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REGISTERED REPORT

Registered report: A coding-independent function of gene and pseudogene mRNAs regulates tumour biology

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REPRODUCIBILITY PROJECT CANCER BIOLOGY

Abstract The Reproducibility Project: Cancer Biology seeks to address growing concerns about reproducibility in scientific research by conducting replications of selected experiments from a number of high-profile papers in the field of cancer biology. The papers, which were published between 2010 and 2012, were selected on the basis of citations and Altmetric scores (***Errington et al., 2014***). This Registered report describes the proposed replication plan of key experiments from 'A coding-independent function of gene and pseudogene mRNAs regulates tumour biology' by ***Poliseno et al. (2010)***, published in *Nature* in 2010. The key experiments to be replicated are reported in Figures 1D, 2F-H, and 4A. In these experiments, Poliseno and colleagues report microRNAs *miR-19b* and *miR-20a* transcriptionally suppress both *PTEN* and *PTENP1* in prostate cancer cells (Figure 1D; ***Poliseno et al., 2010***). Decreased expression of *PTEN* and/or *PTENP1* resulted in downregulated *PTEN* protein levels (Figure 2H), downregulation of both mRNAs (Figure 2G), and increased tumor cell proliferation (Figure 2F; ***Poliseno et al., 2010***). Furthermore, overexpression of the *PTEN* 3′ UTR enhanced *PTENP1* mRNA abundance limiting tumor cell proliferation, providing additional evidence for the co-regulation of *PTEN* and *PTENP1* (Figure 4A; ***Poliseno et al., 2010***). The Reproducibility Project: Cancer Biology is collaboration between the Center for Open Science and Science Exchange, and the results of the replications will be published in *eLife*.
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Introduction

The phosphatase and tensin homolog gene (*PTEN*) functions as a negative repressor of the PI3K/Akt survival pathway and is one of the most frequently deleted tumor suppressor genes in human cancer (***Stambolic et al., 1998***; ***Song et al., 2012***). As a regulator of PI3K signaling, loss of *PTEN* results in over-activation of Akt, leading to unchecked cell proliferation, reduced apoptosis, and elevated tumor angiogenesis (***Stambolic et al., 1998***; ***Carracedo et al., 2008***). In prostate cancer, decreases in PTEN protein expression, either by allelic deletion or functional loss caused by mutation and/or epigenetic modification, can lead to invasive prostate carcinoma (***Trotman et al., 2003***; ***Phin et al., 2013***). In preclinical systems, the genetic restoration of *PTEN* induces apoptosis in cancer cell lines and has a significant negative effect on tumor growth in multiple in vivo models (***Li et al., 1998***; ***Lu et al., 1999***; ***Tian et al., 1999***; ***Chen et al., 2011***). In contrast, clinical efforts to restore *PTEN* functionality have instead focused on targeting kinases in the PI3K pathway, including PI3K, Akt, and the mammalian target of rapamycin (***Hopkins and Parsons, 2014***). However, the development of PI3K targeting drugs has been complicated by the limited tolerability of current pharmacological treatments as well as tumor heterogeneity (***Gerlinger et al., 2012***; ***Bauer et al., 2014***).

It is increasingly apparent that a complex regulatory network exists between the diverse RNA species pervasive in the human transcriptome. MicroRNAs (miRNAs) are small non-coding RNAs that bind to

complementary sequences in the 3′ untranslated regions (UTR) of target messenger RNAs (mRNA), resulting in transcriptional downregulation of the target gene (**Sen et al., 2014**). Meng and colleagues showed that *PTEN* was repressed by *miR-21*, one of the most frequently upregulated miRNAs in cancer, in hepatocarcinoma cells, suggesting that the oncogenic potential of *miR-21* occurs via the downregulation of *PTEN* expression (**Chan et al., 2005**; **Meng et al., 2006**; **Volinia et al., 2006**; **Meng et al., 2007**; **Si et al., 2007**). Several miRNAs that target *PTEN* have since been reported (**Jackson et al., 2014**; **Wang et al., 2015**). While miRNAs play a functional role in silencing target gene expression, it is proposed that miRNAs themselves are subject to regulation by competing endogenous RNA (ceRNA) species, including pseudogenes, long non-coding RNAs, and circular RNAs (**Salmena et al., 2011**; **Cesana and Daley, 2013**). In plants, for example, the non-protein coding gene *IPS1* sequesters miRNAs away from their mRNA targets, thereby leading to an accumulation of target transcripts (**Franco-Zorrilla et al., 2007**). Poliseno and colleagues proposed that pseudogenes, which are non-coding genomic DNA sequences closely related to parental genes, can modulate parental gene expression by influencing the available levels of miRNAs within a cell (**Poliseno et al., 2010**; **Cesana and Daley, 2013**). However, the extent and manner that ceRNAs can exert a consequential effect on the repression of targets for that miRNA is currently unclear (**Broderick and Zamore, 2014**). Recently, Denzler and colleagues analyzed the stoichiometric relationship of miR-122 and target sites in adult mouse liver and reported that the natural abundance of target sites exceeded miRNAs, making the ceRNA hypothesis unlikely (**Denzler et al., 2014**).

PTENP1 is a pseudogene that shares close homology with *PTEN*, including the ability to bind miRNAs (**Fujii et al., 1999**). To determine whether *PTEN* and *PTENP1* expression levels are modulated by miRNA activity, Poliseno and colleagues first established that the *PTEN*-targeting miRNAs *miR-19b* and *miR-20a* were able to target both *PTEN* and *PTENP1* (**Poliseno et al., 2010**). As reported in Figure 1D, overexpression of *miR-19b* and *miR-20a* in prostate cancer cells resulted in a significant decrease in *PTEN* and *PTENP1* mRNA transcription. This is supported by additional studies demonstrating that overexpression of either *miR-19b* or *miR-20a* in cancer cell lines resulted in reduced *PTEN* mRNA levels and protein expression (**Luo et al., 2013**; **Tian et al., 2013**; **Wu et al., 2014**). The ability of *miR-19b* and *miR-20a* to target *PTEN* in prostate cancer was further confirmed by **Tay et al. (2011)**. These key findings established that *PTEN* and *PTENP1* are regulated by interactions with miRNA in multiple cancer cell types and will be replicated in Protocol 1.

In Figure 2F-H, Poliseno and colleagues tested the phenotypic consequences of *PTENP1* downregulation by specifically targeting *PTEN* and/or *PTENP1* expression. Downregulation of *PTENP1* in DU145 prostate cancer cells resulted in a significant decrease in both *PTEN* and *PTENP1* mRNA levels and protein expression (Figure 2G-H; **Poliseno et al., 2010**). Furthermore, downregulation of *PTENP1* profoundly accelerated the proliferation of DU145 cells (Figure 2F), with silencing of both *PTEN* and *PTENP1* having an additive effect (**Poliseno et al., 2010**). These experiments will be replicated in Protocols 2, 3, and 4. Recently, Tay and colleagues reported that *PTEN*-ceRNAs, including *CNOT6L* and *VAPA*, phenocopied *PTENP1* activity, as downregulation of these non-coding transcripts in prostate and colon cancer cells were also able to modulate *PTEN* expression, Akt activity, and cell growth (**Tay et al., 2011**). Additionally, other *PTEN*-ceRNAs that regulate *PTEN* expression have been reported in brain, breast, and skin cancers (**Lee et al., 2010**; **Karreth et al., 2011**; **Sumazin et al., 2011**). Further to this, PTENP1 antisense RNA has been reported to regulate PTEN transcription and mRNA stability, suggesting a model where the PTENP1 pseudogene has biomodal functionality modulating PTEN (**Johnsson et al., 2013**).

As an extension of the findings reported in Figure 2 and further genomic analysis, Poliseno and colleagues demonstrated that the *PTEN* 3′ UTR regulates pseudogene expression, since overexpression of the *PTEN* 3′ UTR was found to de-repress *PTENP1* expression and inhibited DU145 proliferation (Figure 4A) (**Poliseno et al., 2010**). These experiments will be replicated in Protocols 5 and 6. These results were also confirmed by experiments by Yu and colleagues showing that overexpression of either *PTEN* or *PTENP1* suppressed renal cancer cell proliferation (**Yu et al., 2014**). Further to this, the oncosuppressive properties of overexpressing *PTENP1* 3′ UTR have been reported in various cancer cells (**Poliseno et al., 2010**; **Chen et al., 2015**; **Guo et al., 2015**).

Materials and methods

Protocol 1: Quantitative PCR after miR transfection

This experiment utilizes quantitative RT-PCR to analyze the effect of miR-19b or miR-20a on the mRNA levels of *PTEN* and *PTENP1*. It is a replication of Figure 1D.

Sampling

- Experiment to be repeated a total of six times for a minimum power of 88%.
  - See 'Power calculations' section for details.
- Experiment has 5 conditions:
  - Cohort 1: siGENOME non-targeting siRNA #2 (siLUC) transfected DU145 cells.
  - Cohort 2: miR-19b transfected DU145 cells.
  - Cohort 3: miR-20a transfected DU145 cells.
  - Cohort 4: Untransfected DU145 cells (additional negative control).
  - Transfection control: siGLO RISC-free siRNA transfected DU145 cells.
- Quantitative RT-PCR performed in technical triplicate for the following genes:
  - PTEN.
  - PTENP1.
  - *ACTIN* (internal control).
  - *36B4* (additional internal control).

Materials and reagents

Reagent	Type	Manufacturer	Catalog #	Comments
DU145 cells	Cell line	ATCC	HTB-81	–
RPMI 1640 medium	Cell culture	Sigma–Aldrich	R8758	Replaces Invitrogen brand used in original study
Fetal bovine serum (FBS)	Cell culture	Sigma–Aldrich	F2442	Replaces Invitrogen brand used in original study
L-glutamine	Cell culture	Sigma–Aldrich	G7513	Original brand not specified
100× Penicillin/streptomycin	Cell culture	Sigma–Aldrich	P4333	Original brand not specified
0.05% trypsin/0.48 mM EDTA	Cell culture	Sigma–Aldrich	T3924	Original brand not specified
Phosphate buffered saline (PBS), without $MgCl_2$ and $CaCl_2$	Cell culture	Sigma–Aldrich	D8537	Original brand not specified
12 well tissue culture dishes	Labware	Corning	3513	Original brand not specified
siGLO RISC-free siRNA	Nucleic acid	Dharmacon	D-001600-01	–
siGENOME non-targeting siRNA #2 (siLUC)	Nucleic acid	Dharmacon	D-001210-02	–
miRIDIAN microRNA hsa-miR-19b-3p (si-miR-19b)	Nucleic acid	Dharmacon	IH-300489-05-0002	–
miRIDIAN microRNA hsa-miR-20a-5p (si-miR20a)	Nucleic acid	Dharmacon	IH-300491-05-0002	–
Dharmafect 1	Cell culture	Dharmacon	T-2001-01	–
PTENP1 forward and reverse primers	Nucleic acid	Specific brand information will be left up to the discretion of the replicating lab and recorded later		
PTEN forward and reverse primers	Nucleic acid			
ACTIN forward and reverse primers	Nucleic acid			
36B4 forward and reverse primers	Nucleic acid			
TRI reagent	Chemical	Sigma–Aldrich	T9424	Replaces Trizol reagent from Invitrogen
1-bromo-3-chloropropase	Chemical	Sigma–Aldrich	B9673	Reagent needed from TRI reagent protocol
Nuclease free water	Chemical	Sigma–Aldrich	W4502	Reagent needed from TRI reagent protocol
Microscope	Instrument	Zeiss	–	Original brand not specified
Axiovision	Software	Zeiss	–	Original brand not specified
DNAse I amplification grade	Chemical	Sigma–Aldrich	AMPD1	Replaces Invitrogen brand used in original study
First-strand cDNA synthesis kit (includes pd(N)6 random hexamers and NotI-(dT) 18 primers)	Kit	Sigma–Aldrich	GE27-9261-01	Replaces SuperScript II reverse transcriptase from Invitrogen used in original study
QuantiTect Sybr Green PCR kit	Kit	Qiagen	204141	–
Real Time System with a C1000 Thermal Cycler	Instrument	BioRad	CFX 96	Replaces Roche Lightcycler 2.0 used in original study

Procedure

Notes

- Cells will be sent for mycoplasma testing and short tandem repeat (STR) profiling.
- DU145 cells are grown in complete RPMI 1640 supplemented with 2 mM glutamine, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37˚C and 6% CO_2.

1. Seed 1.5 × 10^5 DU145 cells per well in a 12-well dish. Grow overnight.
2. Transfect with 100 nM siLuc, si-miR-19b, and si-miR-20a using 3 µl of Dharmafect 1 according to manufacturer's instructions. Transfect control cells with siGLO RISC-free control siRNA following manufacturer's instructions. Include untransfected control cells. Grow overnight.
3. Confirm that >90% of siGLO transfected control cells show fluorescence, indicating they were successfully transfected.
   a. If transfection is less than 90%, record efficiency for attempt, exclude attempt and do not continue with the rest of the procedure. Repeat procedure until >90% efficiency is obtained.
   b. If modification to transfection (step 2) is needed, record and maintain modified steps for remaining replicates.
4. 24 hr after transfection, extract total RNA from cells directly on the culture dish using TRI reagent and 1-bromo-3-chloropropane according to manufacturer's instructions.
5. Treat RNA with DNAse I following manufacturer's instructions.
   a. Record RNA concentration and purity (A_{280}/A_{260}).
6. Reverse transcribe 1 µg RNA/sample into cDNA using first-strand cDNA synthesis kit with primers following manufacturer's instructions.
7. Perform quantitative PCR reaction using the QuantiTect Sybr Green PCR kit:
   a. Use 2 µl of reverse transcription reaction per 20 µl real-time PCR reaction.
   b. Perform quantitative PCR for *PTEN*, *PTENP1*, *ACTIN*, and *36B4*.
      i. *PTEN* forward primer: 5′-GTTTACCGGCAGCATCAAAT-3′
      ii. *PTEN* reverse primer: 5′-CCCCCACTTTAGTGCACAGT-3′
      iii. *PTENP1* forward primer: 5′-TCAGAACATGGCATACACCAA-3′
      iv. *PTENP1* reverse primer: 5′-TGATGACGTCCGATTTTTCA-3′
      v. *ACTIN* forward primer: 5′-CATGTACGTTGCTATCCAGGC-3′
      vi. *ACTIN* reverse primer: 5′-CTCCTTAATGTCACGCACGAT-3′
      vii. *36B4* forward primer: 5′-GTGTTCGACAATGGCAGCAT-3′
      viii. *36B4* reverse primer: 5′-GACACCCTCCAGGAAGCGA-3′
         i. *36B4* primer sequences reported in ***Fullwood et al. (2009)***.
   c. Do not pre-treat with uracil-N-glycosylase.
   d. All reactions should be optimized and run in technical triplicate.
8. Using *ACTIN* as an internal standard, calculate the relative *PTEN* and *PTENP1* expression for each sample using the comparative Ct method.
   a. Additionally perform normalization using *36B4* as an internal standard (additional control).
9. Repeat independently five additional times.

Deliverables

- Data to be collected:
  - Images of fluorescence and phase/contrast of siGLO transfected cells.
  - Purity ($A_{260/280}$ ratio) and concentration of isolated total RNA from cells.
  - Raw data for all qPCR reactions.
  - Quantification of *PTEN* and *PTENP1* mRNA levels relative to *ACTIN*.
  - Quantification of fold change *PTEN* and *PTENP1* mRNA levels relative to siLuc transfected cells. (Compare to Figure 1D).

Confirmatory analysis plan

This replication attempt will perform the statistical analysis listed below.

- Statistical Analysis:
  - Note: at the time of analysis, we will perform the Shapiro–Wilk test and generate a quantile–quantile plot to assess the normality of the data. We will also perform Levene's test to assess homoscedasticity. If the data appear skewed we will perform the appropriate transformation in

order to proceed with the proposed statistical analysis. If this is not possible we will perform the planned comparisons using the Wilcoxon–Mann Whitney test.
  - One-way MANOVA of normalized *PTEN* or *PTENP1* mRNA fold change in siLuc, 19b, or 20a siRNA transfected cells with the following planned comparisons using the Bonferroni correction:
    1. *PTEN* mRNA levels of siLuc transfected cells compared to 19b transfected cells.
    2. *PTEN* mRNA levels of siLuc transfected cells compared to 20a transfected cells.
    3. *PTENP1* mRNA levels of siLuc transfected cells compared to 19b transfected cells.
    4. *PTENP1* mRNA levels of siLuc transfected cells compared to 20a transfected cells.
- Meta-analysis of effect sizes:
  - Compute the effect sizes of each comparison, compare them against the effect size in the original paper and use a random effects meta-analytic approach to combine the original and replication effects, which will be presented as a forest plot.
- Additional exploratory analysis:
  - The same analysis described above will be performed with *36B4* normalized values, which serves as an independent normalization control not included in the original analysis.

Known differences from the original study

The *PTEN* and *PTENP1* mRNA levels will be normalized with an independent control (*36B4*) in addition to *ACTIN*. All known differences are listed in the materials and reagents section above with the originally used item listed in the comments section. All differences have the same capabilities as the original and are not expected to alter the experimental design.

Provisions for quality control

The cell line used in this experiment will undergo STR profiling to confirm its identity and will be sent for mycoplasma testing to ensure there is no contamination. Transfection efficiency will be recorded for each replicate and any transfection that does not contain >90% efficiency will be excluded and not continue through the rest of the procedure. If the efficiency in the first attempt(s) does not obtain >90%, then any modifications to the transfection protocol will be recorded and the procedure will be maintained for the remaining replicates. The sample purity ($A_{260/280}$ ratio) of the isolated RNA from each sample will be reported. The *PTEN* and *PTENP1* mRNA levels will be normalized with an independent control (*36B4*). All the raw data, including the analysis files, will be uploaded to the project page on the Open Science Framework (OSF) (https://osf.io/yyqas) and made publically available.

Protocol 2: Cell growth assay following siRNA transfection

This experiment tests the effect of siRNA mediated depletion of *PTEN*, *PTENP1*, or both on the growth of DU145 cells. It is a replication of Figure 2F.

Sampling

- Experiment to be repeated a total of five times for a minimum power of 94%.
  - See 'Power calculations' section for details.
- Experiment has 6 conditions:
  - Cohort 1: siGENOME non-targeting siRNA #2 (siLUC) transfected DU145 cells.
  - Cohort 2: siPTEN Smartpool (targets *PTEN* and *PTENP1*) transfected DU145 cells.
  - Cohort 3: siPTEN transfected DU145 cells.
  - Cohort 4: siPTENP1 transfected DU145 cells.
  - Cohort 5: Untransfected DU145 cells (additional negative control).
  - Transfection control: siGLO RISC-free siRNA transfected DU145 cells.
- Each cohort is harvested on the following days performed in technical triplicate:
  - Day 0 (after O/N incubation).
  - Day 1.
  - Day 2.
  - Day 3.
  - Day 4.
  - Day 5.

Materials and reagents

Reagent	Type	Manufacturer	Catalog #	Comments
DU145 cells	Cell line	ATCC	HTB-81	–
RPMI 1640 medium	Cell culture	Sigma–Aldrich	R8758	Replaces Invitrogen brand used in original study
Fetal bovine serum (FBS)	Cell culture	Sigma–Aldrich	F2442	Replaces Invitrogen brand used in original study
L-glutamine	Cell culture	Sigma–Aldrich	G7513	Original brand not specified
100× Penicillin/streptomycin	Cell culture	Sigma–Aldrich	P4333	Original brand not specified
0.05% trypsin/0.48 mM EDTA	Cell culture	Sigma–Aldrich	T3924	Original brand not specified
Phosphate buffered saline (PBS), without $MgCl_2$ and $CaCl_2$	Cell culture	Sigma–Aldrich	D8537	Original brand not specified
12 well tissue culture dishes	Labware	Corning	3513	Original brand not specified
siGLO RISC-free siRNA	Nucleic acid	Dharmacon	D-001600-01	–
siGENOME non-targeting siRNA #2 (siLUC)	Nucleic acid	Dharmacon	D-001210-02	–
siPTEN	Nucleic acid	Dharmacon	Custom	See Supplemental Figure 6 of original paper for sequence
ON-TARGETplus siPTEN Smartpool	Nucleic acid	Dharmacon	L-003023-00	Composed of: J-003023-09; J-003023-10; J-003023-11; J-003023-12
siPTENP1	Nucleic acid	Dharmacon	Custom	See Supplemental Figure 6 of original paper for sequence
Dharmafect 1	Cell culture	Dharmacon	T-2001-01	–
Microscope	Instrument	Olympus	LX81	Original brand not specified
Crystal violet	Dye	Sigma–Aldrich	C0775	Original brand not specified
Formalin	Chemical	Specific brand information will be left up to the discretion of the replicating lab and recorded later		
Acetic acid	Chemical			
Methanol	Chemical			
Spectrophotometer capable of reading at 590 nm (or 595 nm)	Instrument	BioTek Instruments	Synergy 2 (SLFA configuration)	Original brand not specified

Procedure

Note

- All cells will be sent for mycoplasma testing and STR profiling.
- DU145 cells grown in complete RPMI 1640: RPMI 1640 supplemented with 2 mM glutamine, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37˚C and 6% CO_2.

1. Seed 1.5×10^5 DU145 cells per well in a 12-well dish. Grow overnight.
2. Transfect with 100 nM siRNAs (siPTEN, siPTENP1, siPTEN Smartpool (siPTEN and PTENP1), or siLuc in separate wells) using Dharmafect 1 according to manufacturer's instructions or leave untransfected. Transfect control cells with siGLO RISC-free control siRNA according to manufacturer's instructions. Grow overnight.
3. Confirm that >90% of siGLO transfected control cells show fluorescence, indicating they were successfully transfected.
   a. If transfection is less than 90%, record efficiency for attempt, exclude attempt and do not continue with the rest of the procedure. Repeat procedure until >90% efficiency is obtained.
   b. If modification to transfection (step 2) is needed, record and maintain modified steps for remaining replicates.
4. The day after transfection, resuspend 2×10^5 siLuc, siPTEN, siPTENP1, siPTEN/PTENP1, or untransfected cells in 50 ml fresh media. Seed three wells of six sets of 12-well plates with 2 ml of each cell line. Each set of 12 well plates should have three wells of each cell line. Incubate overnight.
5. Fix one plate every 24 hr starting after overnight incubation (the first plate fixed will be called day 0).
   a. Wash wells once in PBS.

b. Fix wells with 10% formalin for 10 min at room temperature.
c. Store plates in PBS at 4˚C.
d. All wells should be fixed by day 6.
6. Stain cells with 0.1% crystal violet, 20% methanol for 15 min. Wash cells.
7. Lyse all wells with 10% acetic acid for 10 min.
8. Read optical density at 590 or 595 nm.
a. Reading can be done at 595 nm if 590 is not available.
9. Repeat independently four additional times.

Deliverables

- Data to be collected:
  - Images of fluorescence and phase/contrast of siGLO transfected cells.
  - Raw data of absorbance from plate reader.
  - Graph of relative cell number for each cell line over time. (Compare to Figure 2F).

Confirmatory analysis plan

This replication attempt will perform the following statistical analysis listed below.

- Statistical Analysis:
  - Note: at the time of analysis, we will perform the Shapiro–Wilk test and generate a quantile–quantile plot to assess the normality of the data. We will also perform Levene's test to assess homoscedasticity. If the data appear skewed we will perform the appropriate transformation in order to proceed with the proposed statistical analysis. If this is not possible we will perform the equivalent non-parametric test.
  - Two-way ANOVA comparing Day 5 absorbance in siLuc, siPTEN, siPTENP1, or siPTEN/PTENP1 transfected cells with the following planned comparisons using the Bonferroni correction:
    1. siLuc compared to siPTEN.
    2. siLuc compared to siPTENP1.
    3. siLuc compared to siPTEN/PTENP1.
    4. siPTEN/PTENP1 compared to siPTEN.
    5. siPTEN/PTENP1 compared to siPTENP1.
  - Two-way ANOVA comparing area under the curve (AUC) measurements (determined from day 0, 1, 2, 3, 4, and 5 for each replicate) from absorbance in siLuc, siPTEN, siPTENP1, or siPTEN/PTENP1 transfected cells with the following planned comparisons using the Bonferroni correction.
    1. siLuc compared to siPTEN.
    2. siLuc compared to siPTENP1.
    3. siLuc compared to siPTEN/PTENP1.
    4. siPTEN/PTENP1 compared to siPTEN.
    5. siPTEN/PTENP1 compared to siPTENP1.
- Meta-analysis of effect sizes:
  - Compute the effect sizes of each comparison, compare them against the effect size in the original paper and use a random effects meta-analytic approach to combine the original and replication effects, which will be presented as a forest plot.

Known differences from the original study

All known differences are listed in the materials and reagents section above with the originally used item listed in the comments section. All differences have the same capabilities as the original and are not expected to alter the experimental design.

Provisions for quality control

The cell line used in this experiment will undergo STR profiling to confirm its identity and will be sent for mycoplasma testing to ensure there is no contamination. Transfection efficiency will be recorded for each replicate and any transfection that does not contain >90% efficiency will be excluded and not continue through the rest of the procedure. If the efficiency in the first attempt(s) does not obtain >90%, then any modifications to the transfection protocol will be recorded and the procedure will be maintained for the remaining replicates. All the raw data, including the analysis files, will be uploaded to the project page on the OSF (https://osf.io/yyqas) and made publically available.

Protocol 3: Quantitative PCR following transfected with siRNA against PTEN and/or PTENP1

This experiment analyzes the effect of depletion of *PTEN*, *PTENP1*, or both on the mRNA expression of *PTEN* or *PTENP1*. Quantitative real time PCR is utilized to assess the levels of expression following transfection of siRNA. This protocol is a replication of Figure 2G.

Sampling

- Experiment to be repeated a total of five times for a minimum power of 89%.
  - See 'Power calculations' section for details.
- Experiment has 6 conditons:
  - Cohort 1: Uninfected DU145 cells (additional negative control).
  - Cohort 1: siGENOME non-targeting siRNA #2 (siLUC) transfected DU145 cells.
  - Cohort 2: siPTEN Smartpool (targets *PTEN* and *PTENP1*) transfected DU145 cells.
  - Cohort 3: siPTEN transfected DU145 cells.
  - Cohort 4: siPTENP1 transfected DU145 cells.
  - Cohort 5: Uninfected DU145 cells (additional negative control).
  - Transfection control: siGLO RISC-free siRNA transfected DU145 cells.
- Quantitative RT-PCR performed in technical triplicate for the following genes:
  - *PTEN*.
  - PTEN1P.
  - *ACTIN* (internal control).
  - *36B4* (additional internal control).

Materials and reagents

Reagent	Type	Manufacturer	Catalog #	Comments
DU145 cells	Cell line	ATCC	HTB-81	–
RPMI 1640 medium	Cell culture	Sigma–Aldrich	R8758	Replaces Invitrogen brand used in original study
Fetal bovine serum (FBS)	Cell culture	Sigma–Aldrich	F2442	Replaces Invitrogen brand used in original study
L-glutamine	Cell culture	Sigma–Aldrich	G7513	Original brand not specified
100× Penicillin/streptomycin	Cell culture	Sigma–Aldrich	P4333	Original brand not specified
0.05% trypsin/0.48 mM EDTA	Cell culture	Sigma–Aldrich	T3924	Original brand not specified
Phosphate buffered saline (PBS), without $MgCl_2$ and $CaCl_2$	Cell culture	Sigma–Aldrich	D8537	Original brand not specified
12 well tissue culture dishes	Labware	Corning	3513	Original brand not specified
siGLO RISC-free siRNA	Nucleic acid	Dharmacon	D-001600-01	–
siGENOME non-targeting siRNA #2 (siLUC)	Nucleic acid	Dharmacon	D-001210-02	–
ON-TARGETplus siPTEN Smartpool	Nucleic acid	Dharmacon	L-003023-00	Composed of: J-003023-09; J-003023-10; J-003023-11; J-003023-12
siPTEN	Nucleic acid	Dharmacon	Custom	See Supplemental Figure 6 of original paper for sequence
siPTENP1	Nucleic acid	Dharmacon	Custom	See Supplemental Figure 6 of original paper for sequence
Dharmafect 1	Cell culture	Dharmacon	T-2001-01	–
Microscope	Instrument	Olympus	LX81	Original brand not specified
ACTIN forward and reverse primers	Nucleic acid	Specific brand information will be left up to the discretion of the replicating lab and recorded later		
PTEN forward and reverse primers	Nucleic acid			
PTENP1 forward and reverse primers	Nucleic acid			
36B4 forward and reverse primers	Nucleic acid			
TRI reagent	Chemical	Sigma–Aldrich	T9424	Replaces Trizol reagent from Invitrogen
1-bromo-3-chloropropase	Chemical	Sigma–Aldrich	B9673	Reagent needed from TRI reagent protocol

Continued on next page

Continued

Reagent	Type	Manufacturer	Catalog #	Comments
Nuclease free water	Chemical	Sigma–Aldrich	W4502	Reagent needed from TRI reagent protocol
DNase I amplification grade	Chemical	Sigma–Aldrich	AMPD1	Replaces Invitrogen brand used in original study
First-strand cDNA synthesis kit (includes pd(N)6 random hexamers and NotI-(dT) 18 primers)	Kit	Sigma–Aldrich	GE27-9261-01	Replaces SuperScript II reverse transcriptase from Invitrogen used in original study
QuantiTect Sybr Green PCR kit	Kit	Qiagen	204141	–
Real-time PCR machine	Instrument	Applied Biosystems	7500	Replaces Lightcycler 2.0 from Roche used in original study

Procedure

Note

- All cells will be sent for mycoplasma testing and STR profiling.
- DU145 cells grown in complete RPMI 1640: RPMI 1640 supplemented with 2 mM glutamine, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37˚C and 6% CO_2.

1. Seed 1.5 × 10^5 DU145 cells per well in a 12-well dish. Grow overnight.
2. Transfect with 100 nM siRNAs (siPTEN, siPTENP1, siPTEN Smartpool (siPTEN/PTENP1), or siLuc in separate wells) using Dharmafect 1 according to manufacturer's instructions or leave untransfected. Transfect control cells with siGLO RISC-free control siRNA according to manufacturer's instructions. Grow overnight.
3. Confirm that >90% of siGLO transfected control cells show fluorescence, indicating they were successfully transfected.
   a. If transfection is less than 90%, record efficiency for attempt, exclude attempt and do not continue with the rest of the procedure. Repeat procedure until >90% efficiency is obtained.
   b. If modification to transfection (step 2) is needed, record and maintain modified steps for remaining replicates.
4. 24 hr after transfection, extract total RNA directly on the culture dish using TRI reagent and 1-bromo-3-chloropropane according to manufacturer's instructions.
5. Treat RNA with DNAse following manufacturer's instructions.
6. Reverse transcribe 1 µg RNA/sample into cDNA using first-strand cDNA synthesis kit with primers following manufacturer's instructions.
   a. Record RNA concentration and purity (A_{280}/A_{260}).
7. Perform quantitative PCR reaction using the QuantiTect Sybr Green PCR kit:
   a. Use 2 µl of reverse transcription reaction per 20 µl real-time PCR reaction.
   b. Perform quantitative PCR for *PTEN*, *PTENP1*, *ACTIN*, and *36B4*.
      i. *PTEN* forward primer: 5′-GTTTACCGGCAGCATCAAAT-3′
      ii. *PTEN* reverse primer: 5′-CCCCCACTTTAGTGCACAGT-3′
      iii. *PTENP1* forward primer: 5′-TCAGAACATGGCATACACCAA-3′
      iv. *PTENP1* reverse primer: 5′-TGATGACGTCCGATTTTTCA-3′
      v. *ACTIN* forward primer: 5′-CATGTACGTTGCTATCCAGGC-3′
      vi. *ACTIN* reverse primer: 5′-CTCCTTAATGTCACGCACGAT-3′
      vii. *36B4* forward primer: 5′-GTGTTCGACAATGGCAGCAT-3′
      viii. *36B4* reverse primer: 5′-GACACCCTCCAGGAAGCGA-3′
         i. *36B4* primer sequences reported in ***Fullwood et al. (2009)***.
   c. Do not pre-treat with uracil-N-glycosylase.
   d. All reactions should be optimized and run in technical triplicate.
8. Using *ACTIN* as an internal standard, calculate the relative *PTEN* and *PTENP1* expression for each sample using the comparative Ct method.
   a. Additionally perform normalization using *36B4* as an internal standard (additional control).
9. Repeat independently four additional times.

Deliverables

- Data to be collected:
  - Images of fluorescence and phase/contrast of siGLO transfected cells.

- Purity ($A_{260/280}$ ratio) and concentration of isolated total RNA from cells.
- Raw data for all qPCR reactions.
- Quantification of *PTEN* and *PTENP1* mRNA levels relative to *ACTIN* or *36B4*.
- Quantification of fold change *PTEN* and *PTENP1* mRNA levels relative to siLuc transfected cells.
- Graph of fold change *PTEN* mRNA expression relative to siLuc. (Compare to Figure 2G, left).
- Graph of fold change *PTENP1* mRNA expression relative to siLuc. (Compare to Figure 2G, right).

Confirmatory analysis plan

This replication attempt will perform the following statistical analysis listed below.

- Statistical Analysis:
  - Note: at the time of analysis, we will perform the Shapiro–Wilk test and generate a quantile–quantile plot to assess the normality of the data. We will also perform Levene's test to assess homoscedasticity. If the data appear skewed we will perform the appropriate transformation in order to proceed with the proposed statistical analysis. If this is not possible we will perform the planned comparisons using the Wilcoxon–Mann Whitney test.
  - One-way MANOVA of *PTEN* and *PTENP1* mRNA levels in siLuc, siPTEN, siPTENP1, or siPTEN/PTENP1 siRNA transfected cells with the following planned comparisons using the Bonferroni correction:
    1. *PTEN* mRNA levels of siLuc transfected cells compared to siPTEN transfected cells.
    2. *PTEN* mRNA levels of siLuc transfected cells compared to siPTENP1 transfected cells.
    3. *PTEN* mRNA levels of siLuc transfected cells compared to siPTEN/PTENP1 transfected cells.
    4. *PTENP1* mRNA levels of siLuc transfected cells compared to siPTEN transfected cells.
    5. *PTENP1* mRNA levels of siLuc transfected cells compared to siPTENP1 transfected cells.
    6. *PTENP1* mRNA levels of siLuc transfected cells compared to siPTEN/PTENP1 transfected cells.
- Meta-analysis of effect sizes:
  - Compute the effect sizes of each comparison, compare them against the effect size in the original paper and use a random effects meta-analytic approach to combine the original and replication effects, which will be presented as a forest plot.
- Additional exploratory analysis:
  - The same analysis described above will be performed with *36B4* normalized values, which serves as an independent normalization control not included in the original analysis.

Known differences from the original study

The *PTEN* and *PTENP1* mRNA levels will be normalized with an independent control (*36B4*) in addition to *ACTIN*. All known differences are listed in the materials and reagents section above with the originally used item listed in the comments section. All differences have the same capabilities as the original and are not expected to alter the experimental design.

Provisions for quality control

The cell line used in this experiment will undergo STR profiling to confirm their identity and will be sent for mycoplasma testing to ensure there is no contamination. Transfection efficiency will be recorded for each replicate and any transfection that does not contain >90% efficiency will be excluded and not continue through the rest of the procedure. If the efficiency in the first attempt(s) does not obtain >90%, then any modifications to the transfection protocol will be recorded and the procedure will be maintained for the remaining replicates. The sample purity ($A_{260/280}$ ratio) of the isolated RNA from each sample will be reported. The *PTEN* and *PTENP1* mRNA levels will be normalized with an independent control (*36B4*). All the raw data, including the analysis files, will be uploaded to the project page on the OSF (https://osf.io/yyqas) and made publically available.

Protocol 4: Western blot of cells transfected with siRNA

This experiment utilizes western blot to assess the protein levels of PTEN after depletion of *PTEN*, *PTENP1*, or both. It is a replication of Figure 2H.

Sampling

- Experiment to be repeated a total of five times for a minimum power of 80%. The original data are qualitative, thus to determine an appropriate number of replicates to initially perform, sample sizes based on a range of potential variance was determined.

- See 'Power calculations' section for details.

- Experiment has 6 conditons:
  - Cohort 1: siGENOME non-targeting siRNA #2 (siLUC) transfected DU145 cells.
  - Cohort 2: siPTEN Smartpool (targets *PTEN* and *PTENP1*) transfected DU145 cells.
  - Cohort 3: siPTEN transfected DU145 cells.
  - Cohort 4: siPTENP1 transfected DU145 cells.
  - Cohort 5: Uninfected DU145 cells (additional negative control).
  - Transfection control: siGLO RISC-free siRNA transfected DU145 cells.
- Western blots performed for:
  - PTEN.
  - Hsp90 (loading control).

Materials and reagents

Reagent	Type	Manufacturer	Catalog #	Comments
DU145 cells	Cell line	ATCC	HTB-81	–
RPMI 1640 medium	Cell culture	Sigma–Aldrich	R8758	Replaces Invitrogen brand used in original study
Fetal bovine serum (FBS)	Cell culture	Sigma–Aldrich	F2442	Replaces Invitrogen brand used in original study
L-glutamine	Cell culture	Sigma–Aldrich	G7513	Original brand not specified
100× Penicillin/streptomycin	Cell culture	Sigma–Aldrich	P4333	Original brand not specified
0.05% trypsin/0.48 mM EDTA	Cell culture	Sigma–Aldrich	T3924	Original brand not specified
Phosphate buffered saline (PBS), without $MgCl_2$ and $CaCl_2$	Cell culture	Sigma–Aldrich	D8537	Original brand not specified
6 well tissue culture dishes	Labware	Corning	3516	Original brand not specified
siGLO RISC-free siRNA	Nucleic acid	Dharmacon	D-001600-01	–
siGENOME non-targeting siRNA #2 (siLUC)	Nucleic acid	Dharmacon	D-001210-02	–
ON-TARGETplus siPTEN Smartpool	Nucleic acid	Dharmacon	L-003023-00	Composed of: J-003023-09; J-003023-10; J-003023-11; J-003023-12
siPTEN	Nucleic acid	Dharmacon	Custom	See Supplemental Figure 6 of original paper for sequence
siPTENP1	Nucleic acid	Dharmacon	Custom	See Supplemental Figure 6 of original paper for sequence
Dharmafect 1	Cell culture	Dharmacon	T-2001-01	–
Microscope	Instrument	Olympus	LX81	Original brand not specified
Rabbit anti-PTEN (clone 138G6) monoclonal antibody	Antibodies	Cell Signaling	9559	–
Mouse anti-Hsp90 (clone 68) antibody	Antibodies	Becton Dickinson	610419	Original catalog number not specified
Secondary antibody (anti-rabbit IgG)	Antibodies	Cell Signaling	7074	Original brand not specified
Secondary antibody (anti-mouse IgG)	Antibodies	Cell Signaling	7076	Original brand not specified
ECL DualVue Western Markers (15–150 kDa)	Western blot reagent	Sigma–Aldrich	GERPN810	Original brand not specified
Tris	Chemical	Specific brand information will be left up to the discretion of the replicating lab and recorded later		
EDTA	Chemical			
$MgCl_2$	Chemical			
NaCl	Chemical			
NP_40	Chemical			
β-glycerophsphate	Chemical			
$NaVO_4$	Chemical			
NaF	Chemical			

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Reagent	Type	Manufacturer	Catalog #	Comments
Protease inhibitor cocktail (mammalian)	Inhibitor	Sigma–Aldrich	P8340	Original brand not specified
Sonifier	Instrument	Branson Digital	n/a	Original brand not specified
Bradford Reagent	Reporter assay	Sigma–Aldrich	B6916	Original brand not specified
TruPAGE LDS sample buffer (4×)	Buffer	Sigma–Aldrich	PCG3009	Original brand not specified
TruPAGE DTT sample reducer (10×)	Buffer	Sigma–Aldrich	PCG3005	–
XCell SureLOCK Mini-cell system	Instrument	Life Technologies	n/a	Original brand not specified
4–12% TruPAGE SDS-PAGE gel	Western blot reagent	Sigma–Aldrich	PCG2003	Replaces NuPage gels
TruPAGE TEA-Tricine SDS running buffer (20×)	Buffer	Sigma–Aldrich	PCG3001	Original brand not specified
Xcell II Blot Module	Instrument	Life Technologies	n/a	Original brand not specified
Hybond ECL nitrocellulose membrane	Western blot reagent	Sigma–Aldrich	GERPN2020D	Original brand not specified
TruPAGE transfer buffer (20×)	Buffer	Sigma–Aldrich	PCG3011	Original brand not specified
Ponceau S solution	Buffer	Sigma–Aldrich	P7170	–
10× Tris buffered saline (TBS)	Buffer	Sigma–Aldrich	T5912	Original brand not specified
ECL Prime Western blotting system	Detection assay	Sigma–Aldrich	GERPN2232	Original brand not specified
Image J	Software	NIH	Version 10.2	–

Procedure

Note

- All cells will be sent for mycoplasma testing and STR profiling.
- DU145 cells grown in complete RPMI 1640: RPMI 1640 supplemented with 2 mM glutamine, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37˚C and 6% CO_2.

1. Seed 3.75 × 10^5 DU145 cells per well in a 6-well dish. Grow overnight.
2. Transfect with 100 nM siRNAs (siPTEN, siPTENP1, siPTEN Smartpool (siPTEN/PTENP1), or siLuc in separate wells) using Dharmafect 1 according to manufacturer's instructions or leave untransfected. Transfect control cells with siGLO RISC-free control siRNA according to manufacturer's instructions. Grow overnight.
3. Confirm that >90% of siGLO transfected control cells show fluorescence, indicating they were successfully transfected.
   a. If transfection is less than 90%, record efficiency for attempt, exclude attempt and do not continue with the rest of the procedure. Repeat procedure until >90% efficiency is obtained.
   b. If modification to transfection (step 2) is needed, record and maintain modified steps for remaining replicates.
4. 48 hr after transfection lyse cells transfected with siRNAs and uninfected cells in lysis buffer on ice for 30 min.
   a. Lysis buffer: 50 mM Tris pH8.0, 1 mM EDTA, 1 mM $MgCl_2$, 150 mM NaCl, 1% NP-40, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF, protease inhibitors.
5. Gently sonicate protein lysate for 3 to 4 bursts for 5 to 10 s. Clear lysate by centrifugation at 10,000×*g* for 10 min at 4˚C.
6. Perform Bradford protein determination assay following manufacturer's instructions.
7. Separate 30 µg of protein (in 1× sample buffer and sample reducer) per lane on a 4–12% Tris Glycine SDS-PAGE gel with protein ladder following manufacturer's instructions.
   a. Sample run per gel:
      i. Protein molecular weight marker.
      ii. Untransfected DU145 cells.
      iii. DU145 cells transfected with siGENOME non-targeting siRNA #2.
      iv. DU145 cells transfected with siPTEN.
      v. DU145 cells transfected with siPTENP1.
      vi. DU145 cells transfected with siPTEN/PTENP1.

8. Transfer to nitrocellulose membrane (pre-wetted with methanol before use) at 25 V constant for 1–2 hr in 1× transfer buffer with 20% methanol following manufacturer's instructions.
   a. After transfer, stain membrane with Ponceau S solution following manufacturer's instructions to visualize transferred protein. Image membrane, then wash out the Ponceau stain (additional quality control step).
9. Perform western blotting with the following antibodies following manufacturer's instructions. Use 1× TBS for washes and blocking reagent recommended by manufacturer.
   a. rabbit anti-PTEN; use at 1:1000 dilution; 54 kDa.
   b. mouse anti-Hsp90; use at 1:1000 dilution; 90 kDa.
10. Detect signal with appropriate HRP conjugated secondary antibody followed by chemiluminescence following manufacturer's instructions.
11. Analyze scanned images using Image J software.
   a. Equal-sized regions of interest (ROI) will be positioned on specific bands.
   b. Background will be located within each individual lane but not occupied by any other discrete band.
   c. Subtract background pixel intensity from ROI pixel intensity.
   d. Normalize PTEN values by Hsp90 values from the same sample.
12. Repeat independently four additional times.

Deliverables

- Data to be collected:
  - Images of fluorescence and phase/contrast of siGLO transfected cells.
  - Images of Ponceau stained membranes and full films for all western blots with ladder. (Compare to Figure 2H).
  - Raw data file of ROI and background pixel intensities.
  - Normalize PTEN values for each sample.

Confirmatory analysis plan

This replication attempt will perform the following statistical analysis listed below.

- Statistical Analysis:
  - Note: at the time of analysis, we will perform the Shapiro–Wilk test and generate a quantile–quantile plot to assess the normality of the data. We will also perform Levene's test to assess homoscedasticity. If the data appear skewed we will perform the appropriate transformation in order to proceed with the proposed statistical analysis. If this is not possible we will perform the equivalent non-parametric test.
  - Two-way ANOVA of normalized PTEN levels in siLuc, siPTEN, siPTENP1, or siPTEN/PTENP1 siRNA transfected cells with the following planned comparisons using the Bonferroni correction:
    1. siLuc compared to siPTEN.
    2. siLuc compared to siPTENP1.
    3. siLuc compared to siPTEN/PTENP1.
    4. siPTEN/PTENP1 compared to siPTEN.
    5. siPTEN/PTENP1 compared to siPTENP1.
- Meta-analysis of effect sizes:
  - The replication data (mean and 95% confidence interval) will be plotted with the original reported data value plotted as a single point on the same plot for comparison.

Known differences from the original study

The original study used 12 well plates seeded with 1.5×10^5 DU145 cells per well, which was increased 2.5× to account for the difference in cell surface area. All known differences are listed in the materials and reagents section above with the originally used item listed in the comments section. All differences have the same capabilities as the original and are not expected to alter the experimental design.

Provisions for quality control

The cell line used in this experiment will undergo STR profiling to confirm their identity and will be sent for mycoplasma testing to ensure there is no contamination. Transfection efficiency will be recorded for each replicate and any transfection that does not contain >90% efficiency will be excluded and not continue through the rest of the procedure. If the efficiency in the first attempt

(s) does not obtain >90%, then any modifications to the transfection protocol will be recorded and the procedure will be maintained for the remaining replicates. Ponceau stained membranes will be used to assess completeness of transfer. All the raw data, including the analysis files, will be uploaded to the project page on the OSF (https://osf.io/yyqas) and made publically available.

Protocol 5: Quantitative PCR following PTEN 3′ UTR transfection

This experiment tests the effect of expressing the 3′ UTR of *PTENP1* on mRNA expression levels of *PTENP1*. It is a replication of the left panel of Figure 4A.

Sampling

- Experiment to be repeated a total of three times for a minimum power of 98%.
  - See 'Power calculations' section for details.
- Experiment has 3 conditions:
  - Cohort 1: pCMV transfected DU145 cells.
  - Cohort 2: pCMV/*PTEN* 3′ UTR transfected DU145 cells.
  - Cohort 3: Uninfected DU145 cells (additional negative control).
- Quantitative RT-PCR performed in technical triplicate for the following genes:
  - *PTENP1*.
  - *ACTIN* (internal control).
  - *36B4* (additional internal control).

Materials and reagents

Reagent	Type	Manufacturer	Catalog #	Comments
DU145 cells	Cell line	ATCC	HTB-81	–
RPMI 1640 medium	Cell culture	Sigma–Aldrich	R8758	Replaces Invitrogen brand used in original study
Fetal bovine serum (FBS)	Cell culture	Sigma–Aldrich	F2442	Replaces Invitrogen brand used in original study
L-glutamine	Cell culture	Sigma–Aldrich	G7513	Original brand not specified
100× Penicillin/streptomycin	Cell culture	Sigma–Aldrich	P4333	Original brand not specified
0.05% trypsin/0.48 mM EDTA	Cell culture	Sigma–Aldrich	T3924	Original brand not specified
Phosphate buffered saline (PBS), without $MgCl_2$ and $CaCl_2$	Cell culture	Sigma–Aldrich	D8537	Original brand not specified
60 mm tissue culture dishes	Labware	Corning	430166	Original brand not specified
Endo-free maxiprep kit	Kit	Sigma–Aldrich	NA0400	–
pCMV (empty vector)	DNA construct	Original lab	n/a	From original lab
pCMV/*PTEN* 3′ UTR	DNA construct	Original lab	n/a	From original lab
Effectene	Cell culture	Qiagen	301425	Original brand not specified
PTENP1 forward and reverse primers	Nucleic acid	Specific brand information will be left up to the discretion of the replicating lab and recorded later		
ACTIN forward and reverse primers	Nucleic acid			
36B4 forward and reverse primers	Nucleic acid			
TRI reagent	Chemical	Sigma–Aldrich	T9424	Replaces Trizol reagent from Invitrogen
1-bromo-3-chloropropase	Chemical	Sigma–Aldrich	B9673	Reagent needed from TRI reagent protocol
Nuclease free water	Chemical	Sigma–Aldrich	W4502	Reagent needed from TRI reagent protocol
DNAse I amplification grade	Chemical	Sigma–Aldrich	AMPD1	Replaces Invitrogen brand used in original study
First-strand cDNA synthesis kit (includes pd(N)6 random hexamers and NotI-(dT) 18 primers)	Kit	Sigma–Aldrich	GE27-9261-01	Replaces SuperScript II reverse transcriptase from Invitrogen used in original study
QuantiTect Sybr Green PCR kit	Kit	Qiagen	204141	–
Real-time PCR system	Instrument	Applied Biosystems	7500 Fast	Replaces Roche Lightcycler 2.0 used in original study

Procedure

Note

- All cells will be sent for mycoplasma testing and STR profiling.
- DU145 cells grown in complete RPMI 1640: RPMI 1640 supplemented with 2 mM glutamine, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37˚C and 6% CO_2.

1. Grow and prepare endotoxin-free plasmid constructs following manufacturer's instructions for an endotoxin-free plasmid maxiprep kit.
   a. pCMV (empty vector).
   b. pCMV/*PTEN* 3′ UTR.
      i. Sequence gene of interest in each plasmid and run whole plasmids on agarose gel to confirm vector integrity.
2. Seed 3.5×10^5 DU145 cells per dish in 6 cm dishes. Grow overnight.
3. Transfect with pCMV or pCMV/*PTEN* 3′ UTR plasmids using Effectene according to manufacturer's instructions and recommended DNA and reagent amounts.
4. 24 hr after transfection, extract total RNA from each cohort directly on the culture dish using TRI reagent and 1-bromo-3-chloropropane according to manufacturer's instructions.
5. Treat RNA with DNase I following manufacturer's instructions.
6. Reverse transcribe 1 µg RNA/sample into cDNA using first-strand cDNA synthesis kit with primers following manufacturer's instructions.
   a. Record RNA concentration and purity (A_{280}/A_{260}).
7. Perform quantitative PCR reaction using the QuantiTect SYBR Green PCR kit:
   a. Use 2 µl of reverse transcription reaction per 20 µl real-time PCR reaction.
   b. Perform quantitative PCR for *PTENP1*, *ACTIN*, and *36B4*.
      i. *PTENP1* forward primer: 5′-TCAGAACATGGCATACACCAA-3′
      ii. *PTENP1* reverse primer: 5′-TGATGACGTCCGATTTTTCA-3′
      iii. *ACTIN* forward primer: 5′-CATGTACGTTGCTATCCAGGC-3′
      iv. *ACTIN* reverse primer: 5′-CTCCTTAATGTCACGCACGAT-3′
      v. *36B4* forward primer: 5′-GTGTTCGACAATGGCAGCAT-3′
      vi. *36B4* reverse primer: 5′-GACACCCTCCAGGAAGCGA-3′
   c. *36B4* primer sequences reported in ***Fullwood et al. (2009)***.
   d. Do not pre-treat with uracil-N-glycosylase.
   e. All reactions should be optimized and run in technical triplicate.
8. Using *ACTIN* as an internal standard, calculate the fold change in *PTEN1P* expression relative to pCMV expressing cells using the comparative Ct method.
   a. Additionally perform normalization using *36B4* as an internal standard (additional control).
9. Repeat independently two additional times.

Deliverables

- Data to be collected:
  - Purity ($A_{260/280}$ ratio) and concentration of isolated total RNA from cells.
  - Raw data for all qPCR reactions.
  - Quantification of *PTENP1* mRNA levels relative to *ACTIN* or *36B4*.
  - Quantification of fold change *PTENP1* mRNA levels relative to pCMV transfected cells. (Compare to Figure 4A, left panel).

Confirmatory analysis plan

This replication attempt will perform the following statistical analysis listed below.

- Statistical Analysis:
  - Note: at the time of analysis, we will perform the Shapiro–Wilk test and generate a quantile–quantile plot to assess the normality of the data. We will also perform Levene's test to assess homoscedasticity. If the data appear skewed we will perform the appropriate transformation in order to proceed with the proposed statistical analysis. If this is not possible we will perform the equivalent non-parametric test.
  - Unpaired two-tailed *t*-test of *PTENP1* mRNA levels of pCMV transfected cells compared to pCMV/*PTEN* 3′ UTR transfected cells.

- Meta-analysis of effect sizes:
  - Compute the effect sizes of each comparison, compare them against the effect size in the original paper and use a random effects meta-analytic approach to combine the original and replication effects, which will be presented as a forest plot.
- Additional exploratory analysis:
  - The same analysis described above will be performed with *36B4* normalized values, which serves as an independent normalization control not included in the original analysis.

Known differences from the original study

The *PTENP1* mRNA levels will be normalized with an independent control (*36B4*) in addition to *ACTIN*. All known differences are listed in the materials and reagents section above with the originally used item listed in the comments section. All differences have the same capabilities as the original and are not expected to alter the experimental design.

Provisions for quality control

The cell line used in this experiment will undergo STR profiling to confirm their identity and will be sent for mycoplasma testing to ensure there is no contamination. The sample purity ($A_{260/280}$ ratio) of the isolated RNA from each sample will be reported. The *PTENP1* mRNA levels will be normalized with an independent control (*36B4*). All the raw data, including the analysis files, will be uploaded to the project page on the OSF (https://osf.io/yyqas) and made publically available.

Protocol 6: Cell growth assay following PTEN 3′ UTR transfection

This experiment tests the effect of expressing the 3′ UTR of PTENP1 on cell growth. It is a replication of the right panel of Figure 4A.

Sampling

- Experiment to be repeated a total of three times for a minimum power of 98%.
  - See 'Power calculations' section for details.
- Experiment has 3 conditions:
  - Cohort 1: pCMV transfected DU145 cells.
  - Cohort 2: pCMV/*PTEN* 3′ UTR transfected DU145 cells.
  - Cohort 3: Uninfected DU145 cells (additional negative control).
- Each cohort is harvested on the following days performed in technical triplicate:
  - Day 0 (after O/N incubation).
  - Day 1.
  - Day 2.
  - Day 3.
  - Day 4.
  - Day 5.

Materials and reagents

Reagent	Type	Manufacturer	Catalog #	Comments
DU145 cells	Cell line	ATCC	HTB-81	–
RPMI 1640 medium	Cell culture	Sigma–Aldrich	R8758	Replaces Invitrogen brand used in original study
Fetal bovine serum (FBS)	Cell culture	Sigma–Aldrich	F2442	Replaces Invitrogen brand used in original study
L-glutamine	Cell culture	Sigma–Aldrich	G7513	Original brand not specified
100× Penicillin/streptomycin	Cell culture	Sigma–Aldrich	P4333	Original brand not specified
0.05% trypsin/0.48 mM EDTA	Cell culture	Sigma–Aldrich	T3924	Original brand not specified
Phosphate buffered saline (PBS), without $MgCl_2$ and $CaCl_2$	Cell culture	Sigma–Aldrich	D8537	Original brand not specified
60 mm tissue culture dishes	Labware	Corning	430166	Original brand not specified
pCMV (empty vector)	DNA construct	Original lab	n/a	From original lab

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Reagent	Type	Manufacturer	Catalog #	Comments
pCMV/*PTEN* 3′ UTR	DNA construct	Original lab	n/a	From original lab
Effectene	Cell culture	Qiagen	301425	Original brand not specified
12 well tissue culture dishes	Labware	Corning	3513	Original brand not specified
Crystal violet	Dye	Sigma–Aldrich	C0775	Original brand not specified
Formalin	Chemical	Specific brand information will be left up to the discretion of the replicating lab and recorded later		
Acetic acid	Chemical			
Methanol	Chemical			
Spectrophotometer capable of reading at 590 nm (or 595 nm)	Instrument	Alamolabs		Original brand not specified

Procedure

Note

- All cells will be sent for mycoplasma testing and STR profiling.
- DU145 cells grown in complete RPMI 1640: RPMI 1640 supplemented with 2 mM glutamine, 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37˚C and 6% CO_2.

1. Seed 3.5×10^5 DU145 cells per dish in 6 cm dishes. Grow overnight.
2. Transfect with pCMV or pCMV/*PTEN* 3′ UTR plasmids using Effectene according to manufacturer's instructions and recommended DNA and reagent amounts.
   a. Plasmids prepped in Protocol 7.
3. 6 hr after transfection, resuspend 2×10^5 pCMV, pCMV/*PTEN* 3′ UTR, and untransfected cells in 50 ml fresh media. Seed three wells of six sets of 12-well plates with 2 ml of each cell line. Each set of 12 well plates should have three wells containing untransfected cells, three wells containing pCMV-transfected cells, and three wells containing pCMV/PTEN 3′ UTR-transfected cells. Incubate overnight.
4. Fix one plate every 24 hr starting after overnight incubation (the first plate fixed will be called day 0).
   a. Wash wells once in PBS.
   b. Fix wells with 10% formalin for 10 min at room temperature.
   c. Store plates in PBS at 4˚C.
   d. All wells should be fixed by day 6.
5. Stain cells with 0.1% crystal violet, 20% methanol for 15 min. Wash cells.
6. Lyse all wells with 10% acetic acid for 10 min.
7. Read optical density at 590 nm.
   a. Reading can be done at 595 nm if 590 nm is not available.
8. Repeat independently two additional times.

Deliverables

- Data to be collected:
  - Raw data of absorbance from plate reader.
  - Relative absorbance for each cohort over time. (Compare to Figure 4A, right panel).

Confirmatory analysis plan

This replication attempt will perform the following statistical analysis listed below.

- Statistical Analysis:
  - Note: at the time of analysis, we will perform the Shapiro–Wilk test and generate a quantile–quantile plot to assess the normality of the data. We will also perform Levene's test to assess homoscedasticity. If the data appear skewed we will perform the appropriate transformation in order to proceed with the proposed statistical analysis. If this is not possible we will perform the equivalent non-parametric test.
  - Unpaired two-tailed *t*-test of Day 5 absorbance of pCMV transfected cells compared to pCMV/*PTEN* 3′ UTR transfected cells.

- Unpaired two-tailed *t*-test of AUC measurements (determined from day 0, 1, 2, 3, 4, and 5 for each replicate) of pCMV transfected cells compared to pCMV/*PTEN* 3′ UTR transfected cells.

- Meta-analysis of effect sizes:
  - Compute the effect sizes of each comparison, compare them against the effect size in the original paper and use a random effects meta-analytic approach to combine the original and replication effects, which will be presented as a forest plot.

Known differences from the original study

All known differences are listed in the materials and reagents section above with the originally used item listed in the comments section. All differences have the same capabilities as the original and are not expected to alter the experimental design.

Provisions for quality control

The cell line used in this experiment will undergo STR profiling to confirm their identity and will be sent for mycoplasma testing to ensure there is no contamination. All the raw data, including the analysis files, will be uploaded to the project page on the OSF (https://osf.io/yyqas) and made publically available.

Power calculations

For additional details on power calculations, please see analysis scripts and associated files on the OSF:

https://osf.io/cd2yq/

Protocol 1

Summary of original data estimated from graph reported in Figure 1D:

siRNA	mRNA	Mean	Stdev	N
siLUC	*PTEN*	1.00	0.239	3
	PTENP1	1.00	0.386	3
19b	*PTEN*	0.286	0.085	3
	PTENP1	0.234	0.065	3
20a	*PTEN*	0.458	0.167	3
	PTENP1	0.250	0.080	3

Test family

- 2 tailed *t* test, Wilcoxon–Mann-Whitney test, Bonferroni's correction: alpha error = 0.0125.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc *PTEN* mRNA	19b *PTEN* mRNA	3.98555	91.9%*	4*	4*
siLuc *PTEN* mRNA	20a *PTEN* mRNA	2.62999	88.3%	6	6
siLuc *PTENP1* mRNA	19b *PTENP1* mRNA	2.76532	80.7%†	5†	5†
siLuc *PTENP1* mRNA	20a *PTENP1* mRNA	2.68908	89.8%	6	6

*6 samples per group will be used based on the siLuc to 20a *PTEN* comparison making the power 99.9%.
†6 samples per group will be used based on the siLuc to 20a *PTEN* comparison making the power 91.6%.

Test family

- Due to the large variance, these parametric tests are only used for comparison purposes. The sample size is based on the non-parametric tests listed above.
- Two-way ANOVA: Fixed effects, special, main effects and interactions: alpha error = 0.05.

○ Due to a lack of raw original data, we are unable to perform power calculations using a MANOVA. We are using a two-way ANOVA to estimate sample size.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

ANOVA F test statistic and partial η^2 performed with R software, version 3.1.2 (***R Development Core Team, 2014***).

Groups	F test statistic	Partial η^2	Effect size *f*	A priori power	Total sample size
siLUC, 19b, 20a (*PTEN* and *PTENP1* mRNA for all)	F(2,12) = 23.1978 (main effect: siRNA)	0.79451	1.96629	96.3%*	9* (6 groups)

*36 total samples (6 per group) will be used based on the planned comparisons making the power 99.9%.

Test family

- Due to the large variance, these parametric tests are only used for comparison purposes. The sample size is based on the non-parametric tests listed above.
- 2 tailed *t* test, difference between two independent means, Bonferroni's correction: alpha error = 0.0125.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc *PTEN* mRNA	19b *PTEN* mRNA	3.98555	94.2%*	4*	4*
siLuc *PTEN* mRNA	20a *PTEN* mRNA	2.62999	90.6%	6	6
siLuc *PTENP1* mRNA	19b *PTENP1* mRNA	2.76532	84.0%†	5†	5†
siLuc *PTENP1* mRNA	20a *PTENP1* mRNA	2.68908	81.6%‡	5‡	5‡

*6 samples per group will be used based on the siLuc to 20a *PTEN* comparison making the power 99.9%.
†6 samples per group will be used based on the siLuc to 20a *PTEN* comparison making the power 93.4%.
‡6 samples per group will be used based on the siLuc to 20a *PTEN* comparison making the power 91.9%.

Protocol 2

Summary of original data estimated from graph reported in Figure 2F:

siRNA	Day	Mean	Stdev	N
siLuc	0	1.000	0	3
	1	0.955	0.108	3
	2	0.928	0.108	3
	3	1.252	0.108	3
	4	1.315	0.108	3
	5	1.604	0.108	3
siPTEN	0	1.000	0	3
	1	1.198	0.108	3
	2	1.306	0.108	3
	3	2.045	0.108	3
	4	2.414	0.108	3
	5	4.153	0.234	3

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siRNA	Day	Mean	Stdev	N
siPTENP1	0	1.000	0	3
	1	1.162	0.108	3
	2	1.099	0.108	3
	3	1.613	0.108	3
	4	1.775	0.108	3
	5	2.613	0.108	3
siPTEN/PTENP1	0	1.000	0	3
	1	1.198	0.108	3
	2	1.387	0.108	3
	3	2.396	0.108	3
	4	3.099	0.108	3
	5	5.414	0.171	3

AUC calculations from estimated values.

Calculations performed with R software 3.1.2 (***R Development Core Team, 2014***).

siRNA	Days	Mean	Stdev	N
siLuc	0, 1, 2, 3, 4, 5	5.752	0.486	3
siPTEN	0, 1, 2, 3, 4, 5	9.541	0.550	3
siPTENP1	0, 1, 2, 3, 4, 5	7.455	0.486	3
siPTEN/PTENP1	0, 1, 2, 3, 4, 5	11.288	0.518	3

Test family

- Two-way ANOVA: Fixed effects, special, main effects and interactions: alpha error = 0.05.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

ANOVA F test statistic and partial η^2 performed with R software, version 3.1.2 (***R Development Core Team, 2014***).

Day 5 values

Groups	F test statistic	Partial η^2	Effect size *f*	A priori power	Total sample size
siLUC, siPTEN, siPTENP1, siPTEN/PTENP1	F(1,8) = 798.9603 (main effect: siPTEN)	0.99009	9.99337	92.0%*	5* (4 groups)
	F(1,8) = 143.7867 (main effect: siPTENP1)	0.94729	4.23948	99.5%*	6* (4 groups)

*16 total samples (4 per group) will be used based on the planned comparisons making the power 99.9%.

AUC values

Groups	F test statistic	Partial η2	Effect size f	A priori power	Total sample size
siLUC, siPTEN, siPTENP1, siPTEN/PTENP1	F(1,8) = 166.9731 (main effect: siPTEN)	0.95428	4.56857	99.8%*	6* (4 groups)
	F(1,8) = 34.2219 (main effect: siPTENP1)	0.81053	2.06827	93.9%*	7* (4 groups)

*16 total samples (4 per group) will be used based on the planned comparisons making the power 99.9%.

Test family

- 2 tailed *t* test, difference between two independent means, Bonferroni's correction: alpha error = 0.01.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

Day 5 values

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc	siPTEN	15.54994	91.1%*	2*	2*
siLuc	siPTENP1	6.15404	91.1%*	3*	3*
siLuc	siPTEN/PTENP1	23.24249	99.9%*	2*	2*
siPTEN/PTENP1	siPTEN	7.69255	99.4%*	3*	3*
siPTEN/PTENP1	siPTENP1	17.08845	94.6%*	2*	2*

*5 samples per group will be used based on the AUC calculation planned comparisons making the power 99.9%.

AUC values

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc	siPTEN	7.41636	99.1%*	3*	3*
siLuc	siPTENP1	3.33339	93.8%	5	5*
siLuc	siPTEN/PTENP1	10.83794	99.9%*	3*	3*
siPTEN/PTENP1	siPTEN	3.42158	81.3%†	4†	4†
siPTEN/PTENP1	siPTENP1	7.50454	99.3%*	3*	3*

*5 samples per group will be used based on the siLuc to siPTENP1 comparison making the power 99.9%.
†5 samples per group will be used based on the siLuc to siPTENP1 comparison making the power 95.0%.

Protocol 3

Summary of original data estimated from graph reported in Figure 2G:

siRNA	mRNA	Mean	Stdev	N
siLUC	*PTEN*	1.000	0.249	3
	PTENP1	1.000	0.155	3
siPTEN	*PTEN*	0.116	0.065	3
	PTENP1	0.543	0.099	3
siPTENP1	*PTEN*	0.381	0.086	3
	PTENP1	0.269	0.094	3
siPTEN/PTENP1	*PTEN*	0.193	0.067	3
	PTENP1	0.482	0.161	3

Test family

- 2 tailed *t* test, Wilcoxon–Mann-Whitney test, Bonferroni's correction: alpha error = 0.008333.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

Test family

- Due to the large variance, these parametric tests are only used for comparison purposes. The sample size is based on the non-parametric tests listed above.
- Two-way ANOVA: Fixed effects, special, main effects and interactions: alpha error = 0.05.

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc *PTEN* mRNA	siPTEN *PTEN* mRNA	4.85884	96.8%*	4*	4*
siLuc *PTENP1* mRNA	siPTEN *PTENP1* mRNA	3.52537	93.1%	5	5
siLuc *PTEN* mRNA	siPTENP1 *PTEN* mRNA	3.32267	89.7%	5	5
siLuc *PTENP1* mRNA	siPTENP1 *PTENP1* mRNA	5.70745	83.9%†	3†	3†
siLuc *PTEN* mRNA	siPTEN/PTENP1 *PTEN* mRNA	4.42658	93.2%‡	4‡	4‡
siLuc *PTENP1* mRNA	siPTEN/PTENP1 *PTENP1* mRNA	3.27585	88.7%	5	5

*5 samples per group will be used based on the siLuc to siPTENP1 *PTEN* comparison making the power 99.8%.
†5 samples per group will be used based on the siLuc to siPTENP1 *PTEN* comparison making the power 99.9%.
‡5 samples per group will be used based on the siLuc to siPTENP1 *PTEN* comparison making the power 99.3%.

○ Due to a lack of raw original data, we are unable to perform power calculations using a MANOVA. We are using a two-way ANOVA to estimate sample size.

Groups	F test statistic	Partial η^2	Effect size *f*	A priori power	Total sample size
siLUC, siPTEN, siPTENP1, siPTEN/PTENP1 (*PTEN* and *PTENP1* mRNA for all)	F(3,16) = 36.6570 (main effect: siRNA)	0.87299	2.62168	94.9%*	11* (8 groups)

*40 total samples (5 per group) will be used based on the planned comparisons making the power 99.9%.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

ANOVA F test statistic and partial η^2 performed with R software, version 3.1.2 (***R Development Core Team, 2014***).

Test family

■ Due to the large variance, these parametric tests are only used for comparison purposes. The sample size is based on the non-parametric tests listed above.

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc *PTEN* mRNA	siPTEN *PTEN* mRNA	4.85884	98.1%*	4*	4*
siLuc *PTENP1* mRNA	siPTEN *PTENP1* mRNA	3.52537	80.8%†	4†	4†
siLuc *PTEN* mRNA	siPTENP1 *PTEN* mRNA	3.32267	92.2%	5	5
siLuc *PTENP1* mRNA	siPTENP1 *PTENP1* mRNA	5.70745	89.1%‡	3‡	3‡
siLuc *PTEN* mRNA	siPTEN/PTENP1 *PTEN* mRNA	4.42658	95.4%§	4§	4§
siLuc *PTENP1* mRNA	siPTEN/PTENP1 *PTENP1* mRNA	3.27585	91.4%	5	5

*5 samples per group will be used based on the siLuc to siPTENP1 *PTEN* comparison making the power 99.9%.
†5 samples per group will be used based on the siLuc to siPTENP1 *PTEN* comparison making the power 95.1%.
‡5 samples per group will be used based on the siLuc to siPTENP1 *PTEN* comparison making the power 99.9%.
§5 samples per group will be used based on the siLuc to siPTENP1 *PTEN* comparison making the power 99.6%.

■ 2 tailed *t* test, difference between two independent means, Bonferroni's correction: alpha error = 0.008333.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

siRNA	Relative PTEN signal
siLuc	1.00
siPTEN	0.50
siPTENP1	0.60
siPTEN/PTENP1	0.10

Protocol 4

Summary of original data reported in Figure 2H:

The original data do not indicate the error associated with multiple biological replicates. To identify a suitable sample size, power calculations were performed using different levels of relative variance.

Test family

- Two-way ANOVA: Fixed effects, special, main effects and interactions: alpha error = 0.05.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

ANOVA F test statistic and partial η^2 performed with R software, version 3.1.2 (***R Development Core Team, 2014***).

2% variance:

Groups	F test statistic	Partial η^2	Effect size *f*	A priori power	Total sample size
siLUC, siPTEN, siPTENP1, siPTEN/PTENP1	F(1,8) = 4629.6 (main effect: siPTEN)	0.99828	24.0564	99.9%	8 (4 groups)
	F(1,8) = 2963.0 (main effect: siPTENP1)	0.99731	19.24404	99.9%	8 (4 groups)

15% variance:

Groups	F test statistic	Partial η^2	Effect size *f*	A priori power	Total sample size
siLUC, siPTEN, siPTENP1, siPTEN/PTENP1	F(1,8) = 82.3050 (main effect: siPTEN)	0.91141	3.20750	99.9%	8 (4 groups)
	F(1,8) = 52.6750 (main effect: siPTENP1)	0.86815	2.56600	99.9%	8 (4 groups)

28% variance:

Groups	F test statistic	Partial η^2	Effect size *f*	A priori power	Total sample size
siLUC, siPTEN, siPTENP1, siPTEN/PTENP1	F(1,8) = 23.6210 (main effect: siPTEN)	0.74700	1.71830	94.5%	8 (4 groups)
	F(1,8) = 15.1170 (main effect: siPTENP1)	0.65394	1.37464	82.4%	8 (4 groups)

40% variance:

Groups	F test statistic	Partial η^2	Effect size *f*	A priori power	Total sample size
siLUC, siPTEN, siPTENP1, siPTEN/PTENP1	F(1,8) = 11.5741 (main effect: siPTEN)	0.59130	1.20281	95.3%	12 (4 groups)
	F(1,8) = 7.4074 (main effect: siPTENP1)	0.48077	0.96225	83.0%	12 (4 groups)

Test family

- 2 tailed *t* test, difference between two independent means, Bonferroni's correction: alpha error = 0.01.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

2% variance:

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc	siPTEN	39.28399	99.9%	2	2
siLuc	siPTENP1	31.42719	99.9%	2	2
siLuc	siPTEN/PTENP1	70.71118	99.9%	2	2
siPTEN/PTENP1	siPTEN	31.42719	99.9%	2	2
siPTEN/PTENP1	siPTENP1	39.28399	99.9%	2	2

15% variance:

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc	siPTEN	5.23782	86.6%	3	3

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Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc	siPTENP1	4.19026	86.6%	4	4
siLuc	siPTEN/PTENP1	9.42808	99.9%	3	3
siPTEN/PTENP1	siPTEN	4.19026	94.6%	4	4
siPTEN/PTENP1	siPTENP1	5.23782	86.6%	3	3

28% variance:

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc	siPTEN	2.80603	82.0%	5	5
siLuc	siPTENP1	2.24482	84.8%	7	7
siLuc	siPTEN/PTENP1	5.05085	84.0%	3	3
siPTEN/PTENP1	siPTEN	2.24482	84.8%	7	7
siPTEN/PTENP1	siPTENP1	2.80603	82.0%	5	5

40% variance:

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
siLuc	siPTEN	1.96419	86.9%	8	8
siLuc	siPTENP1	1.57135	84.7%	12	12
siLuc	siPTEN/PTENP1	3.53554	91.0%	4	4
siPTEN/PTENP1	siPTEN	1.57135	83.6%	12	12
siPTEN/PTENP1	siPTENP1	1.96419	81.0%	8	8

In order to produce quantitative replication data, we will run the experiment five times. Each time we will quantify band intensity. We will determine the standard deviation of band intensity across the biological replicates and combine this with the reported value from the original study to simulate the original effect size. We will use this simulated effect size to determine the number of replicates necessary to reach a power of at least 80%. We will then perform additional replicates, if required, to ensure that the experiment has more than 80% power to detect the original effect.

Protocol 5

Summary of original data estimated from graph reported in Figure 4A, left panel:

Plasmid	Mean	Stdev	N
pCMV	1.000	0.541	3
pCMV/*PTEN* 3′ UTR	3.880	0.707	3

Test family

- 2 tailed *t* test, difference between two independent means, alpha error = 0.05.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
pCMV	pCMV/*PTEN* 3′ UTR	4.57446	98.2%	3	3

Protocol 6

Summary of original data estimated from graph reported in Figure 4A, right panel:

Plasmid	Day	Mean	Stdev	N
pCMV	0	1.000	0	3
	1	1.000	0.119	3
	2	1.238	0.119	3
	3	1.917	0.119	3
	4	5.726	0.119	3
	5	7.167	0.119	3
pCMV/*PTEN* 3′ UTR	0	1.000	0	3
	1	1.000	0.119	3
	2	1.143	0.119	3
	3	1.917	0.119	3
	4	4.155	0.214	3
	5	5.238	0.119	3

AUC calculations from estimated values.
Calculations performed with R software 3.1.2 (***R Development Core Team, 2014***).

Plasmid	Days	Mean	Stdev	N
pCMV	0, 1, 2, 3, 4, 5	13.964	0.536	3
pCMV/*PTEN* 3′ UTR	0, 1, 2, 3, 4, 5	11.333	0.631	3

Test family

- 2 tailed *t* test, difference between two independent means, alpha error = 0.05.

'Power calculations' performed with G*Power software, version 3.1.7 (***Faul et al., 2007***).

Day 5 values

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
pCMV	pCMV/*PTEN* 3′ UTR	16.20000	99.9%	2*	2*

*3 samples per group will be used based on the AUC calculation.

AUC values

Group 1	Group 2	Effect size *d*	A priori power	Group 1 sample size	Group 2 sample size
pCMV	pCMV/*PTEN* 3′ UTR	4.49526	97.9%	3	3

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Competing interests

IK: Alamo Laboratories Inc. is a Science Exchange associated laboratory. JK: Biotechnology Research and Education Program, University of Maryland is a Science Exchange associated laboratory. RP:CB: EI, FT, JL, and NP are employed and holds shares in Science Exchange Inc. The other authors declare that no competing interests exist.

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