Experimental Methods

Reagents, antibodies and plasmids. AGK2 (Selleckchem, S7577), SirReal2 (Sigma Aldrich, SML1514), Tenovin-6 (Axon Medchem, 2249), and Trichostatin A or TSA (Apex Bio, A8183) were all purchased in the highest purity available. TM was synthesized as previously described.[1] Tenovin-6, AGK2, and SirReal2 were dissolved in dimethyl sulfoxide (DMSO), and TM was dissolved in ethanol (EtOH).

The acetyl-p53 (K382) (CST #2525) (used with 1:1000 dilution, overnight at 4˚C), SIRT2 (CST #12650) (used with a 1:1000 dilution, overnight at 4˚C or 3 hr at room temperature), HSP90 (CST #4877) (used with a 1:5000 dilution, 1 hr at room temperature) and anti-rabbit conjugated to horseradish peroxidase (CST #2525) (used with 1:3000 dilution, 1 hr at room temperature) antibodies were purchased from cell signaling technologies. The anti-β-actin conjugated to horseradish peroxidase (sc-47778 HRP) (used with 1:5000 dilution, 1 hr at room temperature) was purchased from Santa Cruz Biotechnologies. Anti-Flag M2 conjugated to horseradish peroxidase (A8592) (used with 1:7500 dilution, 1 hr at room temperature), and the anti-acetyl-α-tubulin (6-11B-1) (MABT868) antibodies were from Sigma-Aldrich. Cy3 Goat-anti-mouse (A10521) was purchased from Life Technologies. Nitrotetrazolium Blue chloride (N6876) and low gelling temperature agarose (A0701) were purchased from Sigma Aldrich.

For ectopic overexpression of SIRT2, pCMV4a-Flag-SIRT2 was cloned as previously described.[1] For lentiviral overexpression of SIRT2, pCDH-Flag-SIRT2 was cloned by inserting SIRT2 into pCDH-CMV-MCS-EF1-Puro between the EcoRI and XhoI restriction sites with a C-terminal Flag tag.

Expression and Purification of Sirtuins. Human SIRT1, SIRT2, SIRT3 and SIRT6 were expressed and purified as previously described.[1-2]

Synthesis of H3K9Ac and Myr peptides. H3K9Ac, H3K9Myr, and H3K9 peptides were all synthesized as previously described using standard solid phase peptide synthesis.[3]

In vitro deacylation assay (with pre-incubation). Various concentrations of AGK2, SirReal2, Tenovin-6 (in DMSO) and TM (in EtOH) (0.0064, 0.032, 0.16, 0.8, 4.0, 20, 100, and 200 μM) were added to solutions containing 20 mM Tris-HCl (pH 8.0), 1 mM NAD, 1 mM dithiothreitol (DTT), and 0.1 μM of SIRT1, 0.2 μM of SIRT2, or 0.4 μM of

SIRT3. The reaction mixtures were incubated at 37˚C for 15 min. Then 10 μM of H3K9Ac peptide or 0.10 μM of H3K9Myr was added to initiate the deacylation reactions. The reactions were incubated at 37°C (3 min reaction for SIRT1, 6 min reaction for SIRT2 with H3K9Ac, 5 min reaction for SIRT2 with H3K9Myr, 10 min reaction for SIRT3 with H3K9Ac, and 75 min reaction for SIRT6 with H3K9Myr). The reaction times were determined to ensure a conversion of no more than 20% from the acylated to free peptide for the control samples. The reactions were stopped by adding an equal volume of acetonitrile. After quenching the reactions, the samples were centrifuged at 17,000 g for 20 min to remove any precipitated proteins. The cleared supernatant was analyzed by HPLC with a reverse phase C18 column (Kinetex XB-C18 100A, 100 mm × 4.60 mm, 2.6 μm, Phenomenex) using a gradient of two solvents (A: 0.1% trifluoroacetic acid in water; B: 0.1% trifluoroacetic acid in acetonitrile): starting with 0% B for 2 min, then 0% to 20% B in 2 min, 20% to 40% B in 13 min, and then 40% to 100% for 2 min at 0.5 mL/min. The peak areas of free H3K9 and either H3K9Ac or H3K9Myr were measured based on the absorbance monitored at 280 nm. The conversion rate was calculated from the peak areas as the fraction of the free H3K9 peptide from the total peptide. AGK2 and Tenovin-6 peaks overlapped with the H3K9Myr peptide peak, and thus the areas of these compounds were subtracted from the H3K9Myr peptide peak area. IC_{50} values were calculated using Prism 7 software.

In vitro deacylation assay (without pre-incubation): Various concentrations of AGK2, SirReal2, Tenovin-6 (in DMSO) and TM (in EtOH) (0.0064, 0.032, 0.16, 0.8, 4.0, 20, 100, and 200 μM) were added to solutions containing 20 mM Tris-HCl (pH 8.0), 1 mM NAD, 1 mM DTT, 10 μM H3K9Ac peptide or 0.10 μM H3K9Myr peptide, and 0.2 μM of SIRT2. The reactions were incubated at 37 °C for 15 min and then quenched and analyzed similarly as described above.

Cell culture and transfection. All cells were cultured in media supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Invitrogen) and at 37˚C with 5% CO_2 unless otherwise specified. MCF-7, MDA-MB-231, MDA-MB-468, HeLa, HME1, and HEK-293T cells were grown in DMEM media (Invitrogen). SK-BR-3, K562, SW948, A549, H520, and ASPC-1 cells were grown in RPMI-1640 media (Invitrogen). HCT116 and HT29 cells were cultured in McCoy's 5A medium, and CF-PAC1 cells were cultured in

IMDM. The MCF-10A cells were cultured in mammary epithelial cell growth medium (MEGM; Lonza) with supplements according to manufacturer's instruction.

To overexpress SIRT2 in MDA-MB-468 cells, the pCMV-tag-4a vector containing SIRT2 or empty pCMV-tag-4a (negative control) were transfected into cells using FuGene 6 (Promega, Madison, WI) according to manufacturer's protocol.

Cell viability assay. To determine the GI_{50} values of the compounds in cells, 1,000-6,000 cells (depending on the cell line) were seeded per well in a 96 well plate in 100 µL of media. The next day, 100 µL of inhibitors in media were added to each well-- so the final concentrations of the inhibitors used were 50, 25, 10, 5, 1, and 0 µM. After 72 hours, CellTiter blue (Promega) was added to each well and the plates were placed at 37˚C for 4 hours. After 4 hours, the cell viability was measured according to the manufactures protocol. The background was subtracted, and the IC_{50} values were calculated using Prism 7 software.

Western blot. Proteins were detected by western blot as previously described.[4] Briefly, proteins were resolved on a 12% SDS-PAGE gel and subsequently transferred to PVDF membranes. After incubation with HRP conjugated primary or secondary antibodies, the proteins of interest were detected using the ECL reagent (Pierce Biotechnology Inc.) and visualized on a Typhoon 9400 Imager (GE Healthcare). Western blots were processed using ImageJ software.[5]

Detection of Ac-p53 levels in cells. MCF-7 cells were treated with the 25 µM of the noted inhibitor and 200 nM of Trichostatin A (TSA) for 6 hours. After treatment, the cells were collected, and washed with ice cold PBS three times. The cell pellet was subsequently lysed with 4% SDS lysis buffer supplemented with protease inhibitor cocktail (Sigma) and universal cell nuclease (Thermo). The protein concentration was determined using the Pierce BCA assay kit (Thermo) following the manufactures protocol, and Ac-p53 and levels were detected by western blot.

Immunofluorescence to detect acetyl-α-tubulin levels in cells. To detect Ac-α-tubulin levels in cells, slight modifications were made to previous protocols. Briefly, 2 x 10^5 MCF-7 cells were seeded in in 35-mm glass bottom dishes (MatTek). After 24 hours, the cells were treated with either the inhibitor at the noted concentration or the control for 6 hours. The cells were washed three times with PBS and fixed with ice cold methanol

(10 minutes). To permeabilize the membrane, the cells were treated with 0.1% Triton-X in PBS. After 10 minutes, the cells were washed three times (5 minute each) with PBS. After the final wash, the cells were blocked with 1% BSA in TBST (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) for 30 minutes and then incubated with the Ac-α-tubulin antibody (1:100) in 1% BSA overnight at 4˚C. After primary antibody incubation, the cells were washed with TBST three times (5 minute each). Subsequently the fluorophore conjugated antibody was added to the cells in 1% BSA in TBST (1:1000) and incubated for 1 hour at room temperature in the dark. After three 5-minute TBST washes, the cells were mounted and the nuclei was stained with DAPI Fluoromount-G® (Southern Biotech, 0100-01), and a coverslip was placed on top of the cells. The cells were imaged using a Zeiss LSM880 inverted confocal microscope set up with a 40x oil objective, using the same settings for all samples, and the images were processed using Fiji software.[5]

Generation of pCDH-Flag-SIRT2 lentivirus. Approximately 1.5 to 2 million WT-HEK-239T cells in a 10cm dish were transfected with 5 μg of empty pCDH-CMV-MCS-EF1-Puro or pCDH-CMV-MCS-EF1-Puro with FLAG-SIRT2, 5 μg of pCMV-ΔR8.2 and 1 μg of pM2D.G. For the transfection, 36μL of FuGene6 was added to 576uL of serum free DMEM. After 5 minutes the plasmids were added and allowed to incubate. After 15 minutes, the transfection mixture was added to cells with 8mL of fresh DMEM (supplemented with 10% FBS). After 12 h, the media was changed to 8mL of fresh FBS supplemented media. After 24, 48 and 72 h the media was harvested, and spun down at 3000 rpm for 10 minutes. The virus was then filtered with a 0.45 μM filter. Virus was stored at -80˚C in the dark until use.

Generation of stable overexpressing SIRT2 HCT116 cells. HCT-116 cells overexpressing SIRT2 or empty vector control were generated by infecting cells with viruses containing either pCDH-Flag-SIRT2 vector or empty pCDH vector. About 1 x 10^5 HCT116 cells were seeded in a 6-well dish. The next day, 1 mL of virus was added to the cells with polybrene at a concentration of 6 μg/mL. After 6 hours, the cells were placed in McCoy's 5A media with 10% FBS. After 72 hours the cells were treated with puromycin for 1 week. After puromycin selection, the cells were allowed to recover. SIRT2 overexpression was confirmed by SIRT2 and Flag western blots.

Soft agar colony formation assay. To each well in a 6-well dish, 2 mL of a 0.6% LMP agar solution was plated. After 30 minutes, 1,000 cells were plated in 1 mL 0.3% LMP agar solution supplemented with the noted inhibitor or vehicle control concentration. After 30 minutes, an additional 1 mL of 0.3% LMP agar solution supplemented with the noted inhibitor concentration was added. The plates were placed in the incubator at 37˚C supplemented with 5% CO_2. An additional 1 mL of inhibitor-supplemented 0.3% LMP agar solution was added after 6 days. After 10-12 days, 200 μL of 1 mg/mL of Nitrotetrazolium Blue chloride in PBS was added to each well. After 12 hours, the plates were placed at 4˚C, and imaged using a chemi-doc imager with the Coomassie blue settings. Colonies were counted using FIJI software.[5]

Cytotoxicity assay with SIRT2 overexpression in MDA-MB-468 cells. MDA-MB-468 were seeded in a 12-well plate with 3 x 10^4 cells/well. After 12 hours, either pCMV4a-Flag-SIRT2 or empty pCMV4a was overexpressed (1 μg of DNA per well of a 12-well dish) using Fugene6 (Promega). After 24 hours, the cells were treated with either ethanol, DMSO or the indicated inhibitor at the noted concentrations. After 12 hours the media was removed, and the cells were washed two times with PBS. To fix the cells, ice cold methanol was added. After 10 minutes, the cells were stained with 0.25% crystal violet (m/v, in 25% methanol) (SIGMA) for 5 minutes. The stained cells were washed with water, and allowed to air dry. The stain was resolubilized in a solution of 0.2% SDS in 50% ethanol, and the absorbance of the solution was read at 550 nm. SIRT2 overexpression was confirmed by western blot. Statistical significance was determined using an unpaired, two tailed, student's t-test in Microsoft Excel.

Cytotoxicity assay with SIRT2 overexpression in stable SIRT2 overexpressing HCT116 cells. HCT116 cells stably over-expressing SIRT2 or empty pCDH vector were seeded in a 12-well plate with 3 x 10^4 cells/well. After 24 hours, the inhibitors were added for 24 hours. The rest of the procedure was the same that used for MDA-MB-468 cells described above.

Supplemental References:

[1] H. Jing, J. Hu, B. He, Y. L. Negron Abril, J. Stupinski, K. Weiser, M. Carbonaro, Y. L. Chiang, T. Southard, P. Giannakakou, R. S. Weiss, H. Lin, *Cancer Cell* **2016**, *29*, 767-768.

[2] X. Zhang, S. Khan, H. Jiang, M. A. Antonyak, X. Chen, N. A. Spiegelman, J. H. Shrimp, R. A. Cerione, H. Lin, *Nat Chem Biol* **2016**, *12*, 614-620.
[3] P. Aramsangtienchai, N. A. Spiegelman, B. He, S. P. Miller, L. Dai, Y. Zhao, H. Lin, *ACS Chem Biol* **2016**, *11*, 2685-2692.
[4] H. Jiang, S. Khan, Y. Wang, G. Charron, B. He, C. Sebastian, J. Du, R. Kim, E. Ge, R. Mostoslavsky, H. C. Hang, Q. Hao, H. Lin, *Nature* **2013**, *496*, 110-113.
[5] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, *Nat Methods* **2012**, *9*, 676-682.

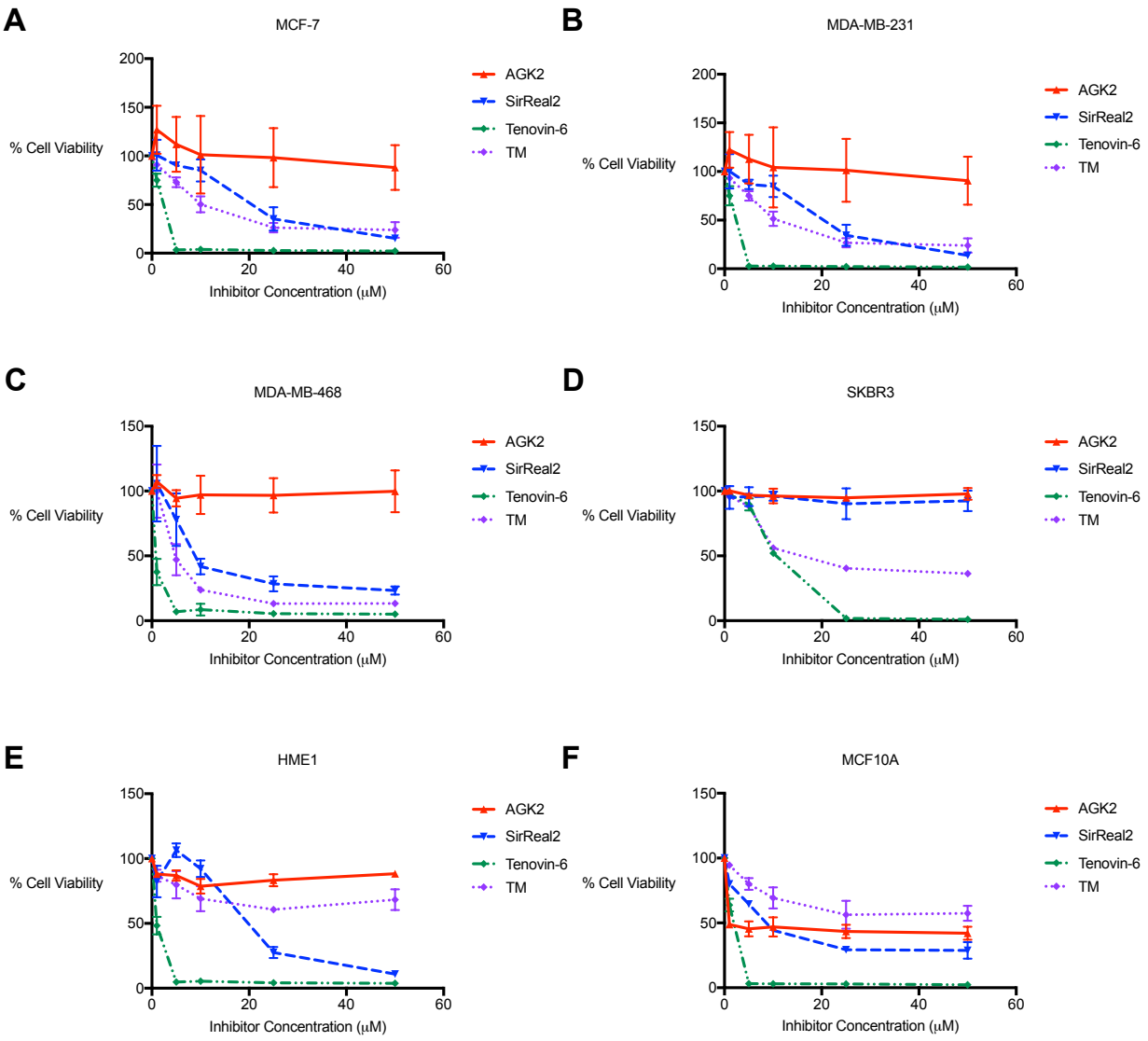
Supplemental Information

Supplemental Table 1. In vitro IC_{50} (μM) values for inhibition of SIRT2 deacetylation and demyristoylation activity without pre-incubating SIRT2 with the inhibitors and NAD^+.

	deacetylation	**demyristoylation**
AGK2	10 ± 8	>200
SirReal2	0.16 ± 0.01	>100
Tenovin-6	24 ± 9	>200
TM	0.04 ± 0.01	>100

Supplemental Table 2. GI_{50} values (in μM) of different SIRT2 inhibitors in various breast cancer and normal breast cell lines. The values are average of three independent experiments each done in duplicate.

	AGK2	SirReal2	Tenovin-6	TM
MCF-7	>50	16.3 ± 0.2	1.24 ± 0.05	10.3 ± 0.7
MDA-MB-231	>50	30 ± 10	1.6 ± 0.4	13 ± 4
MDA-MB-468	>50	13 ± 4	0.61 ± 0.05	4 ± 1
SK-BR-3	>50	>50	9.3 ± 1.7	27 ± 7
HME-1	>50	18 ± 2	1.4 ± 0.2	>50
MCF-10A	>50	11 ± 2	1.7 ± 0.3	>50

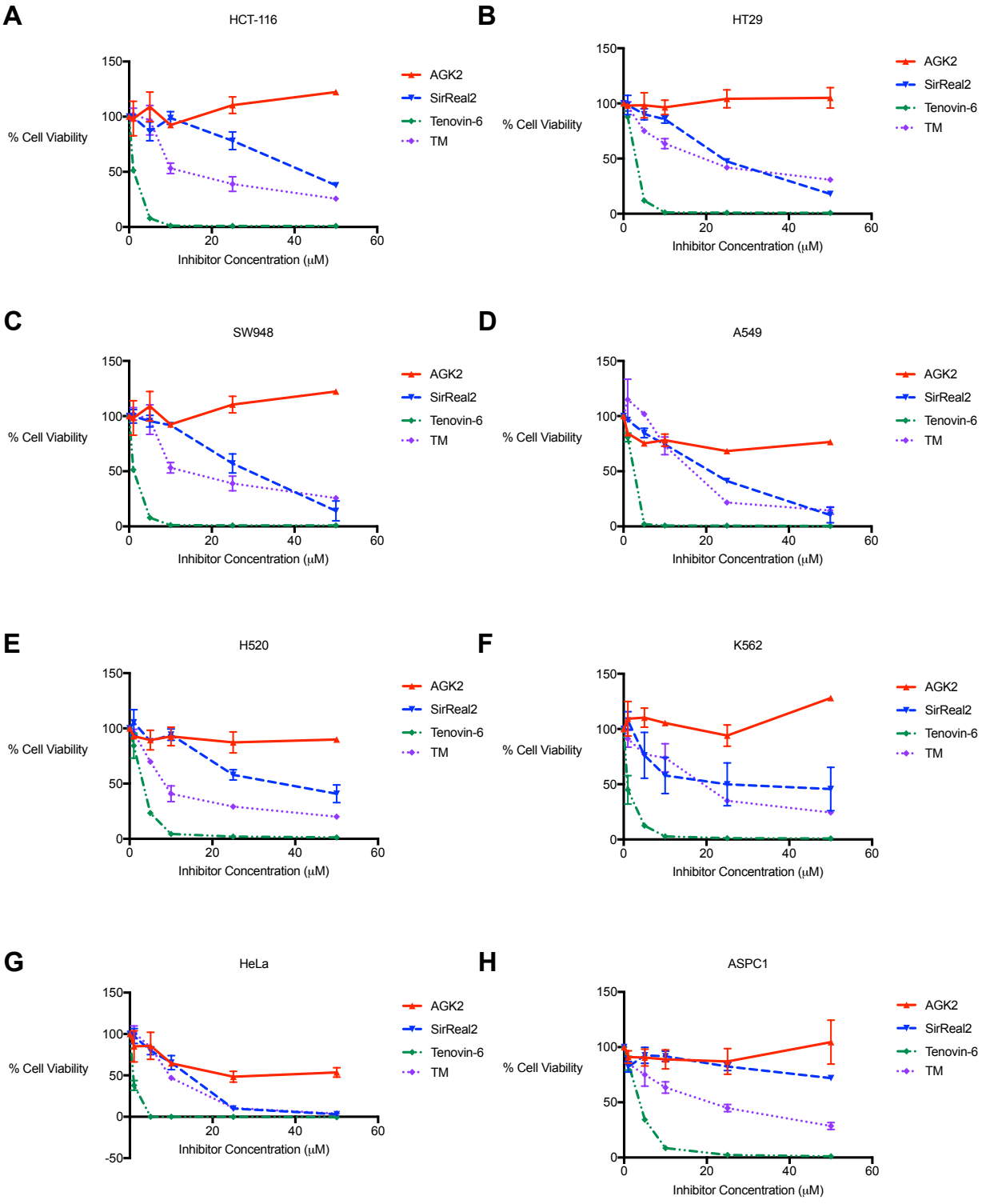


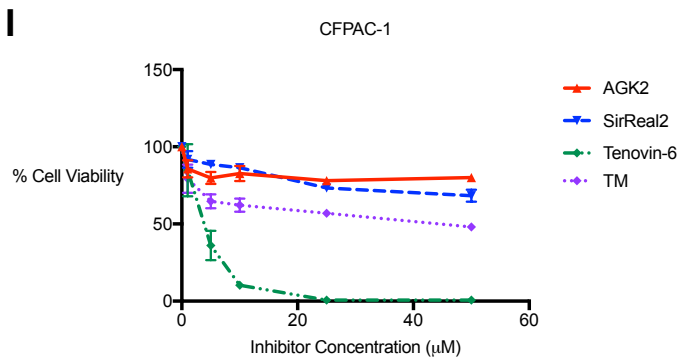
Supplemental Figure 1. Inhibition of cell viability curves from one representative trial of at least three independent experiments. (A) MCF-7 cells, (B) MDA-MB-231 cells, (C) MDA-MB-468 cells, (D) SK-BR-3 cells, (E) HME-1 cells, (F) MCF-10A cells.

Supplemental Table 3. GI_{50} values (μM) of different SIRT2 inhibitors in various cancer cell lines. The values are average and standard deviation of three independent experiments (except those noted) each done in duplicate.

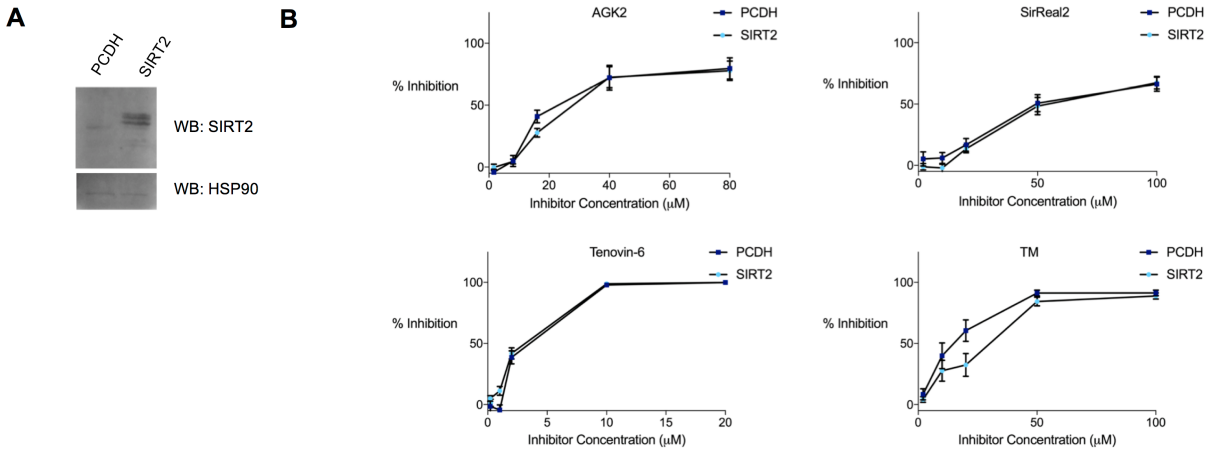
Cancer type	Cell Line	AGK2	SirReal2	Tenovin-6	TM
Colon	HCT116	>50	34 ± 1*	1.34 ± 0.05	19 ± 0.8
	HT29	>50	28 ± 2	1.4 ± 0.2	30 ± 3
	SW948	>50	27.6 ± 0.8*	1.05 ± 0.08	16 ± 2*
Lung	A549	>50	21 ± 3	3 ± 1	10 ± 5
	H520	>50	35 ± 1	2.6 ± 0.2	8.6 ± 1.2
Lukemia	K562	>50	23 ± 6	0.94 ± 0.09	16 ± 2
Cervical	HeLa	>50	11 ± 2	0.7 ± 0.1	9.1 ± 0.8
Pancreatic	ASPC1	>50	>50	3.0 ± 0.3	24 ± 5
	CFPAC1	>50	>50	3.2 ± 0.3	>50

*This value was determined by two independent experiments.

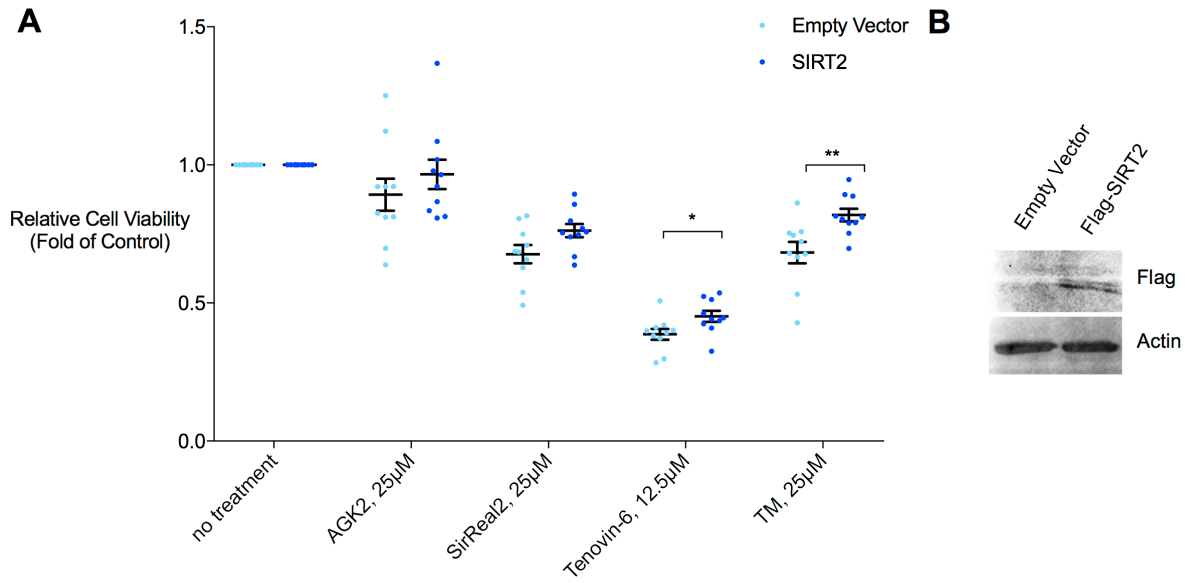

A
HCT-116
B
HT29
C
SW948
D
A549
E
H520
F
K562
G
HeLa
H
ASPC1
% Cell Viability
Inhibitor Concentration (μM)
AGK2
SirReal2
Tenovin-6
TM



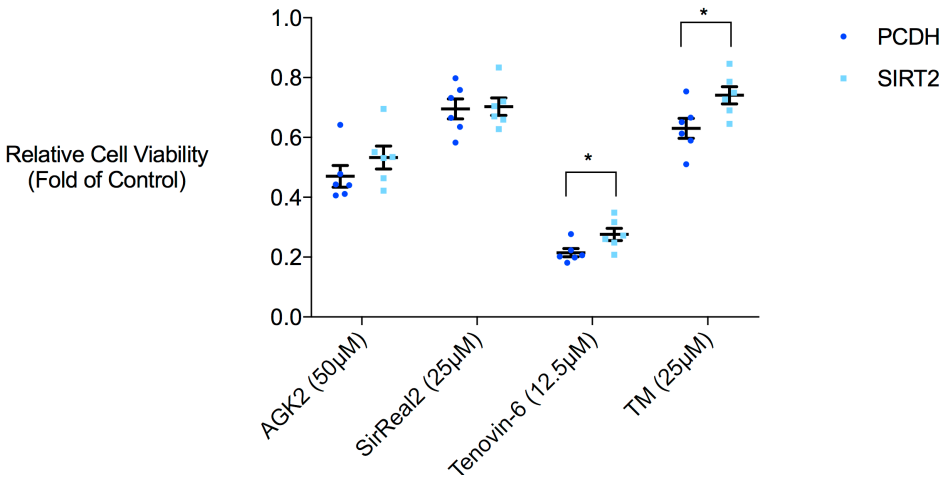
Supplemental Figure 2. Cell proliferation curves from one representative trial of at least three independent experiments. (A) HCT-116 cells (B) HT-29 cells (C) SW948 cells (D) A549 cells (E) H520 cells (F) K-62 cells (G) HeLa cells (H) ASPC1 cells (I) CFPAC1 cells



Supplemental Figure 4. Soft Agar assay showed that SIRT2 overexpression desensitizes HCT116 cells to the effect of TM and to a smaller extent AGK2, but not SirReal2 or Tenovin-6 on anchorage independent growth. (A) Representative western blot confirming SIRT2 overexpression in the pCDH/ SIRT2 HCT116 cells. (B) Inhibition curves used to calculate the GI_{50} values (the mean and SEM (n=12 for AGK2, SirReal2, Tenovin-6 and n=9 for TM) from all individual values from all replicates are presented.



Supplemental Figure 5. The inhibition effect from TM is most sensitive to SIRT2 overexpression. (A) Relative cell viability after 12-h inhibitor treatment in MDA-MB-468 cells overexpressing empty vector or SIRT2. Statistical significance was determined using an unpaired two-tailed student's t test in Excel. Error bars represent the SEM, and the center bar represents the mean. * $P < 0.05$, **= $P < 0.01$. (B) Representative western blot showing SIRT2 overexpression



Supplemental Figure 6. SIRT2 Overexpression desensitizes HCT116 cells to TM and Tenovin-6 treatment. Statistical significance was determined using a two-tailed student's t test in Excel. Error bars represent the SEM, and the center bar represents the mean. *= $P < 0.05$.