SUPPLEMENTAL MATERIALS
(Supplemental Tables and Supplemental Figures)

HTS identifies the DNMT1 inhibitor, 5-azacytidine, as a potent inducer of PTEN: central role for PTEN in 5-azacytidine protection against pathological vascular remodeling

Keith A. Strand[1], Sizhao Lu[1], Marie F. Mutryn[1], Linfeng Li[4], Qiong Zhou[4], Blake T. Enyart[2,3], Austin J. Jolly[1], Allison M. Dubner[1], Karen S. Moulton[3,2], Raphael A. Nemenoff[1,2], Keith A. Koch[2,3], Daniel LaBarbera[4], Mary C.M. Weiser-Evans[1,2,†]

[1]Division of Renal Diseases and Hypertension, Department of Medicine, [2]School of Medicine, Consortium for Fibrosis Research & Translation, [3]Division of Cardiology, Department of Medicine, [4]School of Pharmacy and Pharmaceutical Sciences, University of Colorado, Anschutz Medical Campus, Aurora, CO 80045 USA

Supplemental Table I. Compounds rescreened following initial HTS.

Compound	Description
3,4'-Dimethoxyflavone	
3Alpha-Acetoxydihydrodeoxygedunin	
AC480 (BMS-599626)	
Acepromazine Maleate	sedative
Acesulfame Potassium	sweetener
AEE788 (NVP-AEE788)	
Albendazole	anthelmintic
Albuterol (+/-)	bronchodilator, tocolytic
Almotriptan	5HT 1B/2D receptor agonist
Alpha-Dihydrogedunol	
Altrenogest	progestinantineoplastic
Amiodarone Hydrochloride	adrenergic agonist, coronary vasodilator, Ca channel blocker
Avocadyne	antibacterial, antifungal
Avocatin B	antibacterial, antifungal
AZ 960	
Azacitidine	antineoplastic, pyrimidine antimetabolite
AZD2014	
AZD4547	
AZD5438	
Azelastine Hydrochloride	H1 antihistamine (nonsedating); leukotriene synthesis blocker
Benzydamine Hydrochloride	analgesic, antipyretic, antiinflammatory
Bepridil Hydrochloride	antiarrhythmic
Betaxalol Hydrochloride	antihypertensive, beta-blocker, antianginal
Bisphenol A	endocrine disruptor, plastic monomer
BMS-754807	
Bretylium Tosylate	inhibitor of norepinephrine release
Bromhexine Hydrochloride	expectorant
Bromperidol	antipsychotic
Carvedilol	beta-adrenergic blocker
CAY10505	
Ceforanide	antibacterial
Cefpodoxime Proxetil	antibacterial
Cepharanthine	antineoplastic, hepatoprotectant, radiopropective
Chlorophyllide Cu Complex Na Salt	antineoplastic
Chloroquine Diphosphate	antimalarial, antiamebic, antirheumatic, intercalating agent
Chlorpromazine	antiemetic, antipsychotic
Cinnarazine	H1 antihistamine
Cintriamide	antipsychotic
Ciprofibrate	antihyperlipidemic
Cisapride	peristaltic stimulant
Clotrimazole	antifungal
Clozapine	antipsychotic
Colchicine	antimitotic, antigout agent
Cyclobenzaprine Hydrochloride	muscle relaxant (skeletal)
Cycloheximide	protein synthesis inhibitor
Cyclosporine	immunosuppressant
Cyproheptadine Hydrochloride	H1-antihistamine, antipruritic

Dactinomycin	antineoplastic, intercalating agent
Debrisoquin Sulfate	anti-hypertensive
Deguelin(-)	antineoplastic, antiviral, insecticide
Deoxysappanone B 7,3'-Dimethyl Ether Acetate	
Deoxysappanone B 7,4'-Dimethyl Ether	
Derrusnin	
Desacetylcolforsin	
Desloratidine	H1-antihistamine
Diclazuril	coccidiostat
Dihydromunduletone	
Dihydrorotenone	
Diperodon Hydrochloride	analgesic, anesthetic
Docetaxel	antineoplastic
Docosanol	antiviral
Drofenine Hydrochloride	antispasmodic
Duloxetine Hydrochloride	antidepressant
Ergocalciferol	antirachitic vitamin; LD50 (rat) 56 mg/kg po
Ergosterol Acetate	
Ethanolamine Oleate	sclerosing agent
Ethinyl Estradiol	estrogen, plus progestogen as oral contraceptive
Famprofazone	analgesic, antipyretic, CNS stimulant
Felodipine	vasodilator, Ca channel blocker
Fipronil	GABA Cl channel agonist, antiparasitic
Flavoxate Hydrochloride	smooth muscle relaxant
Fluoxetine	antidepressant
Fluvoxamine Maleate	antiobsessional agent
Gefitinib	antineoplastic
Go 6983	
Haematoporphyrin	antidepressant, antineoplastic
Homidium Bromide	antiprotozoal, intercalcate with DNA
Humulene (Alpha)	
Hycanthone	anthelmintic, hepatotoxic
Imatinib Mesylate (STI571)	
Ipriflavone	anabolic
Itraconazole	antifungal
KU-55933 (ATM Kinase Inhibitor)	
KU-60019	
Lanosterol	
Larixol	
Levobunolol Hydrochloride	beta-adrenergic blocker
Levothyroxine	antihypercholesterimic, thyromimetic
Lidoflazine	calcium channel blocker, anti-anginal
Liothyronine	thyroid hormone blocker
Lithocholic Acid	LD50(mouse) 3900 mg/kg po
Lomerizine Hydrochloride	antimigraine, cerebral vasodilator, Ca channel blocker
Loperamide Hydrochloride	Ca channel blocker
Maprotiline Hydrochloride	antidepressant
Mebendazole	anthelmintic
Meglutol	antihyperlipoproteinemia
Methyl Robustone	
Mexamine	5HT agonist

Mianserin Hydrochloride	5HT antagonist
Milnacipran Hydrochloride	inhibitor of norepinephrine and seritonin uptake, treatment of fibromyalgia
MK-8776 (SCH 900776)	
Molindone Hydrochloride	antipsychotic
N- (9-Fluorenylmethoxycarbonyl)-L-Leucine	antiinflammatory
Niclosamide	anthelmintic, teniacide
Nitarsone	antiprotozoal
Nitrendipine	antihypertensive
Nocodazole	antineoplastic, antimitotic
Octocrylene	sunscreen
Oleanolic Acid Acetate	
Ondansetron	antiallergic, antiemetic, anti-schizophrenic
Orlistat	reversible lipase inhibitor, antiobesity
Oxethazaine	anesthetic (local)
Oxibendazole	anthelmintic
Paramethadione	anticonvulsant
PD184352 (CI-1040)	
Phenformin Hydrochloride	antidiabetic
Picropodophyllin	Insulin growth factor 1 receptor inhibitor, antineoplastic
Picropodophyllin Acetate	
Pizotyline Malate	5HT antagonist, antimigraine
Podofilox	antineoplastic, inhibits microtubule assembly, and human DNA topoisomerase II; antimitotic agent
Pregnenolone Succinate	glucocortcoid, antiinflammatory
Prochlorperazine Edisylate	antiemetic, antipsychotic, treatment of vertigo
Promethazine Hydrochloride	antihistaminic
Protryptyline Hydrochloride	antidepressant
Purpurin	xanthine oxidase inhibitor, irritant
Pyrethrins	insecticide, Na channel toxin
R406 (free base)	
Reserpine	antihypertensive
Ribavirin	antiviral
Ro 31-8220 Mesylate	
Rosolic Acid	diagnostic aid
Rotenonic Acid, Methyl Ether	
Rutilantinone	coccidiostat
S-Ruxolitinib (INCB018424)	
Salmeterol Xinafoate	beta adrenergic agonist
Selegiline Hydrochloride	antidepressant, MAO inhibitor, antiparkinsonian
Simvastatin	antihyperlipidemic, HMGCoA reductase inhibitor
SNS-032 (BMS-387032)	
Sotalol Hydrochloride	beta-adrenergic agonist
Thioridazine Hydrochloride	antipsychotic
Thiothixene	antipsychotic
Tiratricol	thyroid agent
Trimeprazine Tartrate	antipruritic
Troleandomycin	antibacterial
U0126-EtOH	
VE-822	

Vincristine Sulfate	antineoplastic
Vinorelbine	antineoplastic
Vinpocetine	cerebral vasodilator, antimotion
Zoledronic Acid	

Supplemental Table II. Final hit compounds.

Compound	Supplier	Catalog Number	Amount
Azacitidine	Sigma-Aldrich	A2385-100MG	100 mg
Itraconazole	Selleckchem	S2476	100 mg
KU-55933 (ATM Kinase Inhibitor)	Selleckchem	S1092	10 mM/1 mL DMSO
R406 (free base)	Cayman Chemical	11422	5 mg
Lithocholic Acid	Cayman Chemical	20253	5 g
Picropodophyllin Acetate	Selleckchem	S7668	5 mg
Deguelin(-)	Selleckchem	S8132	10 mg
3-O-Acetyloleanolic acid	Ark Pharm Inc	4339-72-4	5 mg
Oxibendazole	Selleckchem	S1851	50 mg
Orlistat	Tocris	3540	10 mg
Humulene (Alpha)	Sigma-Aldrich	53675-1ML	1 mL

Supplemental Table III: Primer sequences for qPCR

Gene	Forward Primer	Reverse Primer
Pten	TGGATTCGACTTAGACTTGACCT	GCGGTGTCATAATGTCTCTCAG
Acta2	CCAGCCAGTCGCCATCAG	AGCATCATCACCAGCAAAGC
Myh11	GTCACAGCTGAGGCCAAGAT	TTGTCAAGTCGCTGACCCTC
Cnn1	CACCAATCATACACAAGTTCA	CTTCTCAGGCTCAAATCTCC
Ccl2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Ccl7	ATGCAGATCTCTGCCGCGCTT	GCTATGGCCTCCTCAACCCAC
Tet2	GGCTGCCCTGTAGGATTTGT	ATGGACAGTCTTGGGAGGGT
Gapdh	GTGGAGATTGTTGCCATCAACGA	CCCATTCTCGGCCTTGACTGT

Major Resources Table

Genetically Modified Animals (in vivo studies)

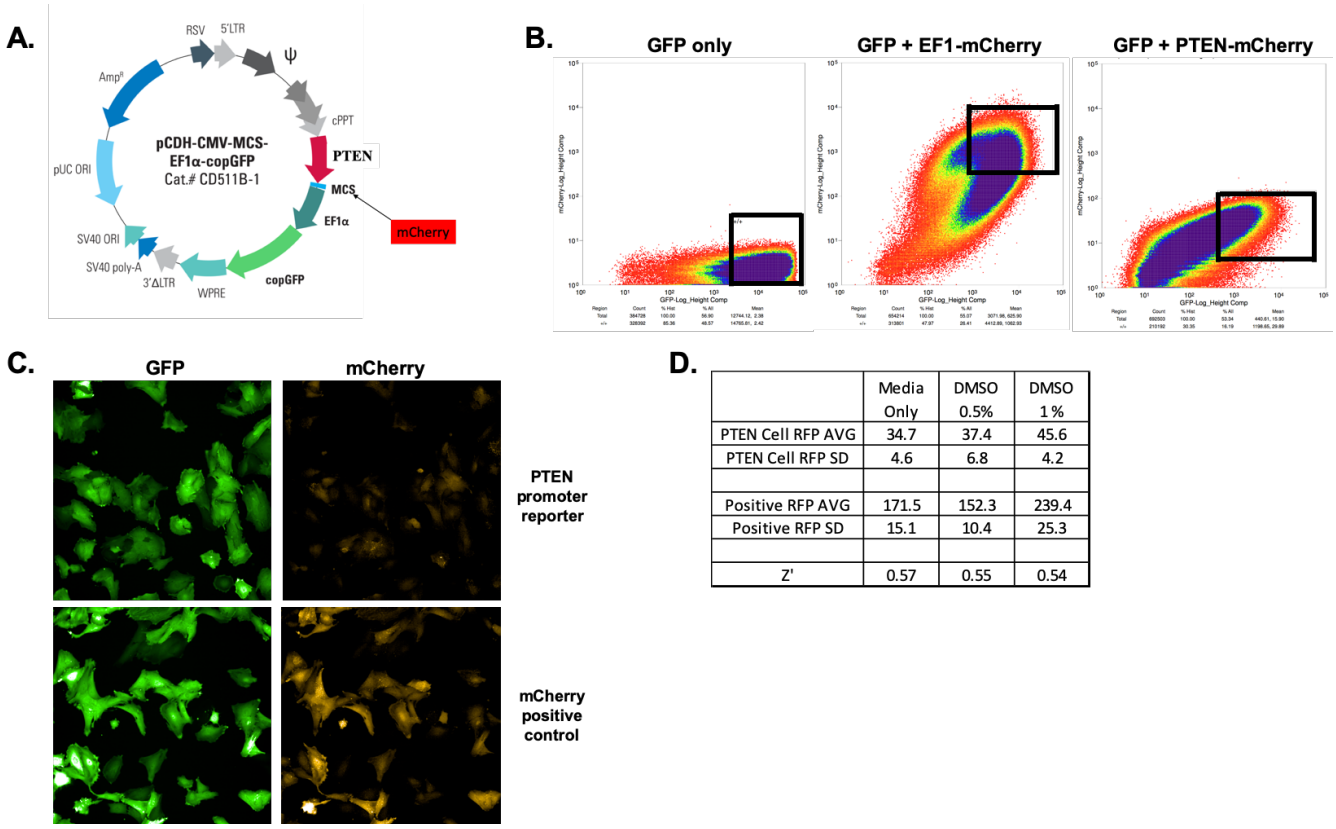
	Species	Vendor or Source	Background Strain	Genotype
PTEN WT	Mouse	In House Breeding	C57/BL6	$PTEN^{+/+}$-*Myh11*-Cre^{ERT2};Rosa26-YFP
PTEN iKO	Mouse	In House Breeding	C57/BL6	$PTEN^{flox/flox}$-*Myh11*-Cre^{ERT2};Rosa26-YFP

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration
PTEN	Cell Signaling	#9559	16 ng/mL (WB)
p-AKT s473	Cell Signaling	#4060S	91 ng/mL (WB)
β-actin	Sigma	#A5441	36.6 ng/mL (WB)
αSMA	Abcam	Ab5694	2 μg/mL (Immunofluorescence)
GFP-FITC	Abcam	Ab6662	5 μg/mL (Immunofluorescence)
SMMHC	Abcam	Ab125884	1.8 μg/mL (Immunofluorescence)
CD68	Bio-Rad	#MCA1957	10 μg/mL(Immunofluorescence)
Alexafluor 647 Goat-anti-rabbit	Invitrogen	A21244	4 μg/mL (Immunofluorescence)
Alexafluor 594 donkey-anti-rat	Invitrogen	A21209	4 μg/mL (Immunofluorescence)
Isotype	Invitrogen	#31235	10 μg/mL(Immunofluorescence)
Isotype	SouthernBiotech	#0108-01	10 μg/mL(Immunofluorescence)
Isotype	BD Biosciences	#550085	10 μg/mL(Immunofluorescence)

Cultured Cells

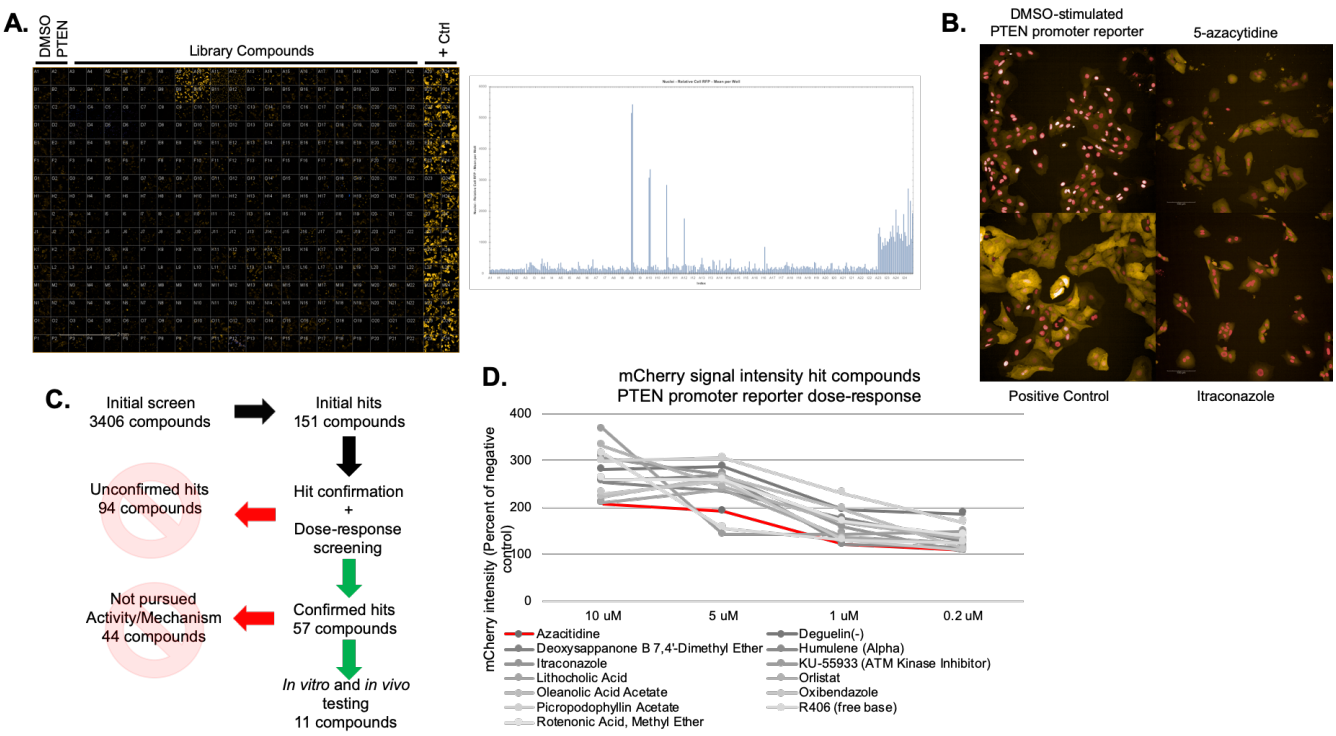
Name	Vendor or Source	Sex (F, M, or unknown)
Rat aortic SMCs	In House Isolation	M
PTEN shRNA SMCs	In House Generation	M
Control shRNA SMCs	In House Generation	M



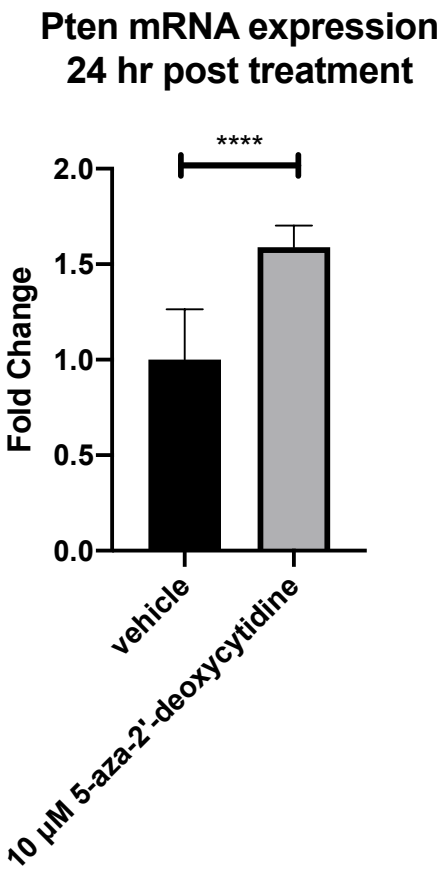
D.

	Media Only	DMSO 0.5%	DMSO 1%
PTEN Cell RFP AVG	34.7	37.4	45.6
PTEN Cell RFP SD	4.6	6.8	4.2
Positive RFP AVG	171.5	152.3	239.4
Positive RFP SD	15.1	10.4	25.3
Z'	0.57	0.55	0.54

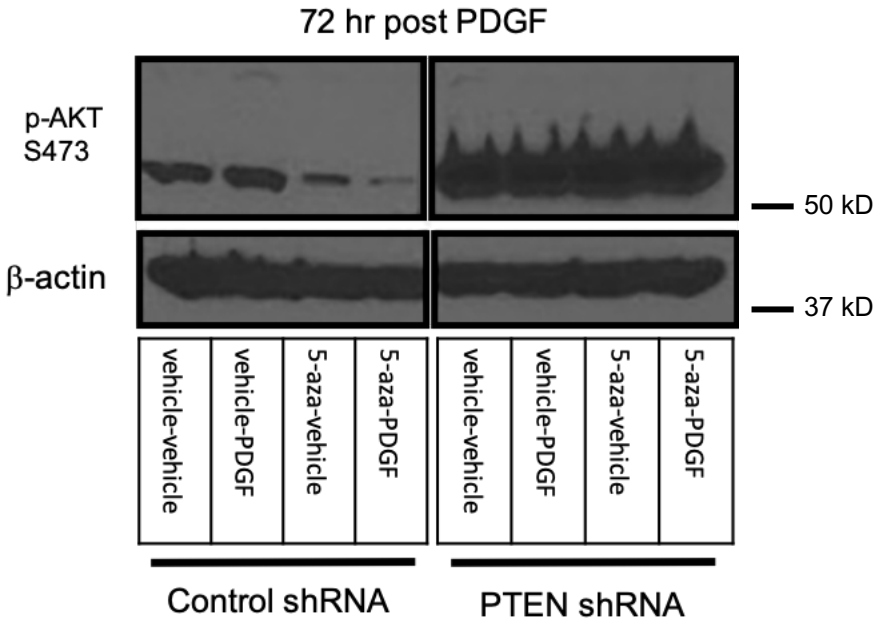
Supplemental Figure I. PTEN promoter-reporter plasmid generation and high throughput screen assay validation. **(A).** Plasmid used to generate PTEN promoter-mCherry reporter construct. Plasmid constructs were generated using a lentiviral pCDH-CMV-MCS-EF1-copGFP backbone. mCherry ORF was inserted to create pCDH-CMV-mCherry-EFI-copGFP, which was used for positive control cells. A 4032-bp fragment of the proximal PTEN promoter and 5'-UTR replaced the CMV promoter to generate pCDH-PTEN-mCherry-EF1-copGFP, which was used for the PTEN promoter-reporter cells. Image from System Biosciences LLC. **(B).** Transduced SMCs were sorted using FACS to obtain a pure population of GFP+ SMCs expressing only GFP (left), constitutively active GFP and mCherry as a positive control (middle), or constitutive GFP and PTEN promoter driven mCherry (right). **(C).** Live cell expression of GFP and mCherry in PTEN promoter-reporter and positive control cells (images taken using Opera Phenix HCS system). **(D).** mCherry intensity from DMSO stimulated plates was quantified and used to calculate a Z' to assess robustness of the assay prior to compound screening. Top rows show mCherry intensity from PTEN promoter-reporter cells, middle rows show mCherry intensity from positive control cells, bottom row shows calculated Z'. A Z' > 0.5 indicates an excellent assay.



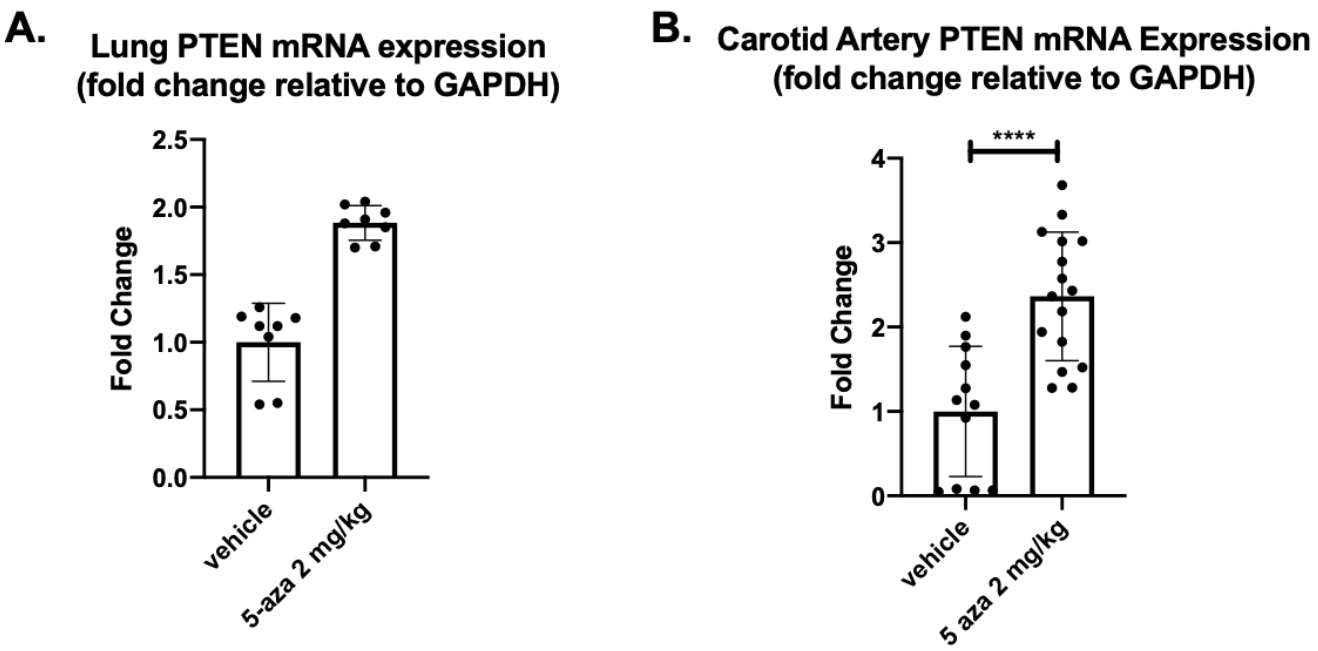
Supplemental Figure II. High throughput screen assay, initial hit identification, dose response testing, and final hit identification. **(A).** (Left panel) Representative mCherry image of all wells in a representative 384-well plate after compound dosing. Left two columns are DMSO-stimulated PTEN promoter-reporter cells used as the negative control, right two columns are positive control SMCs. This image represents only 1 of the 7 images that were taken per well. mCherry signal intensity was calculated as the average mCherry signal intensity per GFP-positive cell, all 7 images were used to calculate average mCherry intensity. (Right panel) mCherry intensity quantified by well expressed in chart format for visualization and analysis. Each peak represents an individual well. **(B).** Representative images of DMSO-stimulated PTEN promoter-reporter cells used as negative control (top left), DMSO-stimulated positive control cells (bottom left) and two hit compounds (right panels) from the initial screen at 10 μM compound concentration. 5-azacytidine and Itraconazole were confirmed as hit compounds during confirmation screening at 10 μM and dose response screening. Red staining = DRAQ5 nuclear stain, yellow staining = mCherry signal. **(C).** The screening library contained 3,406 compounds. 151 compounds were identified as potential hits in the initial screen. These were re-screened in duplicate at 10.0, 5.0, 1.0, and 0.2 μM. A dose-responsive threshold of mCherry induction above negative control was used to identify hits at 5.0, 1.0, 0.2 μM concentrations. 57 compounds exibited confirmed activity in at least 2 doses. 44 compounds were excluded due to lower level of activity or previously known mechanism of action undesirable for potential vascular therapeutics. 11 compounds were identified for further *in vitro* and *in vivo* testing. **(D).** Dose response plots from the 11 final hit compounds that were chosen for *in vitro* and *in vivo* testing. mCherry signal is plotted as percent of average mCherry signal intensity in negative control PTEN promoter-reporter SMCs.



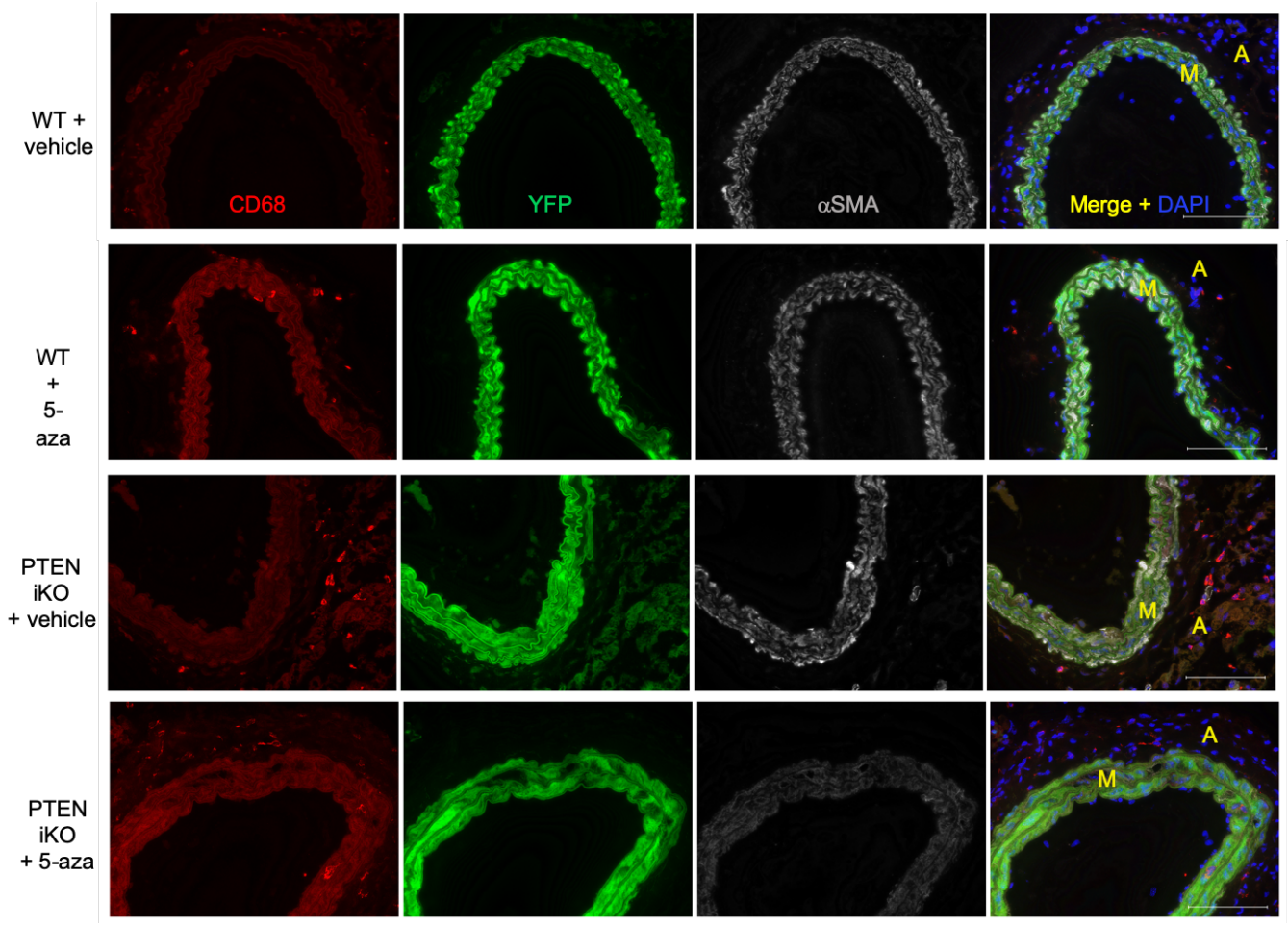
Supplemental Figure III. The DNA methyltransferase-1 inhibitor, 5-aza-2'-deoxycytidine, promotes induction of PTEN. Rat aortic SMCs were plated and treated in triplicate with 10 μM 5-aza-2'-deoxycytidine for 72 hr prior to harvest. PTEN mRNA expression was assessed by RT-qPCR. Paired T-test; ****p<0.0001.



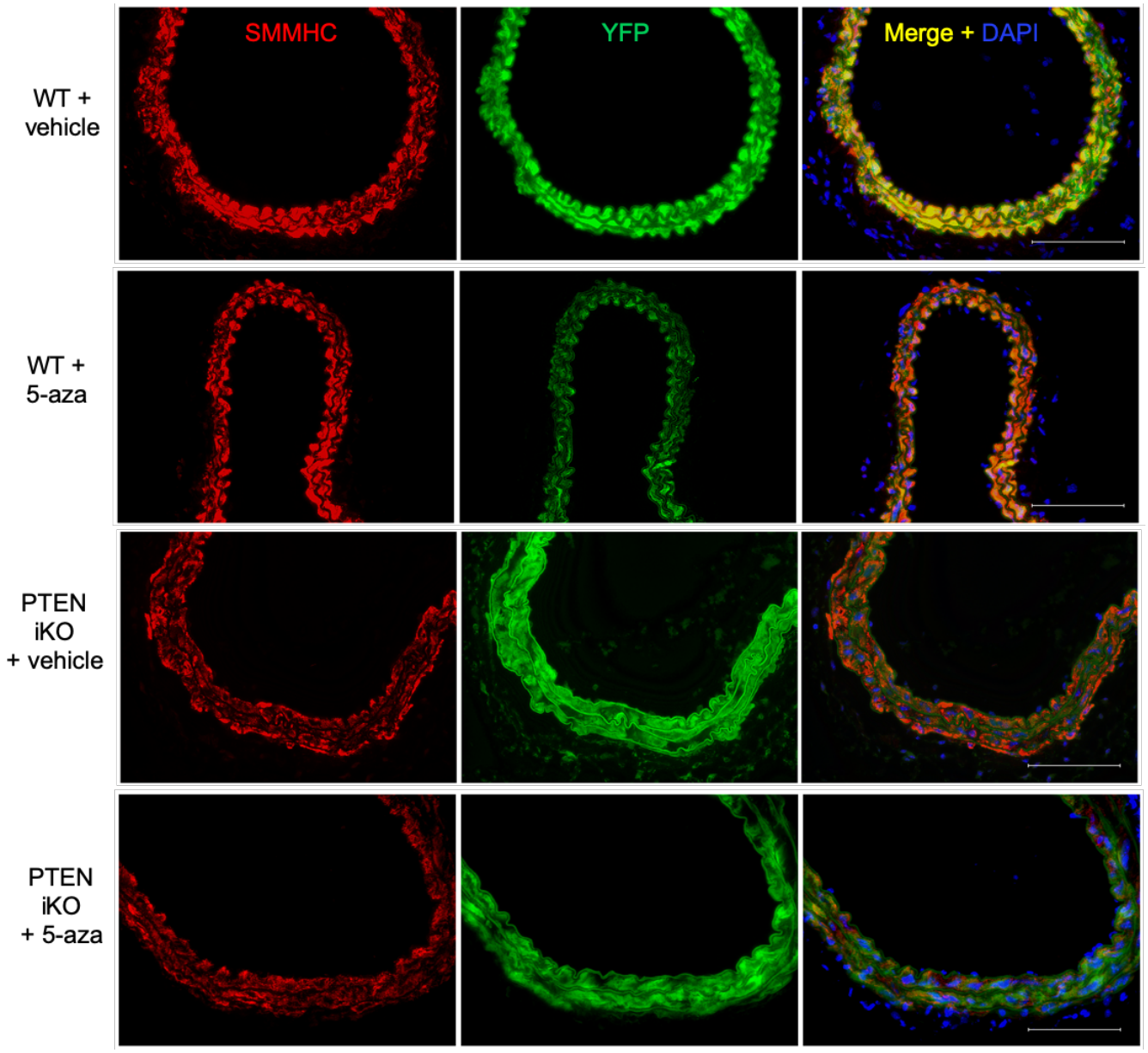
Supplemental Figure IV. Functional loss of PTEN in PTEN-deficient SMCs. Control shRNA and PTEN shRNA SMCs were growth-arrested in 0.1% CS EMEM plus 5-azacytidine or vehicle control for 48 hrs, then stimulated with 20 ng/ml PDGF-BB for 72 hrs. Western blot analysis of phopho^{473}Akt expression; β-actin is shown as a loading control.



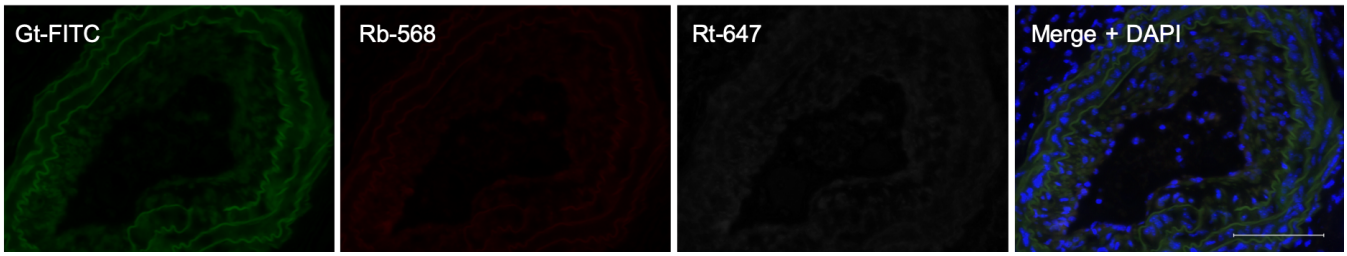
Supplemental Figure V. 5-azacytidine treatment in vivo promotes PTEN induction. Male WT mice underwent left carotid artery ligation as described in Materials and Methods. Mice received daily 2 mg/kg 5-azacytidine or vehicle control i.p. injections for 1 week prior to tissue harvest. Whole lung **(A)** and injured arteries **(B)** were harvested and total RNA extracted for RT-qPCR analysis of PTEN mRNA. N=4 (vehicle, lung), N=3 (vehicle, artery), N=4 (5-azacytidine, lung and artery). Each symbol represents duplicate **(A)** or quadruplicate **(B)** qPCR runs. T test; ****$p<0.0001$.



Supplemental Figure VI. Uninjured carotid arteries from 5-azacytidine or DMSO treated WT or PTEN iKO mice. WT and PTEN iKO mice were treated as described in Figures 6&7. Contralateral uninjured right carotid arteries sections were harvested 3-weeks post-injury and sections immunofluorescently stained for CD68 (red), YFP (green), and αSMA (white); nuclei were stained for DAPI (blue). Representative images from N=7 (WT vehicle), N=6 (WT 5-aza), N=6 (PTEN iKO vehicle), and N=7 (PTEN iKO 5-aza). Scale bars = 100μm.



Supplemental Figure VII. Uninjured carotid arteries from 5-azacytidine or DMSO treated WT or PTEN iKO mice. WT and PTEN iKO mice were treated as described in Figures 6&7. Contralateral uninjured right carotid arteries were harvested 3-weeks post-injury and sections were immunofluorescently stained for SMMHC (red) and YFP (green); nuclei were stained for DAPI (blue). Representative images from N=7 (WT vehicle), N=6 (WT 5-aza), N=6 (PTEN iKO vehicle), and N=7 (PTEN iKO 5-aza). Scale bars = 100μm.



Supplemental Figure VIII. Negative IgG controls for immunofluorescence. Injured arterial sections from WT mice were stained using goat (Gt), rabbit (Rb), or rat (Rt) IgGs followed by respective secondary antibodies as negative controls for immunofluorescence.