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

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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 bi-modal functionality modulating PTEN (Johnson 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                       |
| 100x 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 MgCl₂ and CaCl₂ | 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     | Dharmacon    | T-2001-01   | Specific brand information will be left up to the discretion of the replicating lab and recorded later |
| PTEN forward and reverse primers             | Nucleic acid     | Dharmacon    | T-2001-01   |                                                    |
| ACTIN forward and reverse primers            | Nucleic acid     | Dharmacon    | T-2001-01   |                                                    |
| 36B4 forward and reverse primers             | Nucleic acid     | Dharmacon    | T-2001-01   |                                                    |
| 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% CO₂.

1. Seed 1.5 × 10⁵ 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 (A₂₆₀/A₂₈₀).
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’-GTGTTACCGGCACCATCAAAT-3’
      ii. PTEN reverse primer: 5’-CCCCCACCTTTAATGTCACAGT-3’
      iii. PTENP1 forward primer: 5’-TCAGAACATGGCATACACCAA-3’
      iv. PTENP1 reverse primer: 5’-TGATGACGCAGTTTCTTTCA-3’
      v. ACTIN forward primer: 5’-CATGTACGTTGCTATCCAGGC-3’
      vi. ACTIN reverse primer: 5’-CTCCTTAATGTCACGCACGAT-3’
     vii. 36B4 forward primer: 5’-GTGTTCGACAAATGGGAGCAT-3’
     viii. 36B4 reverse primer: 5’-GACACCTCCAGGAAGCGA-3’
   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 (A₂₆₀/A₂₈₀ 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
(A260/280 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       |
| 100x Penicillin/streptomycin           | Cell culture  | Sigma–Aldrich| P4333     | Original brand not specified       |
| 0.05% trypsin/0.48 mM EDTA             | Cell culture  | Sigma–Aldrich| T9324     | Original brand not specified       |
| Phosphate buffered saline (PBS), without MgCl₂ and CaCl₂ | 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      |              |           | Specified 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% CO₂.

1. Seed 1.5 x 10⁵ 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 x 10⁵ 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 conditions:
  - 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.
  - 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                  |
| 100x 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 MgCl2 and CaCl2 | 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  | Specifi c brand information will be left up to the discretion of the replicating lab and recorded later |
| PTEN forward and reverse primers            | Nucleic acid  | Specifi c brand information will be left up to the discretion of the replicating lab and recorded later |
| PTENP1 forward and reverse primers          | Nucleic acid  | Specifi c brand information will be left up to the discretion of the replicating lab and recorded later |
| 36B4 forward and reverse primers            | Nucleic acid  | Specifi c brand information will be left up to the discretion of the replicating lab and recorded later |
| 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
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% CO₂.

1. Seed 1.5 × 10⁵ 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 (A₂₈₀/A₂₆₀).

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' - CCCCCACCTTTAGTGCACGT - 3'
      iii. PTENP1 forward primer: 5' - TCAAGACATGGCATAACCAA - 3'
      iv. PTENP1 reverse primer: 5' - TGATGACGTCCGATTTTTCA - 3'
      v. ACTIN forward primer: 5' - CATGTACGTTGCTATCCAGGC - 3'
      vi. ACTIN reverse primer: 5' - GTGTTGACAAATGGCAGCAT - 3'
      vii. 36B4 forward primer: 5' - GACACCCCTCCAGGAAGCGA - 3'
      viii. 36B4 reverse primer: 5' - GACACCCCTCCAGGAAGCGA - 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.
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 (A260/280 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 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: 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                                             |
| 100x 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 MgCl₂ and CaCl₂ | 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           |              |             |                                                                          |
| MgCl₂                                            | Chemical           |              |             |                                                                          |
| NaCl                                             | Chemical           |              |             |                                                                          |
| NP₄₀                                            | Chemical           |              |             |                                                                          |
| β-glycerophosphate                               | Chemical           |              |             |                                                                          |
| NaVO₄                                            | Chemical           |              |             |                                                                          |
| NaF                                              | Chemical           |              |             |                                                                          |

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Continued

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% CO₂.

1. Seed 3.75 × 10⁵ 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 MgCl₂, 150 mM NaCl, 1% NP-40, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 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 xg for 10 min at 4°C.

6. Perform Bradford protein determination assay following manufacturer’s instructions.

7. Separate 30 μg of protein (in 1x 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 1x 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 1x 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 x 10^5 DU145 cells per well, which was increased 2.5x 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 publicly 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                  |
| 100x 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 MgCl₂ and CaCl₂ | 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     | NA0040    | From original lab                             |
| 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 pdl(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% CO₂.

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 × 10⁵ 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/A260).
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’-GACACCCTGCCAGGAAGCGA-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 PTENP1 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 (A260/A280 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 (A260/280 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                   |
| 100x 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 MgCl₂ and CaCl₂ | 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                              |

Continued on next page
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% CO₂.

1. Seed 3.5 × 10⁵ 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 × 10⁵ 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 $\eta^2$ 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.
- 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).

### Protocol 2

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

| siRNA | Day | Mean  | Stdev | N |
|-------|-----|-------|-------|---|
| siluc | 0   | 1.000 | 0.108 | 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 | 0.108 | 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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Continued

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 $\eta^2$ performed with R software, version 3.1.2 (R Development Core Team, 2014).

Day 5 values

| Groups | F test statistic | Partial $\eta^2$ | Effect size f | A priori power | Total sample size |
|---|---|---|---|---|---|
| siLUC, siPTEN, siPTENP1, siPTEN/PTENP1 | $F(1,8) = 798.9603$ (main effect: siPTEN) | 0.99009 | 92.0%* | 5* (4 groups) |
| | $F(1,8) = 143.7867$ (main effect: siPTENP1) | 0.94729 | 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 $\eta^2$ | Effect size f | A priori power | Total sample size |
|---|---|---|---|---|---|
| siLUC, siPTEN, siPTENP1, siPTEN/PTENP1 | $F(1,8) = 166.9731$ (main effect: siPTEN) | 0.95428 | 99.8%* | 6* (4 groups) |
| | $F(1,8) = 34.2219$ (main effect: siPTENP1) | 0.81053 | 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 | Stddev | 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.
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 $\eta^2$ 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.

| Groups | F test statistic | Partial $\eta^2$ | 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) |

*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%.

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 $\eta^2$ performed with R software, version 3.1.2 (R Development Core Team, 2014).

  2% variance:

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

  15% variance:

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

  28% variance:

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

  40% variance:

  | Groups                  | F test statistic | Partial $\eta^2$ | Effect size $f$ | A priori power | Total sample size |
  |-------------------------|------------------|------------------|-----------------|-----------------|-------------------|
  | siLUC, siPTEN, siPTENP1 | $F(1,8) = 11.5741$ (main effect: siPTEN) | 0.59130 | 1.20281 | 95.3% | 12 (4 groups) |
  | siLUC, siPTEN, siPTENP1 | $F(1,8) = 7.4074$ (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                   |

Continued on next page
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:

| Group 1   | Group 2          | Effect size $d$ | A priori power | Group 1 sample size | Group 2 sample size |
|-----------|------------------|-----------------|-----------------|----------------------|---------------------|
| siLuc     | siPTEN           | 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.

### 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:
AUC calculations from estimated values. Calculations performed with R software 3.1.2 \citep{RCoreTeam2014}.

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 \citep{FaulEtAl2007}.

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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