

Small-molecule Inhibitors of SETD8 with Cellular Activity

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1. MATERIALS AND GENERAL METHODS

S-Adenosyl-L-[methyl-³H]-methionine (10 Ci/mmol in 9:1 sulfuric acid/ethanol) was purchased from PerkinElmer. The 4 HTS hits, NSC663284 (**SPS8I1**, TOCRIS, 1867), Ryuvidine (**SPS8I2**, TOCRIS, 2609), BVT948 (**SPS8I3**, TOCRIS, 2176) and NSC95397 (TOCRIS, 1027), and their structure-related derivatives (**Chem65**, **Chem73** and **Chem80**) were commercially available and their quality was confirmed by HPLC-MS. All peptide substrates including: H3 aa 1-21 (ARTKQTARKSTGGKAPRKQLA), H3 aa 20-50 (ATKAARKSAPATGGVKKPHRYRPGTVALRE), H3 aa 1-40 (ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHR), RGG peptide (GGRGGFGGRGGFGGRGGFG) p53 aa 359-393 (GGSRAHSSHLKSKKGQSTSRHKLMFKTEGPDSD-GGK-biotin) and H4 aa 10-30 (LGKGGAKRHRKVLRLDNIQGIT) were synthesized by the Proteomics Resource Center at Rockefeller University via standard Fmoc-protected solid-phase peptide synthesis and purified by HPLC with the gradient of 0~40% acetonitrile in 0.1% trifluoroacetic acid/H₂O. Integrity of purified peptides was confirmed by MALDI-MS. P-81 phosphocellulose filter paper was purchased from Whatman. Other reagents were obtained from available commercial sources unless otherwise mentioned. All the enzymatic reactions were carried out at ambient temperature (22 °C) unless noticed otherwise. HEK293T cells were cultured in DMEM (Cellgro) with 10% heat-inactivated FBS (Gemini) supplemented with penicillin, streptomycin, L-glutamine and NEAA (Lonza) according to a standard protocol.

2. EXPRESSION AND PURIFICATION OF PROTEIN METHYLTRANSFERASES (PMTS)

Plasmids and Constructs. The plasmids for protein expression of the various PMTs were generous gifts of several laboratories: N-terminal His6-tagged human SETD2 (residues 1347-1711), human GLP (residues 951-1235), human G9a (residues 913-1193), human SMYD2 (a full-length construct with SNP coded for G165E variant) and human PRMT3 (residues 211-531) from the Min lab at the University of Toronto,¹ N-terminal His6-tagged human PRMT1 (residues 10-352) from the Thompson lab at Scripps Florida; N-terminal His6-tagged human CARM1 (residues 19-608) from the Frankel laboratory at University of British Columbia; N-terminal

His6-tagged human SETD7 (full-length) and human SETD8 (residues 191-352) from the Trievel lab at University of Michigan. Expression and purification of these proteins (except human SMYD2, see below for details) were carried out according to previously established protocols.^{S1-}

^{S3} The expressed proteins were flash-frozen in liquid nitrogen and stored at -80 °C prior to use. These proteins are stable under these storage conditions for at least 6 months.

Expression and Purification of SMYD2. The full-length SMYD2 with an N-terminal His-tag in a pET28-MHL vector was transformed into BL21 RIL Codon+ competent cells. Cells were grown at 37°C to OD₆₀₀ = 0.6 in LB growth media containing Kanamycin (50 µg/ml) and ZnSO₄ (25 µM final concentration). Protein expression was induced with 0.2 mM IPTG for 20 h at 17 °C. Cells were lysed by French-press and purified with Ni-NTA agarose resin (Qiagen). Bound protein was washed with a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 25 mM imidazole and 10% (v/v) glycerol, and eluted with a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 400 mM imidazole and 10% (v/v) glycerol. The eluted fractions containing SMYD2 (analyzed by SDS-PAGE) were combined and subject to a 5 ml HiTrap Q ion-exchange column (GE HealthCare) to afford active SMYD2.

Expression and Purification of SETD8 and its C270S Mutant. SETD8 C270S mutant was generated from by QuickChange site-directed mutagenesis (Stratagene) from N-terminal His6-tagged human SETD8 (residues 191-352) following manufacturer's instructions (verified by DNA sequencing). The protocol for expressing and purifying SETD8 C270S mutant was the same as described previously for native SETD8.^{S2, S3} Briefly, the SETD8 plasmids were transformed into the *E. coli* Rosseta-2(DE3) strain (Novagen), and the expression of SETD8 and its C270S mutant was induced by 0.5 mM IPTG at 17°C for overnight. The crude protein was first purified with a gradient of 300 mM imidazole (50 mM Hepes, pH = 8.0, 50 mM NaCl, 25-300 mM imidazole and 10% (v/v) glycerol) on Ni-sepharoseTM 6 Fast Flow resin (GE Healthcare). Native SETD8 and its C270S mutant were then subject to the Sephacryl S-200 size-exclusion column (GE HealthCare). The desired protein samples were flash-frozen in liquid nitrogen and stored at -80 °C before their use. SETD8 and its mutants are stable under these conditions for at least 6 months.

3. VALIDATION OF HTS HITS AS SETD8 INHIBITORS

Summary of Prior HTS Conditions.^{S2} Here 0.75 μM (0.15 μCi) [$^3\text{H-Me}$]-SAM, 1.5 μM (30 pmol) biotinylated-H4 (10-30 aa) peptide, 1.5 μM for SETD8 and 0.2 mg of scintillation proximity imaging assay beads were used for the HTS screening.² For SETD8, the chemical library used for screening contains 5,632 compounds in a 384-well format. "High control" with 1% DMSO (v/v) for no enzymatic inhibition and "low control" with 10 mM of HClO_4 for total inhibition served as the quality controls and were included to measure assay robustness. For the two HTS screenings of SETD8, the readout of "high control" is 192~203 with the standard derivative of 4~5; the readout of "low control" is 66~82 with the standard derivative of 6~7. The percent coefficients of variation for "high" and "low" control of the two HTS assays were 6~7 % and 3%, respectively. The average signal-to-background ratios are 2.5~2.9. The resulting Z' values are 0.74~0.78. With a threshold of 30% inhibition, 18 positive hits were identified with the overall hit rate of 0.32%. Here the high concentration of SETD8 was used to give the desired Z' values for HTS. However, such a condition may miss certain low-affinity inhibitors and may rationalize the low hit rate of 0.32% of the HTS.

Validation of HTS Hits with a Secondary Assay. The dose response curves of the HTS hits for SETD8 inhibition were determined by a secondary radioactivity filter paper assay as documented previously.^{S3} Various concentrations of the compounds in DMSO stocks were incubated for 10 min with 2 μM SETD8 in a reaction buffer (50 mM Hepes-HCl (pH = 8.0), Tween-20 0.005% (v/v), 5 $\mu\text{g/mL}$ BSA and 1 mM TCEP) with the final concentrations of 0~200 μM compounds (mixture A). A 20 μL methylation reaction was then carried out by mixing 10 μL of Mixture A with 10 μL of Mixture B containing 50 mM Hepes-HCl (pH = 8.0), Tween-20 0.005% (v/v), 5 $\mu\text{g/mL}$ BSA, 1 mM TCEP, 3 μM of peptide substrates, 1.5 μM of [$^3\text{H-Me}$]-SAM and 20% (v/v) DMSO (this DMSO concentration was shown previously not to affect the methylation reaction).^{S3} The reactions were incubated for a designated period of time and the resulting reaction products were assayed for methylation activity by immobilizing the peptides onto P81 phosphocellulose filter paper, proceeded by washing and finally, quantification with a liquid scintillation counter.^{S3} Briefly, 6 μL of the methylation reaction mixture was spotted onto Whatman P-81 phosphocellulose filter paper ($1.2 \times 1.2 \text{ cm}^2$) to immobilize [$^3\text{H-methyl}$]-labeled peptides. After drying in air at ambient temperature (22 $^{\circ}\text{C}$) for 20 min, filter paper was

immersed in 20 mL of 50 mM Na₂CO₃/NaHCO₃ buffer (pH = 9.2), and shaken for 10 min/time, the wash step was repeated 5 times. The filter paper was then transferred to a 10 mL scintillation vial containing 500 µL of distilled water followed by addition of 5 mL of Ultima Gold scintillation cocktail (PerkinElmer). β-emission of the immobilized peptides was quantified by a Beckman LS6000IC liquid scintillation counter. The CPM values of the β-emission (Partial) were plotted as the percent of inhibition relating to the DMSO control (Full) after subtracting background CPM (Background for CPM of nonenzyme control) according to the following equation: $[\%Inhibition] = \left(1 - \frac{[Partial] - [Background]}{[Full] - [Background]}\right) \times 100$. The IC₅₀ values were obtained by fitting $[\%Inhibition]$ versus the concentrations of inhibitors with GraphPad Prism6 software.

Dose Response Curves of SPS8I1-3 against SETD8 C270S Mutant. The dose response curves of SPS8I1~3 against SETD8 C270S mutant were determined similarly as native SETD8 with the secondary radioactivity filter paper assay described above, except that native SETD8 was replaced by SETD8 C270S mutant.

Dose Response Curves of SPS8I1-3 against Other PMTs. The filter-paper assay described above was implemented in obtaining the dose response curves of **SPS8I1~3** against other PMTs including 5 protein lysine methyltransferases (SETD2, GLP, G9a, SMYD2 and SETD7) and 3 protein arginine methyltransferases (CARM1, PRMT1 and PRMT3). Experiments were run in triplicate with the final concentrations of **SPS8I1~3**: 0, 0.1, 0.2, 0.5, 1.5, 3, 6, 12.5, 25, 50 and 100 µM. Here the assay format is similar to that of SETD8 with optimized assay parameters (see Table S2 for the optimized assay parameters). Briefly, Mixture A as described above was preincubated with **SPS8I1~3** for 10 min, followed by the addition of Mixture B containing 50 mM Hepes-HCl (pH = 8.0), Tween-20 0.005% (v/v), 5 µg/mL BSA, 1 mM TCEP, 3 µM peptide substrate and 1.5 µM [³H-Me]-SAM. The IC₅₀ values of **SPS8I1~3** against the panel of 8 PMTs were obtained by fitting $[\%Inhibition]$ versus the log of the concentrations of inhibitors with GraphPad Prism6 software as described above for SETD8 inhibition.

4. SUBSTRATE/SAM-DEPENDENT DOSE RESPONSE CURVES OF SPS8I1~3 AGAINST SETD8

To assess the substrate/SAM dependence of SETD8 inhibition by **SPS8I1~3**, the dose response curves of each compound were measured by varying the concentrations of [³H-Me]-SAM (0.5 – 2.5 μ M) or the H4K20 peptide substrate (5 – 100 μ M). Briefly, stock solutions of each inhibitor (final concentrations of: 0, 0.1, 0.2, 0.3, 0.5, 1.5, 3, 6, 12.5, 50 and 100 μ M) were incubated for 1 h with the reaction buffer (50 mM Hepes-HCl (pH = 8.0), Tween-20 0.005% (v/v), 5 μ g/mL BSA and 1 mM TCEP) to afford a reaction mixture containing 4 \times fold concentrations of inhibitors (in comparison with the final assay conditions). Into the 4 \times fold inhibitor stock, SETD8 was added to a final concentration of 1 μ M. The resultant mixture was then incubated for another 10 min to afford a 2 \times fold reaction mixture containing an inhibitor and SETD8. For each examined concentration of substrate or SAM, 10 μ L of the 2 \times fold inhibitor-enzyme stock was mixed with 10 μ L of Mixture B containing (a) 20 μ M fixed-concentration of the H4K20 peptide substrate and varied concentrations of [³H-Me]-SAM (SAM-dependent IC₅₀); (b) 4 μ M fixed concentration of [³H-Me]-SAM and varied concentrations of the H4K20 peptide (substrate-dependent IC₅₀). These reaction mixtures (triplicate) were allowed to proceed for 2 h, and then quenched by depositing the reaction samples onto P81 phosphocellulose filter paper. Beta-emission of immobilized peptides was quantified by a Beckman LS6000IC liquid scintillation counter. The dose response curves (IC₅₀ values) were obtained according to the equation:

$$[\%Inhibition] = \left(1 - \frac{[Partial] - [Background]}{[Full] - [Background]} \right) \times 100 \text{ as described above. It is worth noting that herein}$$

less SETD8 and shorter reaction time were applied. These assay parameters are different from those used to validate HTS hits. The current assay parameters allowed the reactions to process in a linear range even with increased concentrations of SAM or substrates. The IC₅₀ values were plotted as a ratio to those with the lowest concentration of SAM (0.5 μ M) or substrate (5 μ M).

5. TIME-DEPENDENT, SLOW-ONSET INACTIVATION OF SETD8 BY SPS8I1~3

Reaction buffer containing 50 mM Hepes-HCl (pH = 8.0), Tween-20 0.005% (v/v), 5 μ g/mL BSA, 1 mM TCEP and 50 μ M SETD8 was mixed with 100 μ M of each inhibitor (**SPS8I1~3** with a concentration higher than 20 \times IC₅₀) or DMSO as a control (10% (v/v) DMSO as the final

concentration for all the reactions). The mixture was incubated at ambient temperature (22 °C) over an increasing period of time (0, 0.5, 1, 1.5, 3, 6 and 16 h). At each time interval, an aliquot of the mixture was subject to a 100-fold dilution with the reaction buffer to a final mixture containing 0.5 μ M SETD8 and 1 μ M inhibitor (final concentration) or DMSO control. Into this diluted mixture was added an equivalent volume of the reaction buffer containing 4 μ M [3 H-Me]-SAM and 20 μ M peptide substrate. Initial velocities of SETD8-catalyzed methylation were measured over a period of 30 min with the filter paper assay as described above. Results were presented as the percentage of inhibition with the ratios of the initial velocities in the presence of the inhibitors versus the DMSO control as a function of the time of the preincubation with the inhibitors.

Dilution Assay to Characterize SPS8I1~3 as Irreversible Inhibitors of SETD8. A reaction buffer containing 50 mM Hepes-HCl (pH = 8.0), Tween-20 0.005% (v/v), 5 μ g/mL BSA, 1 mM TCEP and 50 μ M SETD8 was incubated for 6 h with 100 μ M of each inhibitor (**SPS8I1~3** with a concentration higher than $20 \times \text{IC}_{50}$) or DMSO as a control (10% (v/v) DMSO as the final concentration for all the reactions). According to the results of the time-dependent inactivation of SETD8 (see Figure 2d), the 6-h preincubation results in the inactivation of most of the enzyme. This mixture was then diluted 100-fold with the reaction buffer to final concentrations of 0.5 μ M SETD8 and 1 μ M inhibitor (or DMSO as a control). Into this diluted mixture was added an equivalent volume of reaction buffer with 4 μ M [3 H-Me]-SAM and 20 μ M peptide substrate at different time intervals (1, 2, 4, 8 and 20 h). The resulting reaction mixture was allowed to react for 2 h. The activities of inhibitor-treated SETD8 and the DMSO-treated control were quantified by the level of the substrate methylation at the 2-h time interval with the filter paper assay described above. The level of SETD8 inhibition (or the level of recovery of SETD8's activity) was plotted as the percentage of inactivated SETD8 versus the DMSO control as a function of the time after the 100-fold dilution step.

Total Progression Curves of SPS8I1~3 to Measure k_{inact}/K_i . To obtain k_{inact}/K_i values of slow onset, irreversible inhibitors **SPS8I1~3**, the total progression curves of each compound were measured with fixed concentrations of [3 H-Me]-SAM (2 μ M), the H4K20 peptide substrate (10 μ M) and SETD8 (50 nM), and with varied concentrations of **SPS8I1~3** (0 – 100 μ M, triplicates) over a 3-hour period of time (a linear range for the progression of SETD8's methylation in the

absence of inhibitors). Briefly, stock solutions of each inhibitor were incubated for 1 h with the reaction buffer (50 mM Hepes-HCl (pH = 8.0), Tween-20 0.005% (v/v), 5 µg/mL BSA and 1 mM TCEP) to afford a reaction mixture containing 4×fold concentrations of individual inhibitors (in comparison with the final concentrations after mixing with other components). Into each of the 4×fold inhibitor stocks, 4 µM [³H-Me]-SAM and 20 µM H4K20 peptide substrate were added to afford a 2×fold reaction mixture containing an inhibitor and the substrate with a final volume of 140 µL. The enzymatic reaction was initiated by the addition of 140 µL 2×enzyme mixture (100 nM SETD8 in 50 mM Hepes-HCl (pH = 8.0), Tween-20 0.005% (v/v), 5 µg/mL BSA and 1 mM TCEP) into the substrate/inhibitor mixture prepared above. At each time interval (5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 150 and 180 min), 3×6 µL of the final reaction mixture was spotted onto P81 phosphocellulose filter paper to quench the reaction. After collecting the samples of time progression, the filter paper was processed as described above for the filter paper assay and β-emission of immobilized peptides was quantified by a Beckman LS6000IC liquid scintillation counter. The total progression curves were obtained by plotting the total CPM readouts (after background subtraction at 0 min) as a function of time. The curves were then fit with eq. S1 for a slow-onset mechanism (Fig. S1), in which [P] is the concentration of accumulated product as the reaction progresses, V_i is the initial velocity, t is the time of progression, and k_{obs} is the rate constant for the fit progression curve.^{S4}

$$[P] = \frac{V_i}{k_{obs}} [1 - e^{(-k_{obs} \times t)}] \quad (1)$$

To obtain values of k_{inact} (the inactivation rate constant) and K_i (the binding constant of inhibitors), the k_{obs} values obtained for **SPS8I1~3** were plotted as a function of the concentration of the inhibitors according to eq. 2 (Fig. S1), in which [I] is the concentration of inhibitors. The data were fit to eq. S2 with PRISM (GraphPad Software, Inc.) to afford k_{inact} and K_i values.^{S4}

$$k_{obs} = \frac{k_{inact} [I]}{K_i + [I]} \quad (2)$$

6. DIFFERENTIAL SCANNING FLUORIMETRY ASSAY WITH SYPRO ORANGE.

Differential Scanning Fluorimetry (DSF) assay was carried out to measure the melting curves of inhibitor-bound SETD8 as previously described for other proteins.^{S5} Briefly, 2 μ M of SETD8 was incubated with 6 μ M of **SPS8I1~3** or DMSO for 6 h in a buffer containing 50 mM Hepes-HCl (pH = 8.0), Tween-20 0.005% (v/v), 5 μ g/mL BSA and 1 mM TCEP. SYPRO Orange Protein Gel Stain stock (Sigma Aldrich) was diluted by 1000-fold and added into the enzyme mixtures. To examine the samples, 40 μ L/well (triplicate) of the protein-SYPRO Orange mixture above was transferred into a Low 96-well Clear Multiplate PCR Plate (Bio-Rad). The plate was covered with Microseal 'B' Film (Bio-Rad) and was equilibrated at 25 °C for 5 min, followed by Real-time thermal cycling (CFX96 Real-Time System, Bio-Rad). Here the temperature was increased by 1 °C/5s to afford a melting curve from 25 °C to 100 °C. Protein thermal stability was then quantified in terms of Hex fluorescence at each temperature. After exporting raw data files in the format of Microsoft Office Excel by the CFX software. Data were processed by including the low and high values that flank the melting region as the percentage of the values of the highest plateau phase. T_m values were calculated as the inflection point of the resulting sigmoidal curve using PRISM (GraphPad Software, Inc.).

8. CELL VIABILITY, WESTERN BLOTS AND CELL CYCLE ANALYSIS

Cell Viability and Western Blot Analysis. After seeding 10^5 HEK293T cells/well in 6-well plates for 24 h, various concentrations of **SPS8I1~3** or DMSO control were added. At each time interval (24 h, 48 h or 72 h), a whole well of the treated cells were collected and their viability was determined by a standard trypan blue staining assay (triplicate). The remaining cells were subject to Western blot analysis. Briefly, the lysate of approximately 5×10^4 HEK293T cells (for each lane of Western blot) was resolved by SDS-PAGE. The proteins were transferred to a PVDF membrane, and then blocked with 5% nonfat milk in TBST buffer at 4°C for 1 h, followed by blotting with a corresponding primary antibody overnight. After washing the blotted PVDF membrane with TBST buffer, HRP-conjugated secondary antibodies (Jackson ImmunoResearch; 1:10,000) in 5% nonfat milk in TBST buffer were incubated for 2 h at ambient temperature (22 °C). The membrane was then washed with TBST buffer, followed by the addition of ECL Plus (GE Healthcare) for film exposure. Antibodies and their dilution

conditions: anti-H4K20me antibody (Active Motif, 39175 rabbit polyclonal, 1:150,000), anti-H4K20me2 (Active Motif, 39173 rabbit polyclonal, 1:100,000), anti-H4K20me3 (Active Motif, 39180 rabbit polyclonal, 1:30,000), anti-H3K9me (Millipore, 07-450 rabbit polyclonal, 1:10,000), anti-H4 (Millipore, 07-108 rabbit polyclonal, 1:100,000) and anti-H3 (Millipore, 06-755 rabbit polyclonal, 1:100,000). Densitometry analysis was carried out with ImageJ program.

Flow Cytometry. HEK293T cells treated with inhibitors or DMSO control. For each condition, approximately 10^6 HEK293T cells were washed with 5 ml of ice-cold PBS and resuspended in 0.5 ml of ice-cold PBS. The cells were slowly dropped into 4.5 ml of vortexing ice-cold 70% ethanol for rapid dispersion. The sample was incubated on ice for 45 min and then fixed at -20°C overnight. The fixed cells were centrifuged at 4°C at $300\times g$ for 10 min. The resultant cell pellet was resuspended to 10^6 cells/mL in the master mix (40 μL of $10\mu\text{g}/\text{ml}$ propidium iodide (PI), 10 μL of 0.2 mg/ml RNase A and PBS 950 μL). The PI-treated cells were incubated at 37°C for 30 min and then analyzed by a flow cytometry (BD LSR II). The ModFit software version 3.2 (Verity Software House) was used for cell cycle analysis.

9. SUPPLEMENTARY TABLES

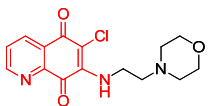
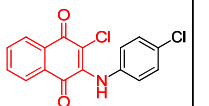
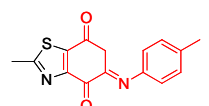
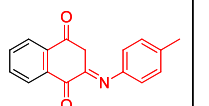
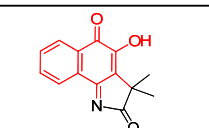
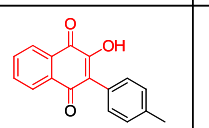
Table S1. IC₅₀ values of **SPS8I1~3** against a representative panel of protein methyltransferases (PMTs).

IC ₅₀ /μM	SETD2	GLP	G9a	SETD8	SMYD2	SETD7	CARM1	PRMT1	PRMT3
SPS8I1	1.5 ± 0.2	> 100	6.5 ± 0.4	0.21 ± 0.03	0.5 ± 0.2	> 100	1.3 ± 0.3	> 100	1.5 ± 0.2
SPS8I2	2.3 ± 0.2	4.7 ± 0.3	3.1 ± 0.2	0.5 ± 0.2	2.0 ± 0.2	>100	1.5 ± 0.2	>100	1.8 ± 0.2
SPS8I3	1.0 ± 0.7	> 100	3.2 ± 0.1	0.7 ± 0.2	3 ± 1	> 100	2 ± 1	> 100	1.1 ± 0.6

Table S2. List of assay parameters to measure IC₅₀ of inhibitors against PMTs examined in the present work.

PKMT	Enzyme Concentration [nM]	Peptide substrate	Reaction time [h]
SETD2	250	H3 (aa 20-50)	4
GLP1	10	H3 (aa 1-21)	1
G9a	20	H3 (aa 1-21)	1
SETD8	1000	H4 (aa 10-30)	8
SMYD2	50	p53 (aa 359-393)	4
SETD7	150	H3 (aa 1-21)	3
CARM1	50	H3 (1-40)	7
PRMT1	100	RGG	1.5
PRMT3	100	RGG	3

Table S3. Structures and IC₅₀ values of compounds related to **SPS8I1~3**.

ID	Structure	IC ₅₀ [μM]	ID	Structure	IC ₅₀ [μM]
SPS8I1		0.21 ± 0.03	Chem80		> 100
SPS8I2		0.5 ± 0.2	Chem65		>100
SPS8I3		0.7 ± 0.2	Chem73		>100

10. SUPPLEMENTARY FIGURE (S1 – S7)

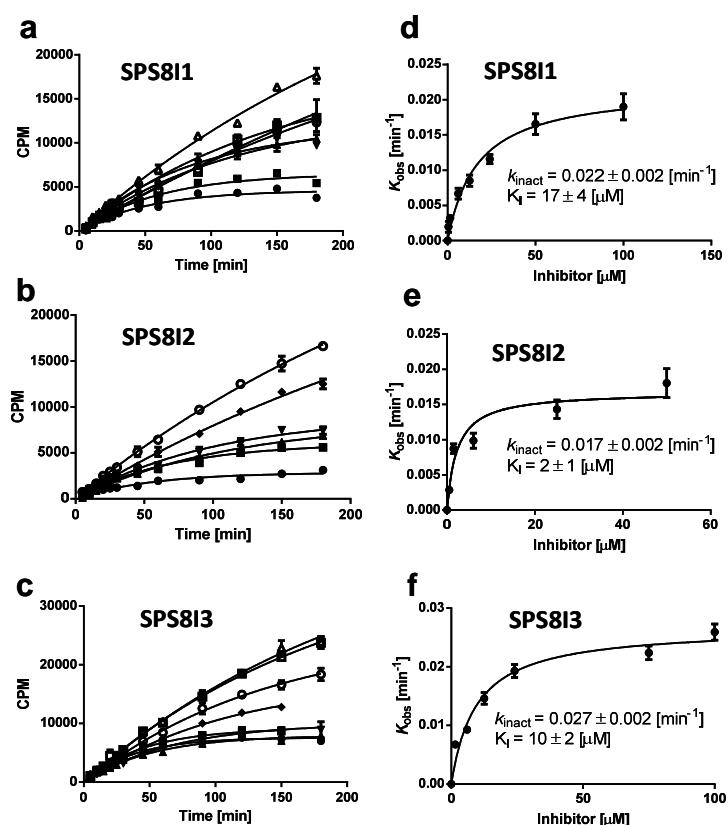


Figure S1. Total progression curves for **SPS8I1-3**. (a-c) Total progression curves for **SPS8I1** (a) **SPS8I2** (b) and **SPS8I3** (c) with the formation of the products (CPM) as a function of time (3 h). The curves were generated with 2 μM [$^3\text{H-Me}$]-SAM, 10 μM H4K20 peptide substrate and 50 nM SETD8 enzyme, and varied concentrations of **SPS8I1-3**: **SPS8I1** (Δ , DMSO, \blacksquare 0.5, \odot 1.5, \blacklozenge 6, \blacktriangledown 12.5, \blacktriangle 24, \blacksquare 50, and \bullet 100 μM), **SPS8I2** (\odot DMSO, \blacklozenge 0.5, \blacktriangledown 1.5, \blacktriangle 6, \blacksquare 25 and \blacktriangle 50), and **SPS8I3** (Δ , DMSO, \blacksquare 0.5, \odot 1.5, \blacklozenge 6, \blacktriangledown 12.5, \blacktriangle 24, \blacksquare 75 and \bullet 100 μM). The data were fitted to eq. S1. (d-f) Concentration-dependent k_{obs} for **SPS8I1-3**. The k_{obs} values were obtained from (a-c) and plotted as a function of the concentrations of **SPS8I1** (d) **SPS8I2** (e) and **SPS8I3** (f) according to eq. S2.

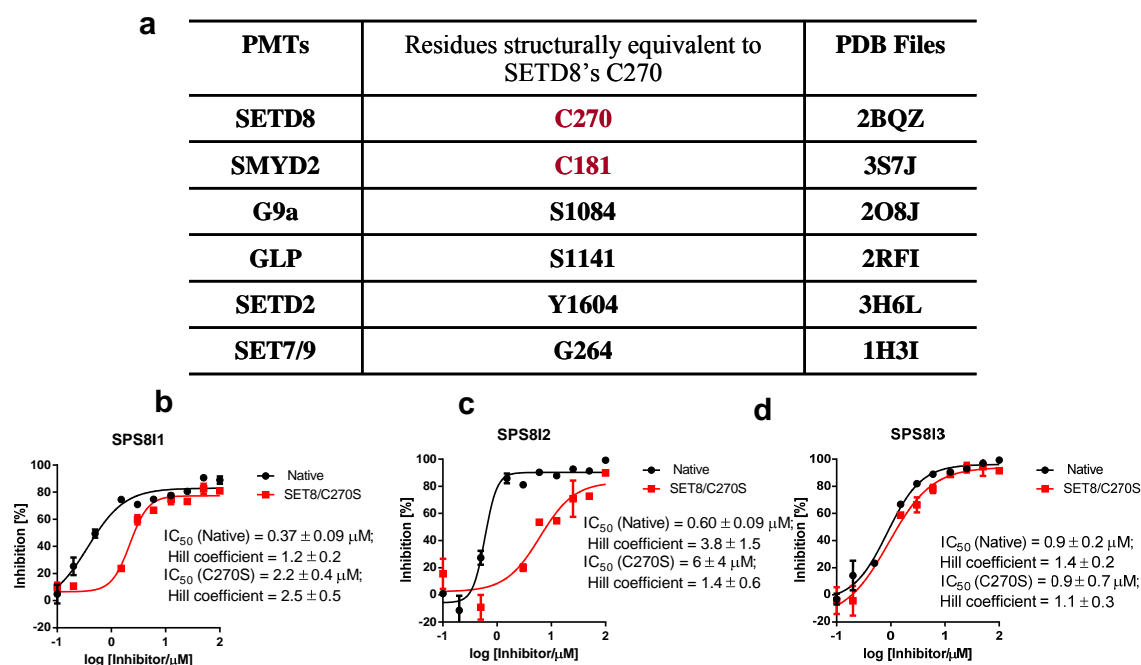


Figure S2. Evaluation of potential interaction of SETD8's C270 with **SPS8I1~3**. (a) Residues of PKMTs equivalent to SETD8's C270. The residues were identified by aligning the structures of SMYD2, G9a, GLP, SETD2, and SET7/9 with SETD8 according to the PDB files listed on the right. (b-c) Dose response curves for native and C270S SETD8 against **SPS8I1~3**. Here 1 μM SETD8 or its C270S variant was incubated with 1.5 μM H4K20 peptide substrate and 0.75 μM [3H -Me]-SAM in the presence of **SPS8I1~3** (0, 0.1, 0.2, 0.5, 1.5, 3, 6, 12.5, 25, 50 and 100 μM) for 8 h. **SPS8I1~3** display different sensitivity towards the C270S mutant by IC_{50} and Hill coefficients values.

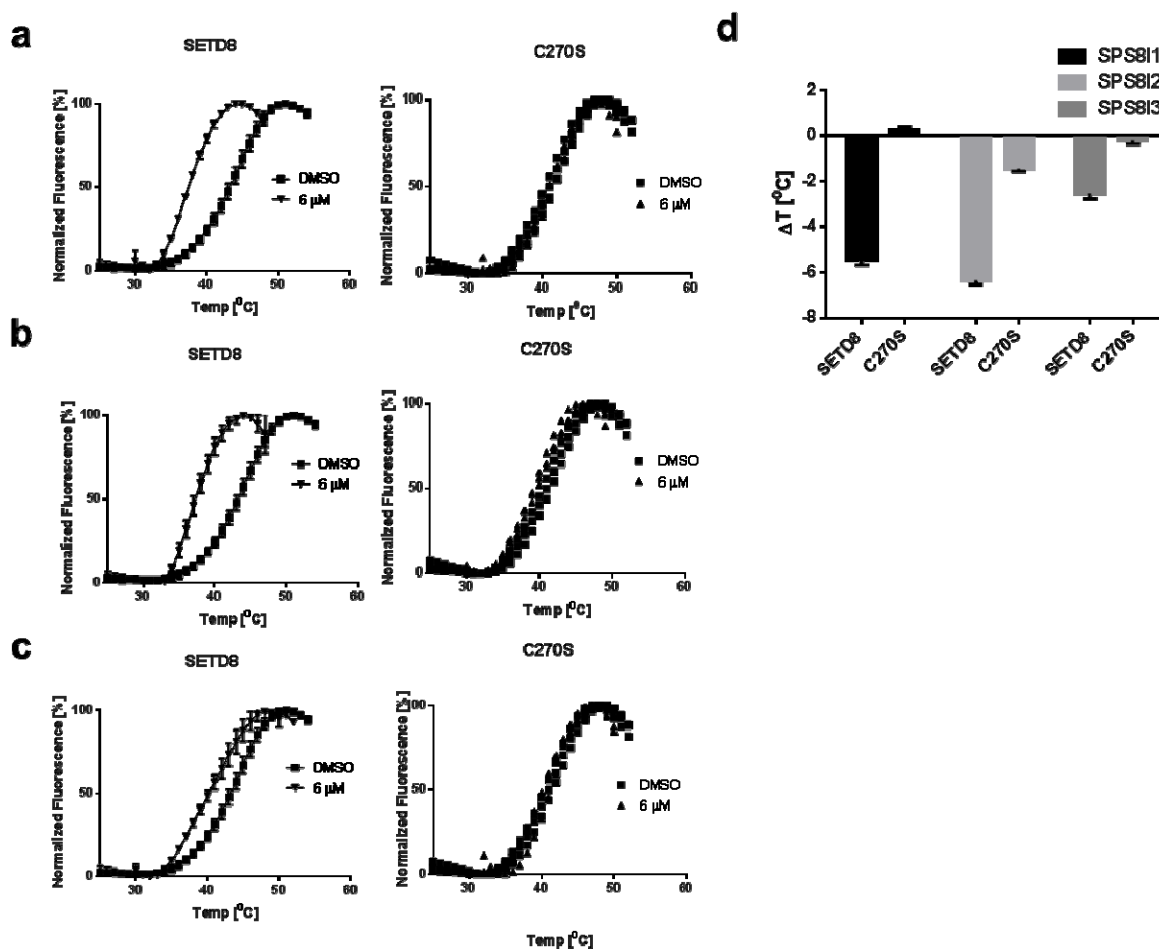
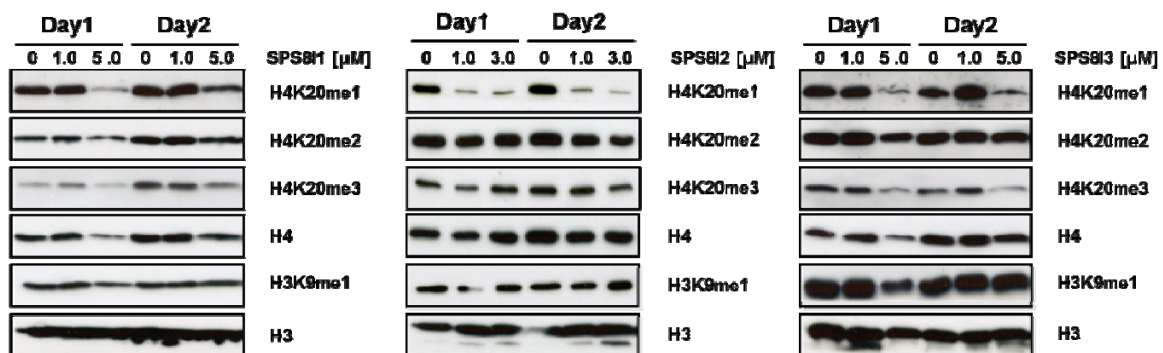


Figure S3. Temperature-dependent melting curves for **SPS8I1~3**. (a-c) Differential Scanning Fluorimetry (DSF) assay of **SPS8I1~3** with SYPRO Orange. To assess inhibitor-bound SETD8, 6 μM of **SPS8I1~3** and a DMSO control were incubated with 2 μM SETD8 or SETD8 C270S mutant for 6 h. The thermal cycle was carried out by gradually increasing the temperature with the fluorescence readout monitored for (a) **SPS8I1**, (b) **SPS8I2** and (c) **SPS8I3**. (d) Bar graph representation of the difference of melting temperatures of native and C270S SETD8 before and after the treatment of **SPS8I1~3**.

a



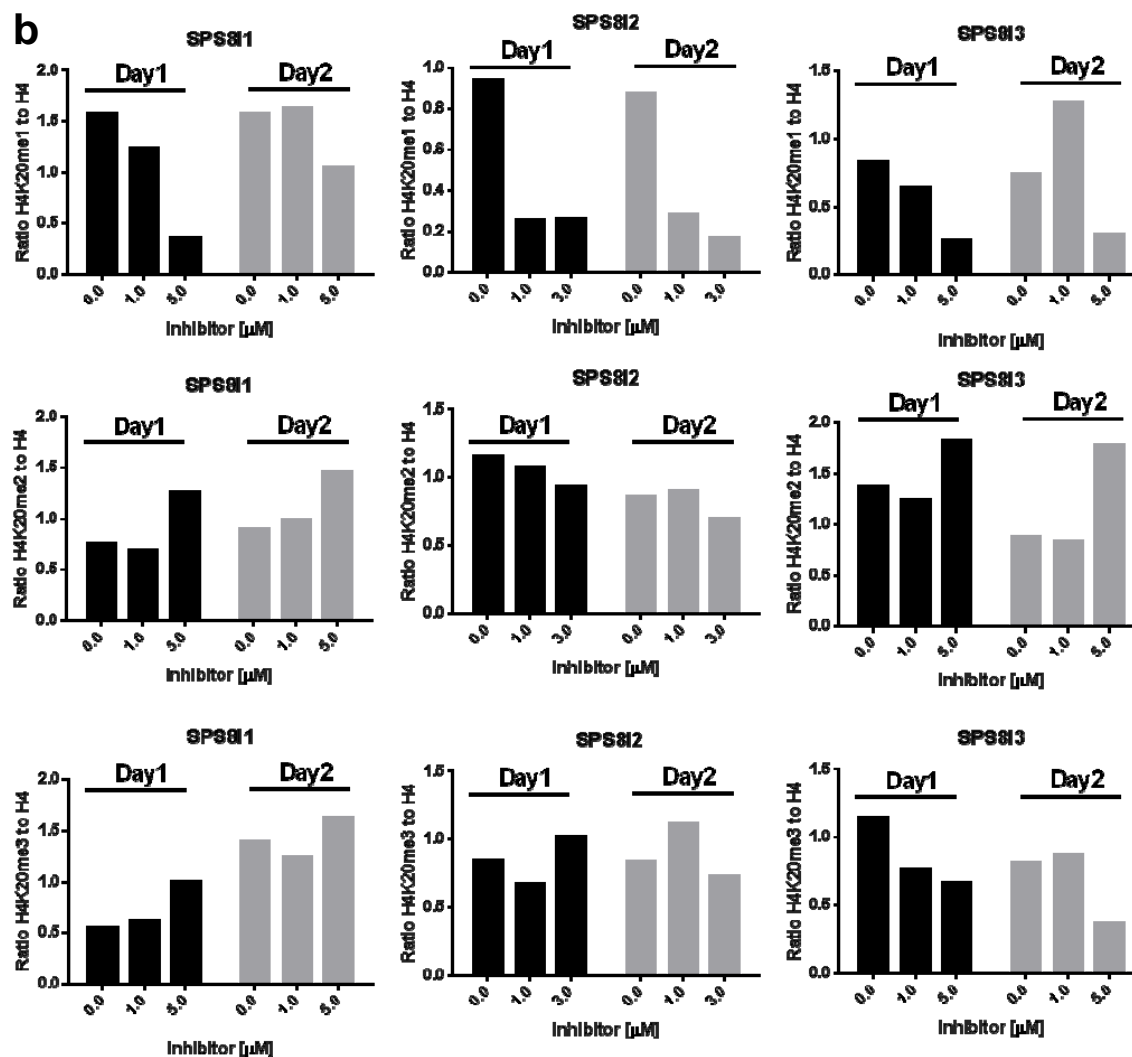


Figure S4. Western blots of H4K20me and other marks after treatment with **SGS8I1~3**. (a) The HEK293T cells were treated with varied concentrations of **SGS8I1~3** for 2 days. The level of H4K20me was examined as a cellular mark of SETD8's methyltransferase activity with the levels of H3, H4, H4K20me2/3 and H3K9me as controls. (b) Densitometry analysis of the methylation marks (H4K20me1/2/3, Figure **S1a**) plotted as a ratio to histone H4 as the loading control.

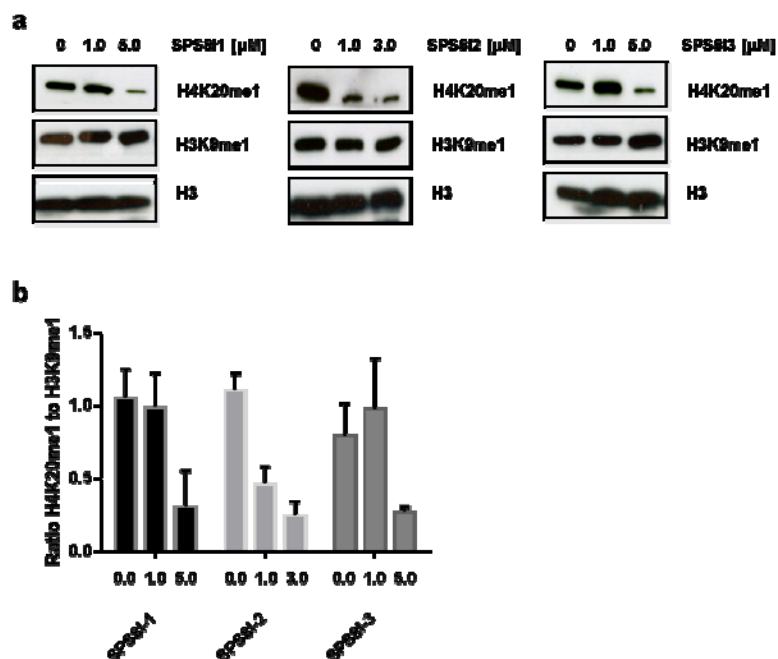
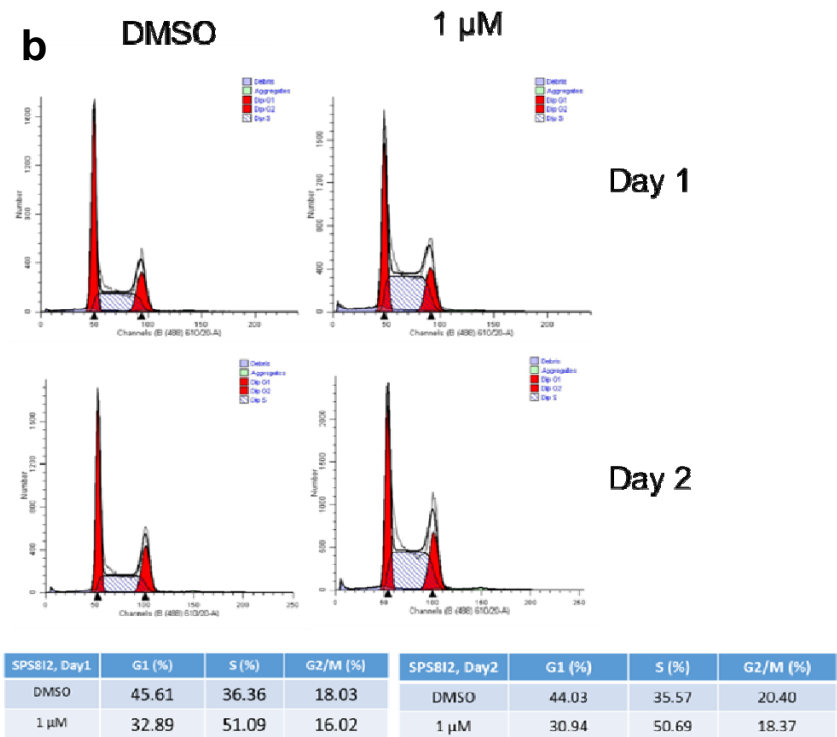
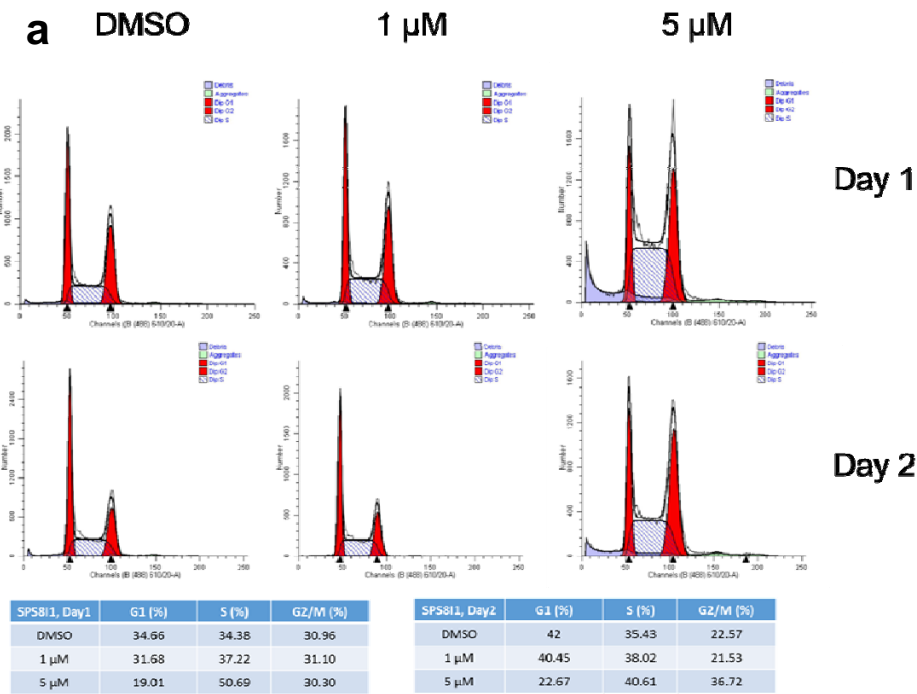


Figure S5. Western blots of H4K20me and other histone marks upon **SGS8I1~3** treatment. (a) HEK293T cells were treated at a single dose with varied concentrations of **SGS8I1~3** for 3 days. The level of H4K20me was examined as a cellular mark of SETD8's methyltransferase activity with the levels of H3, and H3K9me as controls. (b) Average ratios of densitometry of H4K20me versus H3K9me1 for Day 1, Day 2 and Day 3 (Figure **S4a**, **5a**). Here the treatment of **SGS8I1~3** led to 80% decreases of H3K9me1.



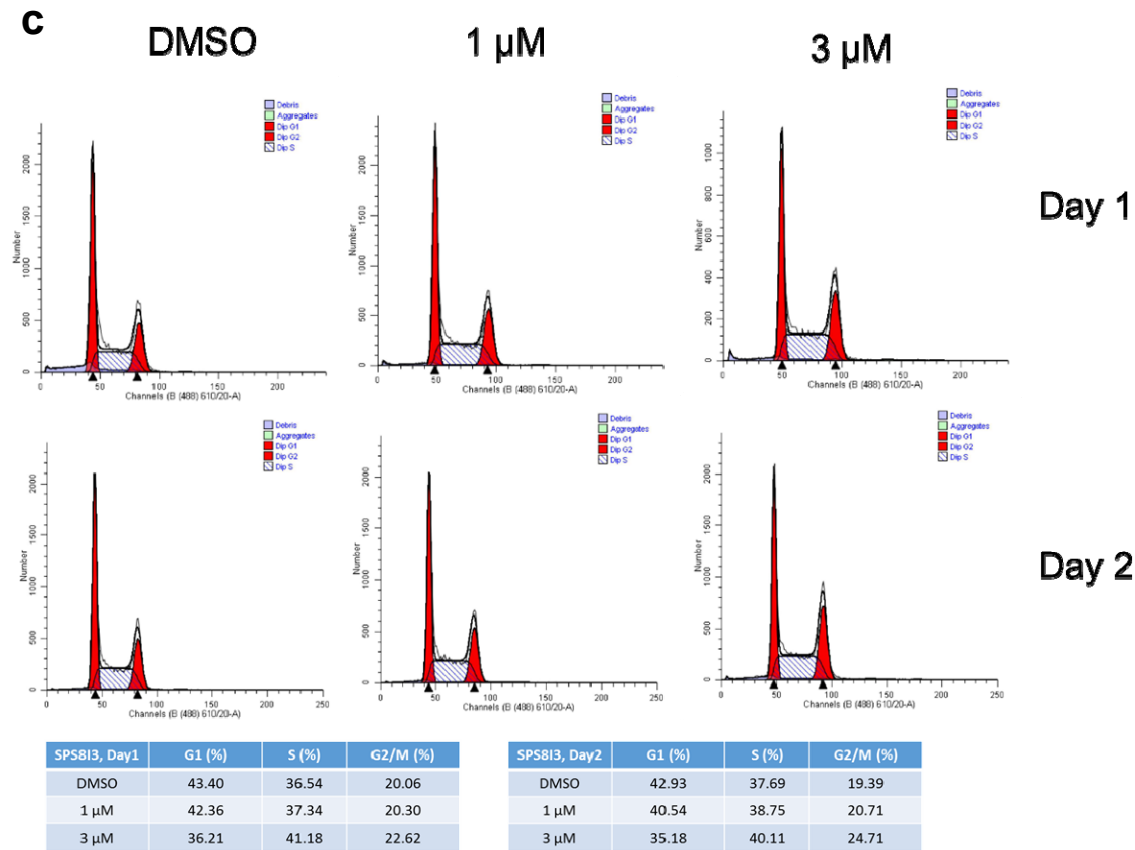


Figure S6. Cell cycle phenotypes associated with **SPS8I1~3**. (a) Cell cycle distribution of HEK 293T cells treated with DMSO control, or 1 and 5 μ M of **SPS8I1** over a period of 2 days. (b) Cell cycle distribution of HEK 293T cells treated with DMSO control, or 1 μ M **SPS8I2** a period of 2 days. (c) Cell cycle distribution of HEK 293T cells treated with DMSO control, or 1, 3 μ M **SPS8I3** over a period of 2 days.

10. SUPPLEMENTARY REFERENCES

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