***Purification of His_6-Cks1 from* E. coli**

pGEX-4T-1 vector containing GST-Thrombin-His_6-TEV-Cks1 was used to purify recombinant Cks1. BL21 *E.Coli* cells were used to generate recombinant protein. The cells were lysed by sonication in Buffer A (50 mM Tris pH 7.4, 200 mM NaCl, 2.5 mM PMSF, 5 mM DTT). Cleared lysate was allowed to bind to 500 μg of glutathione-charged sepharose beads (Amersham Biosciences). After 5 washes in Buffer A with 500 mM NaCl, GST-His_6-Cks1 was eluted with 10 mM glutathione. 10 Units of thrombin per 1 mg of GST-Cks1 were added for overnight cleavage at 4ºC in Buffer A in the presence of 2.5 mM of $CaCl_2$. The mixture was loaded onto a glutathione column to remove cleaved GST and uncut GST-Cks1. His_6-Cks1 was separated from thrombin by running the mixture through a Ni-NTA column and eluted with imidazole. A Superdex 75 gel fitration column was used to exchange His_6-Cks1 into a suitable storage buffer (150 mM NaCl, 1mM DTT, 50 mM Tris pH 7.5). SDS-PAGE followed by Coomassie blue staining was carried out to determine the concentration and purity of protein. The overall yield was 10 mg from 4 liters of bacterial culture.

***Expression and Purification of GST-Skp2/Skp1 from* E. coli**

The plasmid was transformed into *E. coli* BL21 and grown in LB at 37 ºC to an OD_{600} of 1.0. IPTG was added to a working concentration of 1 mM, and the bacterial culture was induced for 20 hours at 16°C. Cells were harvested by centrifugation and lysed by sonication in 50 mM Tris-HCl (pH 7.6), 200 mM NaCl, 2.5 mM PMSF, and 5 mM DTT. 10 mL of lysis buffer was used for each liter of culture. The clarified lysate was incubated with glutathione beads for 2 hours at 4°C. The beads were transferred into a gravity column and washed with 20 CV of the lysis buffer. The GST-Skp2/Skp1 complex was eluted with 10 mM reduced glutathione in 50 mM Tris-HCl

(pH 8.0), 200 mM NaCl, and 5 mM DTT. This was followed by anion exchange chromatography using a 30 mL Q-Sepharose fast flow (Sigma) gravity column. After washing the column with 10 CV of low salt buffer (25 mM Tris-HCl pH 7.6, 50 mM NaCl, 5 mM DTT,), the sample was applied to the column. The column was washed again with 20 CV of low-salt buffer, and the protein was eluted using a gradient of low-to-high salt (25 mM Tris-HCl pH 7.6, 400 mM NaCl, 5 mM DTT). The purification scheme was finished by buffer exchange through a 120 mL Superdex 200 size exclusion column on an AKTA FPLC system, using a storage buffer (50 mM Tris-HCl pH 7.6, 200 mM NaCl, 5 mM DTT). The fractions containing the purified complex were pooled, concentrated to 5 µg/µL, and rapidly frozen in liquid nitrogen. The yield was 4 mg of protein per liter of *E. coli* culture.

Western Blotting

For western blot analysis, total protein extracts were prepared by lysing Kip16 cells in lysis buffer (50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride). 50 µg of total soluble proteins were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and the membrane was blocked for 1 hour with 4% nonfat milk, followed by overnight incubation at 4°C with primary antibodies against p27 (1:1000, Santa Cruz, SC-53871) and Ezrin (1:5000, Sigma, E-8897), whose expression was used as a loading control. Membranes were then incubated with peroxidase-conjugated secondary antibodies for one hour at room temperature. Detection was performed using Super Signal WestDura Substrate (Thermo Scientific).

SUPPLEMENTARY FIGURE LEGENDS

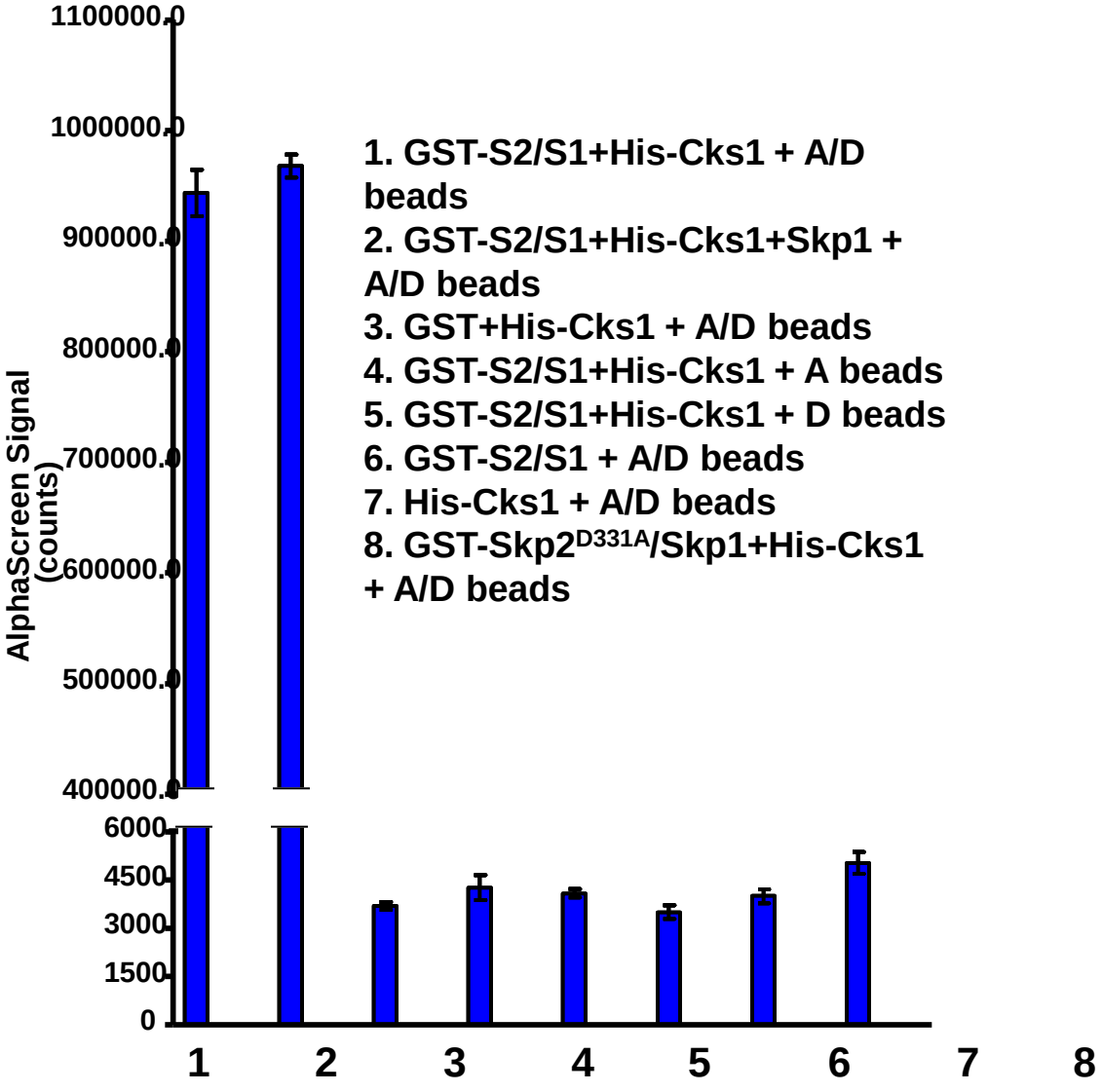
Supplementary Fig. 1. Detection of specific GST-Skp2/Skp1 and His_6-Cks1 interaction *in vitro*. 50 nM GST-Skp2/Skp1 was incubated with 125 nM Cks1 for 1 hour in AlphaScreen reaction buffer. A mixture of donor/acceptor beads was added into each well to a final concentration of 20 μg/mL in a reaction volume of 25 μL. After 2 hours of incubation, the 384-well plate was read on an Envision plate reader from PerkinElmer. Data are presented as mean +/- SD. Each experiment was performed in triplicates. A robust signal is generated upon GST-Skp2/Skp1-His_6-Cks1 interactions (bar 1). Equimolar addition of Skp1 does not affect Skp2-Cks1 binding activity (bar 2). AlphaScreen signal is significantly reduced when GST-Skp2/Skp1 is replaced with GST alone (bar 3), or when one of the reagents is omitted (donor beads, acceptor beads, GST-Skp2/Skp1, His_6-Cks1 – bars 4, 5, 6, 7, respectively).

Supplementary Fig. 2. Correlation of two independent AlphaScreen runs and corresponding statistical parameters. (A) Table of results that show statistical parameters Z', S/B ratio, and the number of hits identified in the screen. (B) Correlation plot of 1,600 compounds identified from runs 1 and 2 expressed as percent activity. Forty five hits, that were positive for both runs, were identified.

Supplementary Fig. 3. Immunoblotting of GFP-p27 from cell lysates prepared from the mink lung epithelial cell line Kip16 [28] using an anti-p27 antibody (Santa Cruz). Kip16 cells were incubated with 1 μM MG132 or NSC689857 or NSC681152 (0.1 μM, 1 μM, 2.5 μM and 5 μM) as indicated for 24 hours, and the cell extracts were resolved on SDS-PAGE followed by detection with p27 antibodies. The expression of ezrin was used as loading control.

Supplementary Fig. 4. Results of NCI 60 cell screens using NSC689857 and NSC681152. The results were downloaded from the DTP website (http://dtp.nci.nih.gov/).

Supplementary Fig. 5. Counterscreen of hits identified by the primary screen. (A) The effects of 45 hits on a biotinlyated GST control that artificially brings donor and acceptor beads together. The compounds were identified by their NSC numbers. 0.5 nM of biotinlyated GST and a mixture of donor/acceptor beads (20 μg/ml final concentration) were added into each well in a reaction volume of 25 μl with 10 μM of each compound. (B) The effect of NSC689857 and its analogs on a biotinlyated GST control that artificially brings donor and acceptor beads together. Data are presented in mean +/- SD form. Each experiment was done in triplicates.

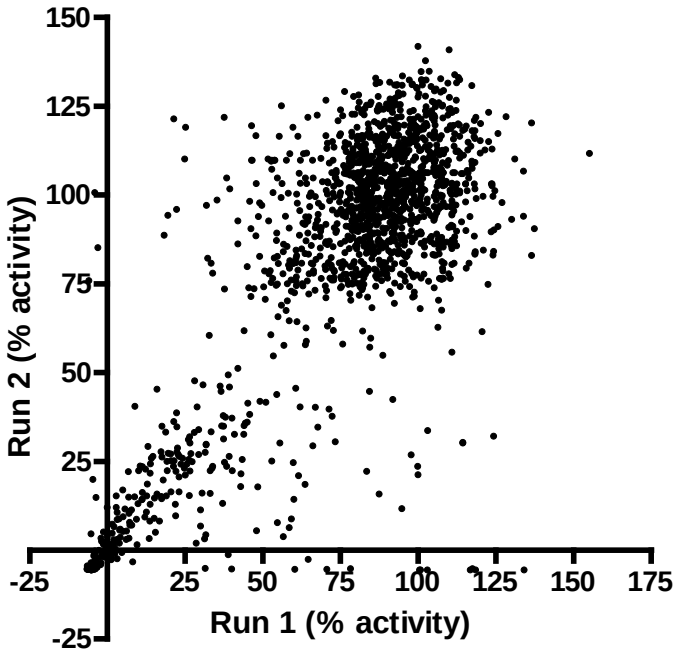


Supplemental Figure 1

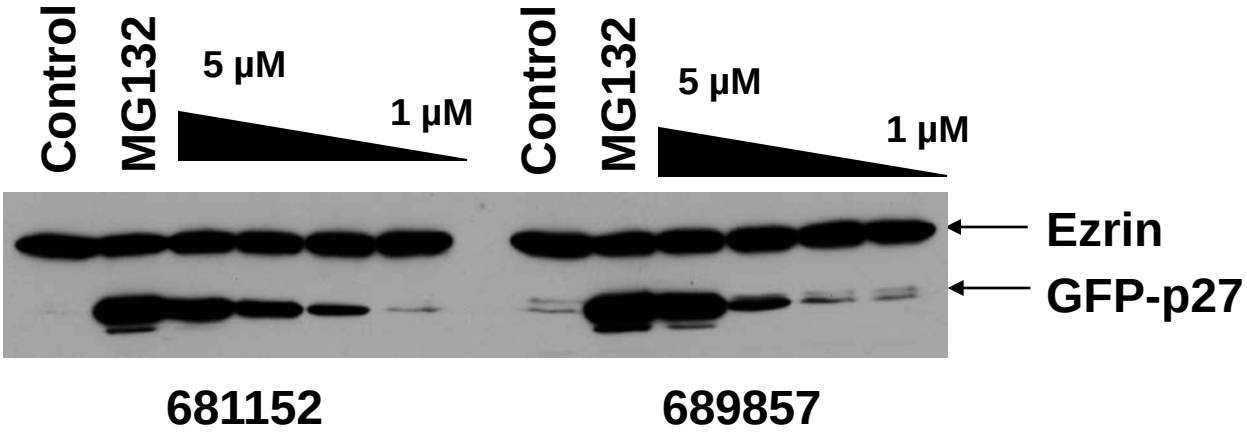
A.

Plate number	1	2	3	4	5
Z' trial 1	0.66	0.59	0.59	0.67	0.69
Z' trial 2	0.76	0.70	0.72	0.61	0.59
S/B trial 1	16	19	18	22	21
S/B trial 2	18	18	18	18	18
Hits	14	25	29	8	14

B.



Supplemental Figure 2



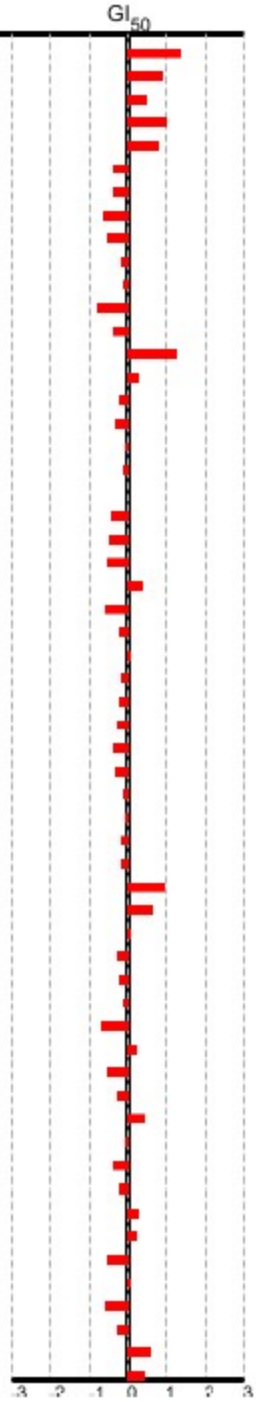
Supplemental Figure 3

GI_{50} Mean Graph for Compound 689857

NCI Cancer Screen Current Data

Average GI_{50} over all cell lines is 1.25E-6 M

Cell Panel	Cell Line	Log GI_{50}
Leukemia	CCRF-CEM	-7.3
	HL-60(TB)	-6.8
	K-562	-6.4
	MOLT-4	-6.9
	RPMI-8226	-6.7
Non-Small Cell Lung	A549/ATCC	-5.6
	EKVX	-5.6
	HOP-62	-5.3
	HOP-92	-5.4
	NCI-H226	-5.8
	NCI-H23	-5.8
	NCI-H322M	-5.1
	NCI-H460	-5.6
	NCI-H522	-7.2
Colon	COLO 205	-6.2
	HCC-2998	-5.7
	HCT-116	-5.6
	HCT-15	-5.9
	HT29	-5.8
	KM12	-5.9
	SW-620	-5.5
Central Nervous System	SF-268	-5.5
	SF-295	-5.4
	SF-539	-6.3
	SNB-19	-5.4
	SNB-75	-5.7
	U251	-6.0
Melanoma	LOX IMVI	-5.8
	M14	-5.7
	MDA-MB-435	-5.7
	MDA-N	-5.6
	SK-MEL-2	-5.6
	SK-MEL-28	-5.8
	SK-MEL-5	-5.9
	UACC-257	-5.8
	UACC-62	-5.8
Ovarian	IGROV1	-6.9
	OVCAR-3	-6.5
	OVCAR-4	-6.0
	OVCAR-5	-5.7
	OVCAR-8	-5.7
	NCI/ADR-RES	-5.8
	SK-OV-3	-5.2
Renal	786-0	-6.2
	A498	-5.4
	ACHN	-5.7
	CAKI-1	-6.3
	RXF 393	-5.9
	SN12C	-5.6
	TK-10	-5.7
	UO-31	-6.2
Prostate	PC-3	-6.1
	DU-145	-5.5
Breast	MCF7	-6.0
	MDA-MB-231/ATCC	-5.4
	HS 578T	-5.7
	BT-549	-6.5
	T-47D	-6.3



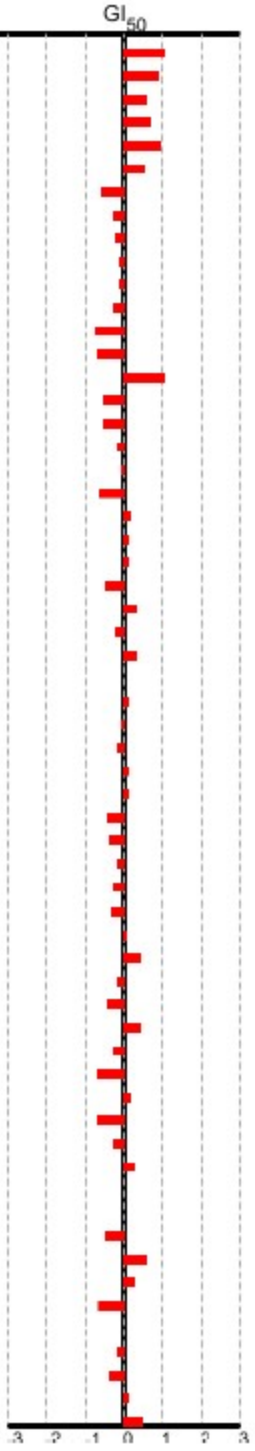
Supplemental Figure 4A

GI_{50} Mean Graph for Compound 681152

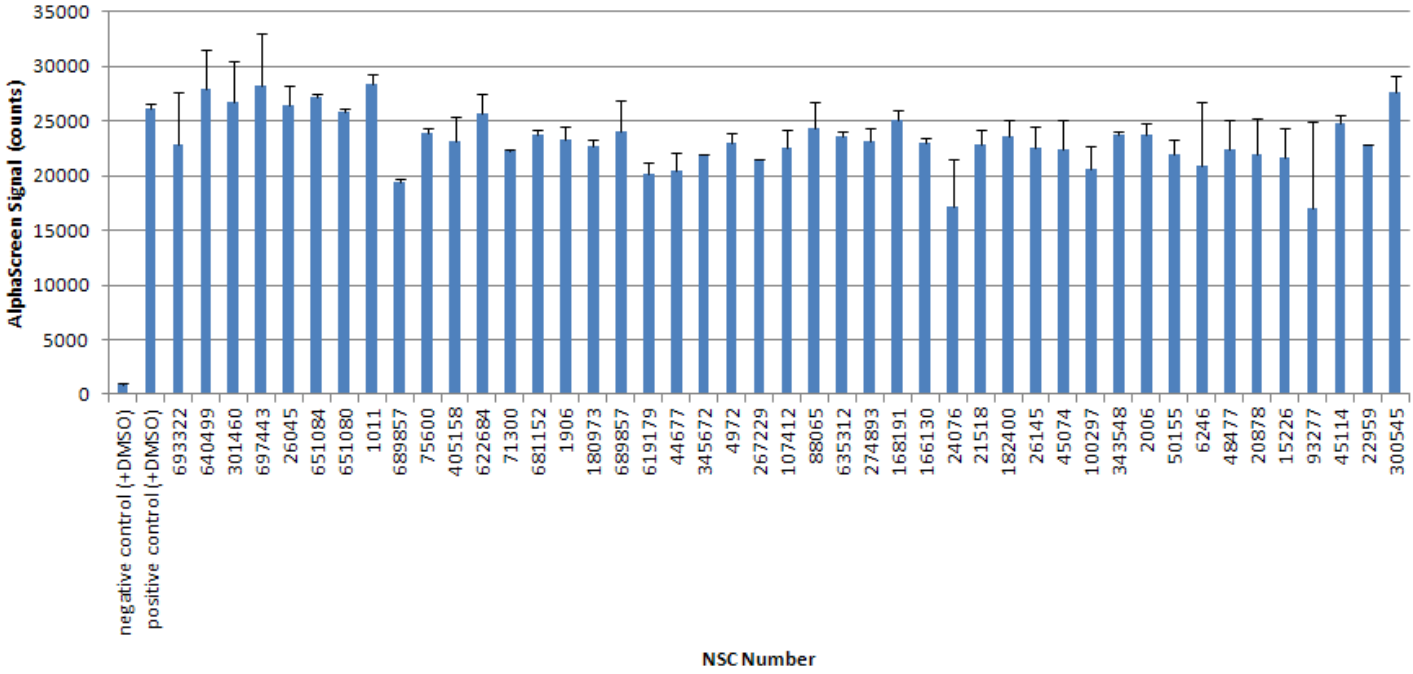
NCI Cancer Screen Current Data

Average GI_{50} over all cell lines is 7.97E-6 M

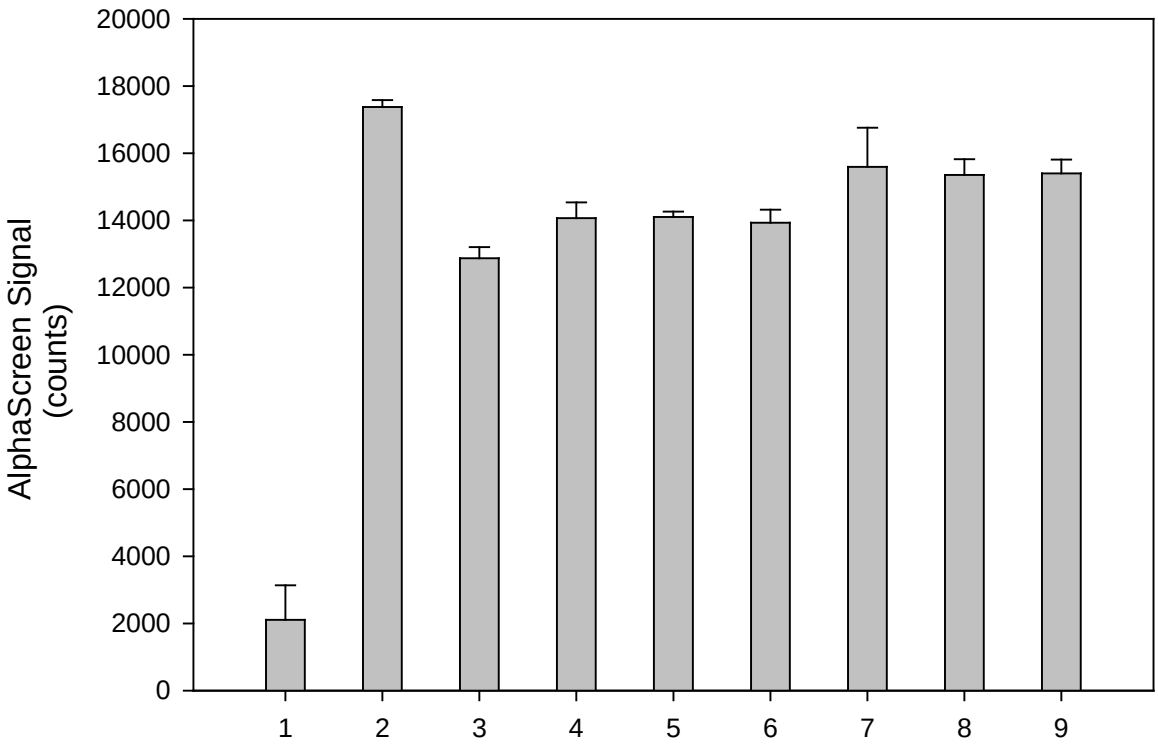
Cell Panel	Cell Line	Log GI_{50}
Leukemia	CCRF-CEM	-6.2
	HL-60(TB)	-6.0
	K-562	-5.7
	MOLT-4	-5.8
	RPMI-8226	-6.1
	SR	-5.6
Non-Small Cell Lung	A549/ATCC	-4.6
	EKVX	-4.9
	HOP-62	-4.9
	HOP-92	-5.0
	NCI-H226	-5.0
	NCI-H23	-4.9
	NCI-H322M	-4.4
	NCI-H460	-4.4
	NCI-H522	-6.2
Colon	COLO 205	-4.6
	HCC-2998	-4.6
	HCT-116	-5.0
	HCT-15	-5.1
	HT29	-4.5
	KM12	-5.3
	SW-620	-5.2
Central Nervous System	SF-268	-5.2
	SF-295	-4.7
	SF-539	-5.4
	SNB-19	-4.9
	SNB-75	-5.4
	U251	-5.1
Melanoma	LOX IMVI	-5.2
	MALME-3M	-5.1
	M14	-5.0
	MDA-MB-435	-5.2
	MDA-N	-5.2
	SK-MEL-2	-4.7
	SK-MEL-28	-4.8
	SK-MEL-5	-5.0
	UACC-257	-4.9
	UACC-62	-4.8
Ovarian	IGROV1	-5.2
	OVCAR-3	-5.5
	OVCAR-4	-5.0
	OVCAR-5	-4.7
	OVCAR-8	-5.5
	NCI/ADR-RES	-4.9
	SK-OV-3	-4.4
Renal	786-0	-5.3
	A498	-4.4
	ACHN	-4.9
	CAKI-1	-5.4
	RXF 393	-5.1
	SN12C	-5.1
	TK-10	-4.6
	UO-31	-5.7
Prostate	PC-3	-5.4
	DU-145	-4.5
Breast	MCF7	-5.1
	MDA-MB-231/ATCC	-5.0
	HS 578T	-4.8
	BT-549	-5.2
	T-47D	-5.6



Supplemental Figure 4B



Supplemental Figure 5A



1: A/D beads
2: Biotinylated-GST + A/D beads
3: 857 + Biotinylated-GST + A/D beads
4: Q857 + Biotinylated-GST + A/D beads
5: A857 + Biotinylated-GST + A/D beads
6: E857 + Biotinylated-GST + A/D beads
7: MHQ + Biotinylated-GST + A/D beads
8: BQ + Biotinylated-GST + A/D beads
9: NSC681152 + Biotinylated-GST + A/D beads

Supplemental Figure 5B