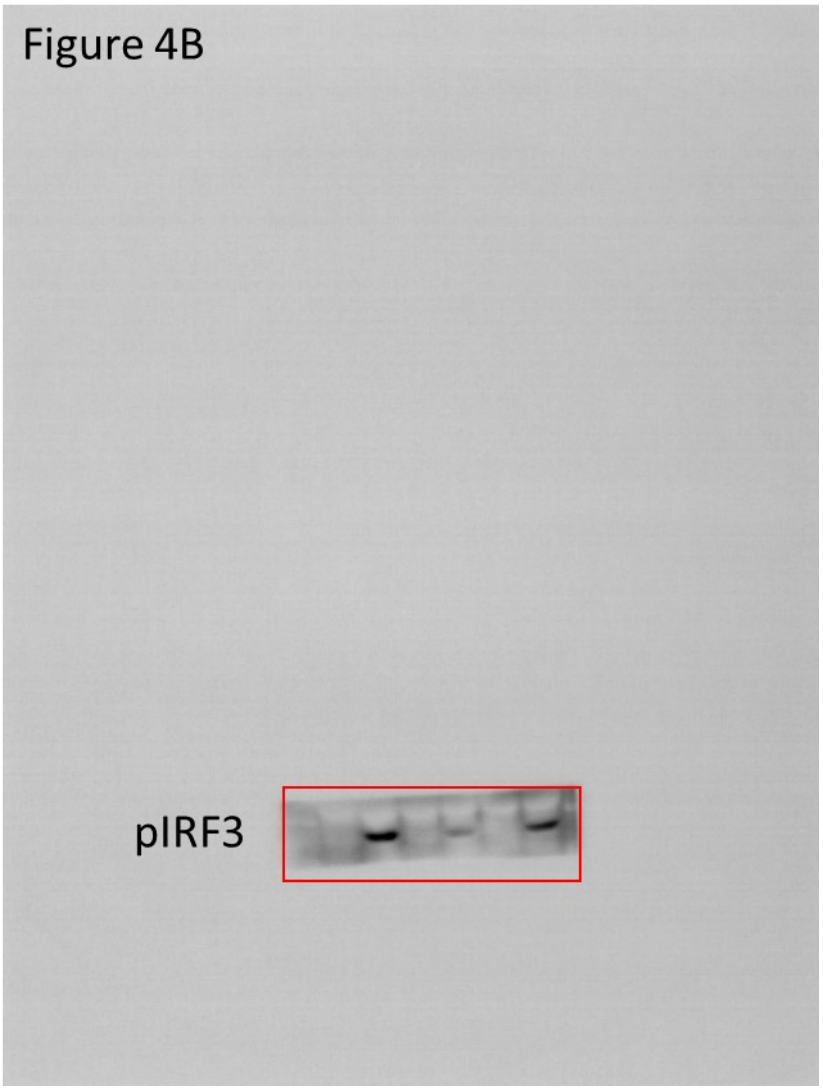
Figure 4B
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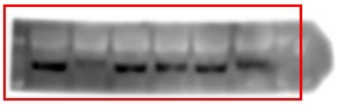
Figure 4B

IRF3



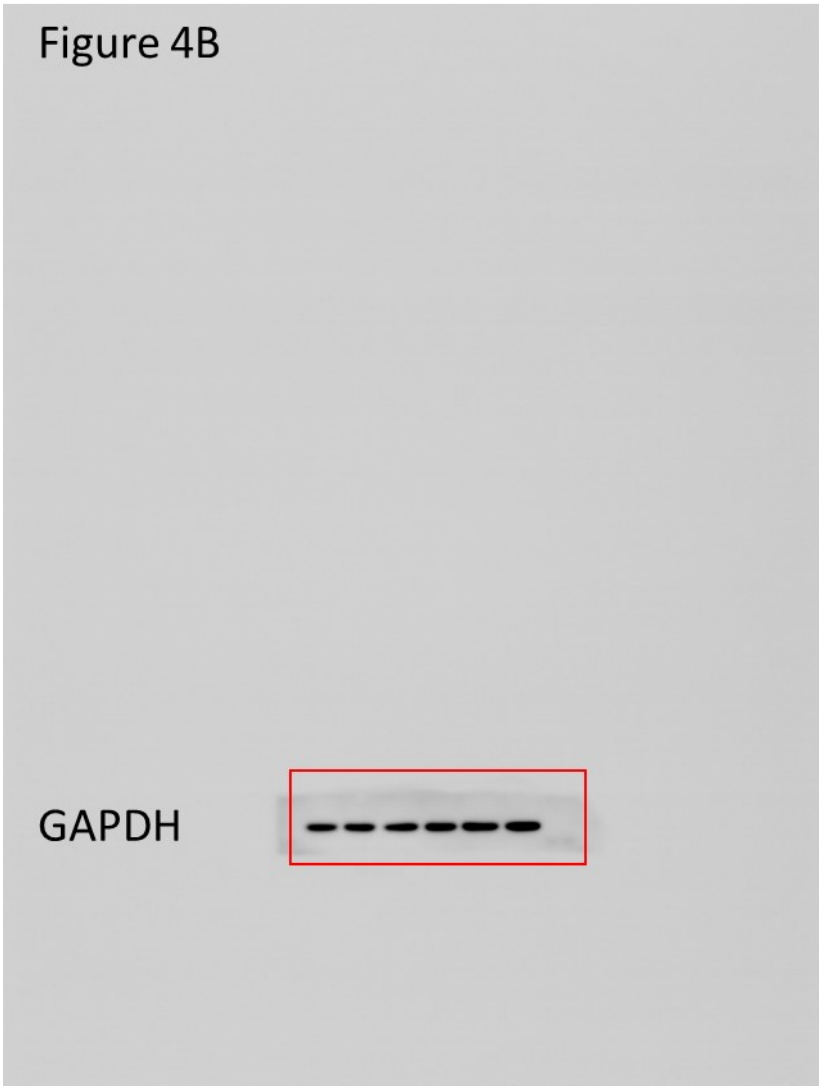
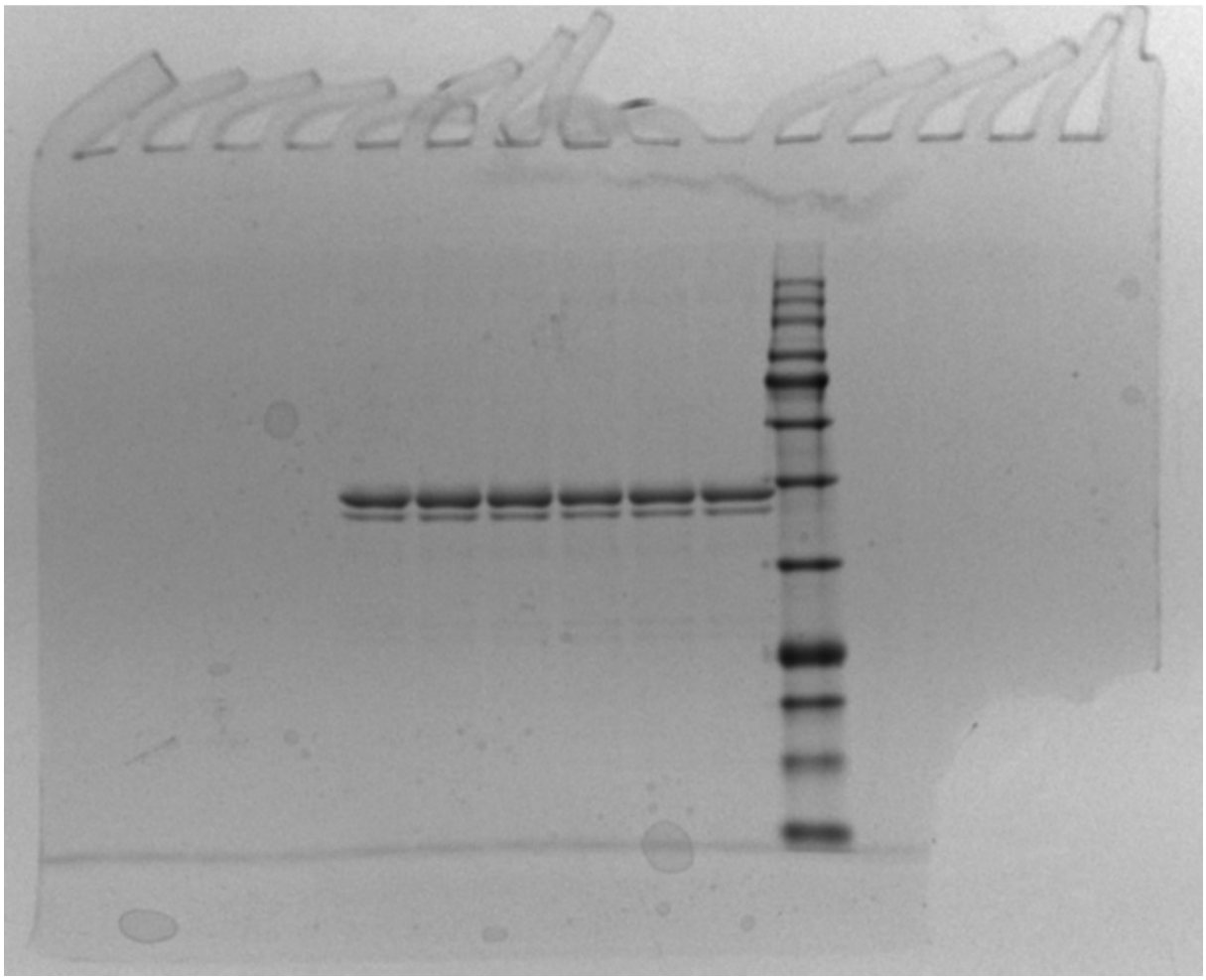


Figure 4B
GAPDH

Figure S2B



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