Vitiello et al. Journal of Hematology & Oncology (2020) 13:73
https://doi.org/10.1186/s13045-020-00894-2

Journal of Hematology & Oncology

RAPID COMMUNICATION Open Access

PTENP1 is a ceRNA for *PTEN*: it's CRISPR clear

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Abstract

Here we apply state-of-the-art CRISPR technologies to study the impact that *PTENP1* pseudogene transcript has on the expression levels of its parental gene *PTEN*, and hence on the output of AKT signaling in cancer. Our data expand the repertoire of approaches that can be used to dissect competing endogenous RNA (ceRNA)-based interactions, while providing further experimental evidence in support of the very first one that we discovered.

Keywords: *PTENP1*, *PTEN*, ceRNA, CRISPR, CasRx-mediated knock-down, Cas9-mediated knock-in

Main text

In our 2010 paper entitled "A coding independent function of gene and pseudogene mRNAs regulates tumor biology", we provided the first evidence that RNA molecules, including non-coding RNAs (such as pseudogenes) and mRNAs, may be endowed with a biological function that specifically relies on their ability to compete for microRNA binding [1].

Our findings have contributed to an evolving microRNA-RNA interaction paradigm, where RNAs are not only "passive" targets of microRNAs, but also "active" regulators of microRNA availability, through a mechanism termed competing endogenous RNA (ceRNA) [2, 3]. Since our publication, a plethora of mRNAs and non-coding RNAs (lincRNAs, pseudogenes, circular RNAs) have been reported to function as ceRNAs in vitro and in animal models. Furthermore, ceRNA functions have been demonstrated to go beyond individual RNA-RNA interactions and extend into complex transcript interaction networks that can be severely dysregulated in cancer [4, 5].

Subsequent to our 2010 publication, many studies independently confirmed *PTENP1* pseudogene as a ceRNA for *PTEN* in prostate cancer, in other cancer types (e.g., bladder cancer, breast cancer, clear cell renal cell carcinoma, endometrial carcinoma, gastric cancer, head and neck squamous cell carcinoma, hepatocellular carcinoma), and in other physio-pathological conditions (see Supplementary references for a list). Nonetheless, a number of articles published in prestigious journals have repeatedly raised concerns about this functional interaction. Herein, we wish to address those concerns raised regarding the techniques we used to modulate *PTENP1* expression and show its impact on PTEN expression.

To rule out potential non-specific effects associated with (1) supra-physiological expression of a 3′UTR [6–9] and (2) congestion of RNA interference machinery caused by siRNA transfection [7], we have chosen to downregulate *PTENP1* expression at the transcriptional or post-transcriptional level, taking advantage of CRISPR technology.

To begin, we successfully replicated results reported in our original paper, in spite of the fact that the source of DU145 cells and the batch of siRNAs against *PTEN* and *PTENP1* were different, and that the experiments were performed in a different lab (Fig. 1).

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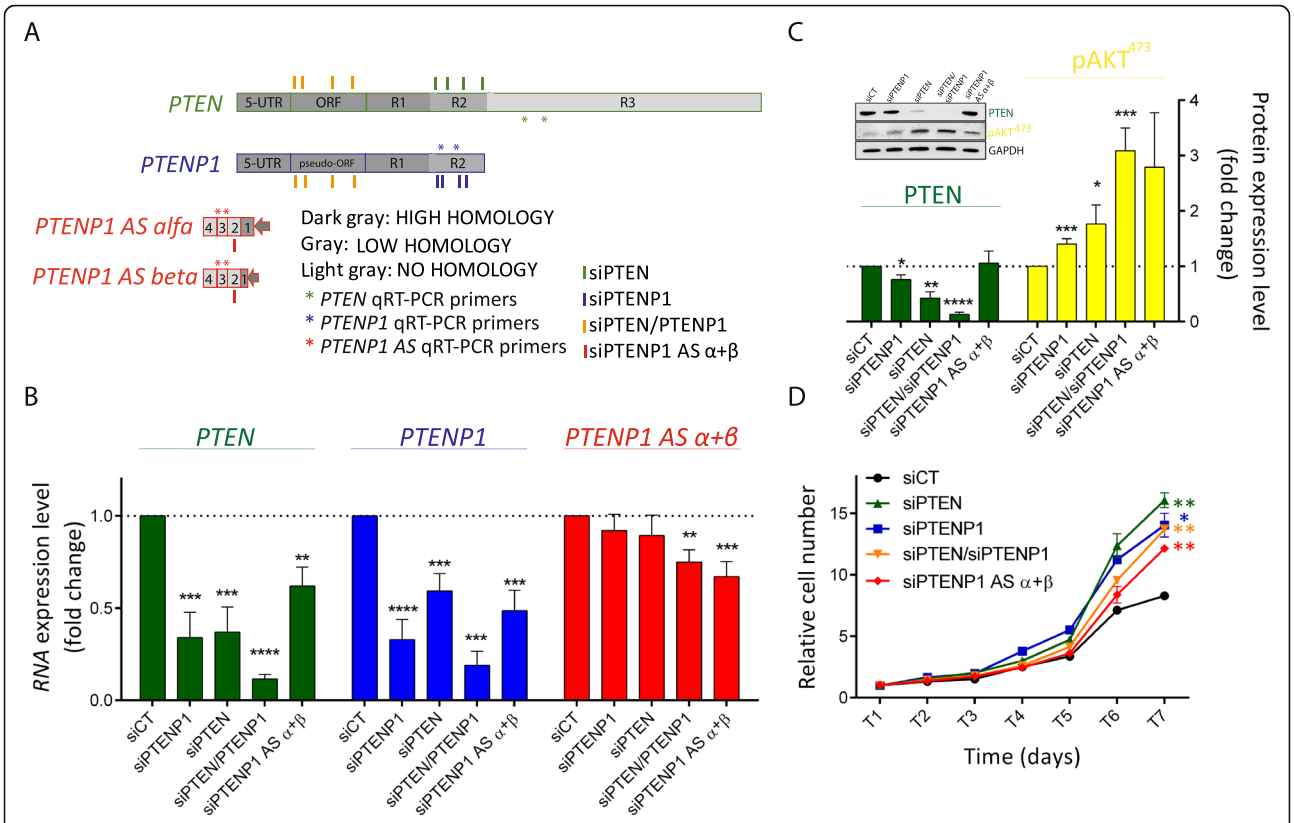

Fig. 1 siRNA-mediated knock-down of *PTENP1* RNA results in the downregulation of PTEN expression and in an increase in DU145 cell proliferation. **a** Schematic representation of the location of the siRNAs used to downregulate *PTEN* (green), *PTENP1* (blue), *PTEN* + *PTENP1* (orange), and *PTENP1 AS α* + *β* (red) transcripts. The 5′UTR and open reading frame of *PTEN* and *PTENP1* are highly homologous (dark gray). Their 3′UTRs are composed of an R1 high homology region (dark gray), an R2 low homology region (gray), and an R3 region with no homology (light gray). The first exon of *PTENP1 AS α* and *β* is transcribed in antisense compared to the 5′UTR of *PTENP1*. The location of the siRNAs is chosen so that only the intended transcript(s) are selectively knocked down. The location of qRT-PCR primers used to detect *PTEN*, *PTENP1*, and *PTENP1 AS α* + *β* is indicated with green, blue, and red asterisks, respectively. **b** qRT-PCR quantification of *PTEN*, *PTENP1*, and *PTENP1 AS α* + *β* transcripts performed 24 h after the transfection of the indicated siRNAs. **c** (upper left) Representative western blot detection of PTEN, pAKT, and GAPDH proteins, performed 48 h after the transfection of the indicated siRNAs. (lower) Quantification of protein levels. **d** Growth curve of DU145 cells transfected with the indicated siRNAs. T1/2/3/4/5/6/7: days after the transfection. The results reported in **b**–**d** confirm the data reported in [1]: the knock-down of *PTENP1* negatively affects PTEN expression. Conversely, *PTEN* knock-down negatively affects *PTENP1* expression. As a consequence, AKT gets hyper-phosphorylated and cell proliferation increases. The graphs represent the mean ± SEM of three independent experiments. Statistically significant differences are indicated with asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Next, in order to downregulate *PTENP1* post-transcriptionally, we used the recently reported CRISPR/CasRx system [10]. For this, we utilized 4 gRNAs designed on the same sequence of the 4 siRNAs composing the siPTENP1 mix (Fig. 2a, b) and we tested them for their ability to decrease the expression of a reporter construct in which *PTENP1* 3′UTR is cloned downstream of Luciferase coding sequence. As shown in Fig. 2c, the gRNAs work similarly to the corresponding siRNAs, with the mix of all 4 gRNAs working best. Therefore, we decided to use the combination of all 4, as we had done with siRNAs. In Fig. 2d–h, we show the results obtained upon the transient transfection of the gRNA mix in GFP-sorted DU145 prostate cancer cells that stably express CasRx-eGFP (Fig. 2d, e). Consistent with the RNA interference approach (Fig. 1), the gRNA mix caused a downregulation of the intended target *PTENP1* RNA, as well as of *PTEN* mRNA (Fig. 2f). The decrease in mRNA level was mirrored by a decrease in PTEN protein level and accompanied by increases in pAKT levels (Fig. 2g) and cell proliferation (Fig. 2h).

We also adapted the CRISPR/Cas9-based gene replacement strategy [11] in order to achieve the downregulation of *PTENP1* at the transcriptional level. Specifically, we engineered an sgRNA-mediated cut between the promoter and the transcribed region of *PTENP1* gene. Then, by exploiting homology-mediated recombination, we "knocked-in" a GFP expression cassette in the reverse orientation, which interferes with *PTENP1* transcription (Fig. 3a). Using this strategy, we identified 11 GFP-positive KI clones (Fig. 3b), of which 7 harbored correct recombination of both homology arms

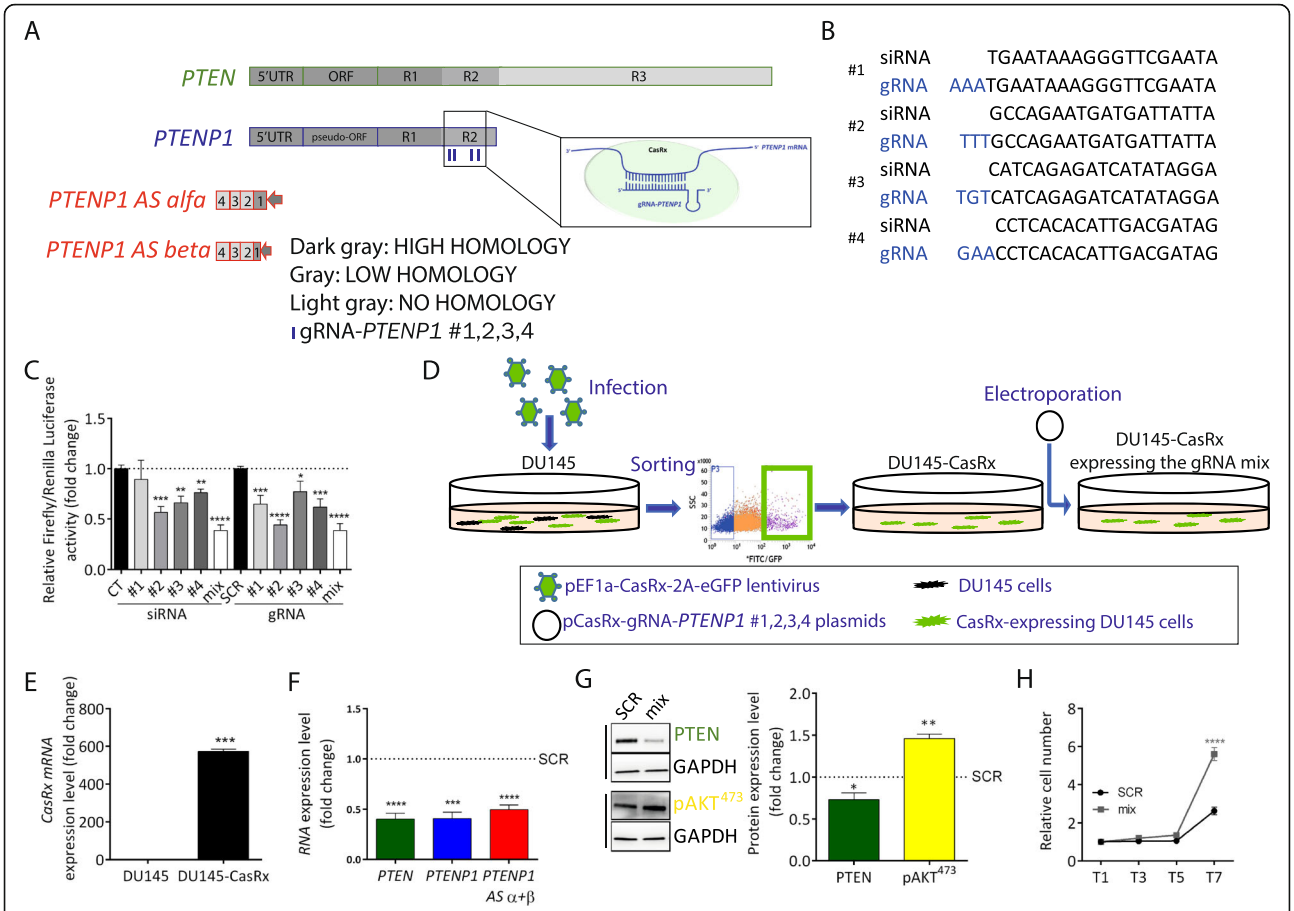

Fig. 2 CRISPR-mediated knock-down of *PTENP1* RNA results in the downregulation of PTEN expression and in an increase in DU145 cell proliferation. **a**, **b** Schematic representation of the location of the 4 gRNAs used to knock down *PTENP1* expression (**a**). The sequence of *PTENP1* gRNAs (#1 to #4) corresponds to the sequence of the siRNAs described in Fig. 1, extended by 3 nt (blue, **b**). **c** Luciferase assay performed in HEK293T cells to compare the efficiency of single siRNAs and gRNAs, as well as their mixes, in downregulating the expression of *PTENP1*. The assay was performed 36 h after the co-transfection of pGLU/ψ3′UTR plasmid with the indicated siRNAs (siCT; siPTENP1 #1,2,3,4) or gRNA-expressing plasmids (pCasRx-gRNA (SCR); pCasRx-gRNA-*PTENP1* #1,2,3,4). In pGLU/ψ3′UTR plasmid, the R1 high homology and the R2 low homology regions of *PTENP1* 3′UTR are cloned downstream of Luciferase coding sequence [1]. **d** Cartoon summarizing the experimental protocol used to test the gRNAs in DU145 cells. DU145 cells were stably infected using the lentiviral vector pEF1a-CasRx-2A-eGFP, then sorted for high eGFP expression and, finally, electroporated with pCasRx-gRNA plasmid or the mix of pCasRx-gRNA-*PTENP1* #1,2,3,4 plasmids. **e** qRT-PCR quantification of *CasRx* mRNA in DU145 cells that were stably infected with pEF1a-CasRx-2A-eGFP and then sorted for high eGFP expression (DU145-CasRx). **f** qRT-PCR quantification of the indicated transcripts 24 h after the transfection of pCasRx-gRNA (SCR) or pCasRx-gRNA-*PTENP1* #1,2,3,4 mix in DU145-CasRx cells. **g** (left) Representative western blot detection of PTEN/GAPDH and pAKT/GAPDH proteins, performed 48 h after the transfection of pCasRx-gRNA (SCR) or pCasRx-gRNA-*PTENP1* #1,2,3,4 mix in DU145-CasRx cells. (right) Quantification of protein levels. **h** Growth curves of DU145-CasRx cells transfected with pCasRx-gRNA (SCR) or pCasRx-gRNA-*PTENP1* #1,2,3,4 mix. T1/3/5/7: days after the transfection. The results reported in **f**–**h** confirm the data obtained using siRNAs: the knock-down of *PTENP1* negatively affects PTEN expression. As a consequence, AKT gets hyper-phosphorylated and cell proliferation increases. The graphs represent the mean ± SEM of three independent experiments. Statistically significant differences are indicated with asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

and 5 showed the expected drop in *PTENP1* mRNA levels (clones #A, 2, 5, 8, and 13 reported in Fig. 3c, d). In these clones, we also observed a decrease in both PTEN mRNA and protein levels (Fig. 3d, e). In addition, clones #A, 2, and 13 had accompanying increases in pAKT levels (Fig. 3e) and cell proliferation (Fig. 3f). Crucially, in Fig. 3g, we show that endogenous *PTEN* mRNA levels are rescued in clone #13, if *PTENP1* 3′UTR is reintroduced by means of a plasmid that expresses it downstream of Luciferase coding sequence.

In summary, using 2 CRISPR-based technologies (Figs. 2 and 3), we confirmed our results achieved using RNA interference (ref. [1] and Fig. 1): knock-down of *PTENP1* leads to the repression of *PTEN* expression, hence the hyperactivation of oncogenic AKT signaling. In addition, we confirmed that siRNA-mediated knock-down of *PTENP1* antisense alpha + beta transcripts results in a downregulation of *PTENP1* and *PTEN* transcripts (Fig. 1b), as previously reported in [12]. Conversely, we showed that the knock-down of *PTEN* plus *PTENP1*

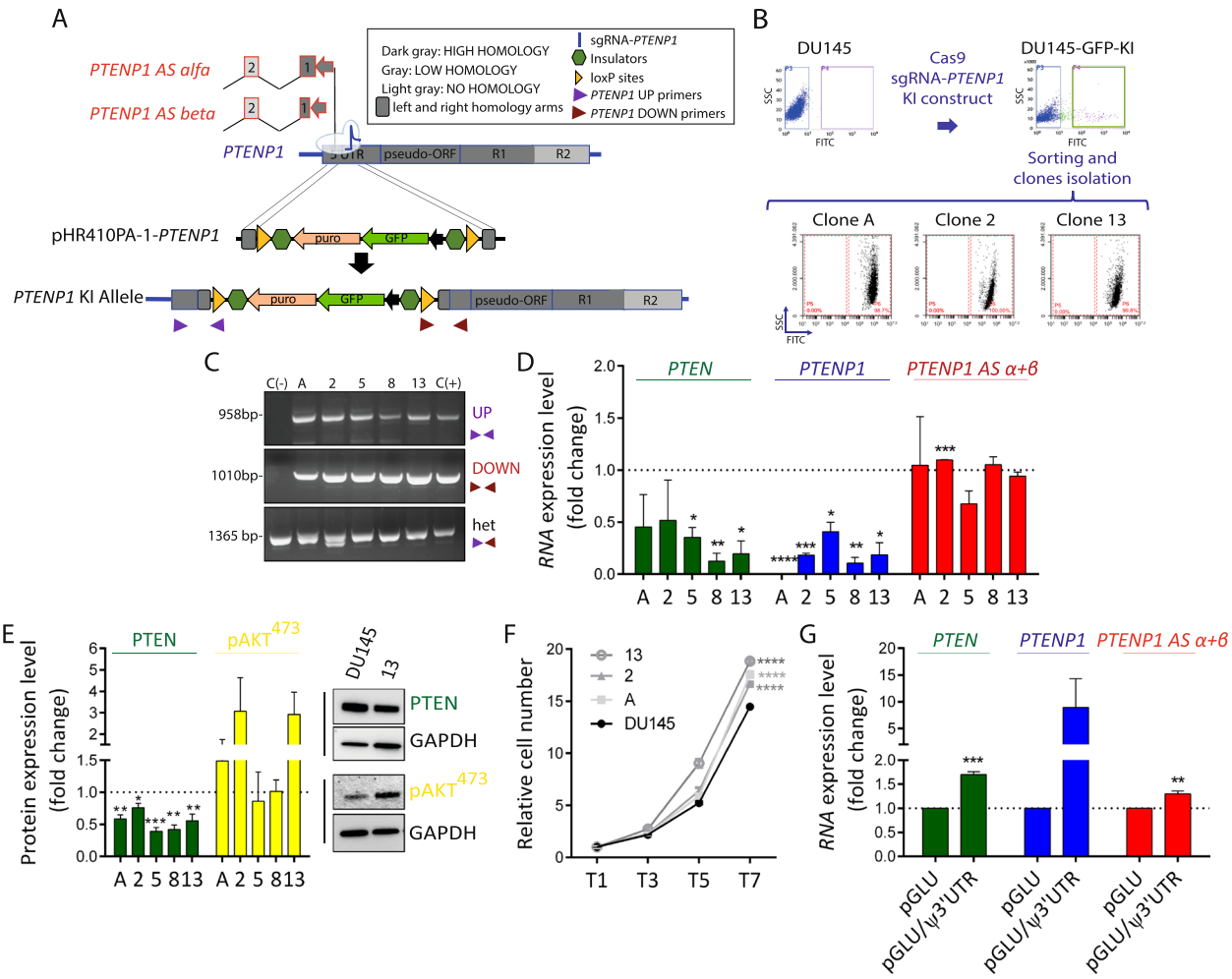

Fig. 3 CRISPR-mediated knock-in of a GFP expression cassette in *PTENP1* gene results in the downregulation of PTEN expression and in an increase in DU145 cell proliferation. **a** Schematic representation of the CRISPR/Cas9-mediated cleavage of *PTENP1* gene region, followed by the homology recombination-mediated knock-in of a GFP expression cassette. Being oriented in the opposite direction, the cassette interferes with the transcription of *PTENP1* itself. The double-strand cut of genomic DNA produced by Cas9/sgRNA-*PTENP1* (blue) is located within the 5'UTR region of *PTENP1*, upstream of the promoter of *PTENP1 AS α* and *β*. Therefore, the transcriptional unit of the antisense transcripts is preserved. Besides the arms required for homology recombination and the GFP expression cassette, the knock-in construct contains 2 insulators and 2 loxP sites. **b** Cartoon summarizing the experimental protocol used. DU145 that constitutively express Cas9 and sgRNA-*PTENP1* were electroporated with pHR410PA-1-*PTENP1* plasmid that contains the knock-in construct. Then, Cas9-mediated cleavage of *PTENP1* genomic DNA was induced by adding doxycycline. After waiting 10–14 days in order to allow the dilution of non-integrated plasmid, cells that stably express GFP were sorted and seeded at 1-cell/96-well density to obtain individual knock-in clones. **c** PCR-based screening of the clones that are correctly knocked in. The junction regions located upstream and downstream of the knock-in construct were amplified using the primers shown in panel **a** as purple and dark red arrows, respectively. The purple forward and the dark red reverse primers (they give a PCR band only on wt alleles, not on knocked-in alleles) were also used to test whether the integration of the construct occurred on all chromosome 9 copies or not (het). The genomic DNA extracted from parental DU145 cells was used as negative control (C–). The genomic DNA extracted from DU145-GFP-KI cells right before 1-cell/96-well seeding was used as positive control (C+). Clones #A, 2, 5, 8, and 13 all show the correct integration of the construct, although non-knocked-in copies of chromosome 9 remain. **d** qRT-PCR quantification of the indicated transcripts in DU145 cells (taken as control) and clones #A, 2, 5, 8, and 13. **e** (left) Quantification of PTEN/GAPDH and pAKT/GAPDH protein levels in DU145 cells (taken as control) and in clones #A, 2, 5, 8, and 13. (right) Representative western blot detection of PTEN, pAKT and GAPDH proteins in DU145 cells and in clone #13. **f** Growth curves of DU145 cells and clones #A, 2, and 13. T1/3/5/7: days after seeding. **g** qRT-PCR quantification of the indicated transcripts 24 h after the electroporation of 1.5 μg of pGLU empty plasmid or of pGLU/ψ3'UTR plasmid in clone #13. The results reported in **d**–**g** confirm what obtained by RNA interference and CRISPR/CasRx: the knock-down of *PTENP1* negatively affects PTEN expression. As a consequence, AKT gets hyper-phosphorylated and cell proliferation increases. The graphs represent the mean ± SEM of three independent experiments. Statistically significant differences are indicated with asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

transcripts by RNA interference (Fig. 1b) and of *PTENP1* by CRISPR/CasRx technology (Fig. 2f) represses the expression of *PTENP1* antisense transcripts, whereas the upregulation of *PTENP1* transcript elicits the opposite effect (Fig. 3g). In sum, we provide evidence that uncovers a dynamic cross-talk between *PTENP1* and *PTEN* sense transcripts on one side and antisense *PTENP1* transcripts on the other.

In the decade since our discovery, numerous groups have independently validated the regulatory interaction between *PTENP1* and *PTEN*. Altogether, these data provide a persuasive body of work to support the existence of a robust and reproducible functional interaction between this gene-pseudogene pair [13]. Finally, the new data presented herein further reinforces the *PTENP1-PTEN* paradigm and highlights the utility of CRISPR technologies for investigations of pseudogene-parental gene transcript relationships in cancer and other diseases.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13045-020-00894-2.

Additional file 1. Supplementary references.

Additional file 2. Supplementary methods.

Additional file 3. Supplementary **Figure 1. a** sgRNA-*PTENP1* sequence. **b** (left) *PTENP1* genomic sequence recognized by sgRNA-*PTENP1* (bold), and PAM sequence (5'-TGG-3', underlined). (right) Orthologous *PTEN* genomic sequence. sgRNA-*PTENP1* cannot mediate the cleavage of *PTEN* because of 4 mismatches (red), one of which falls in the PAM sequence. **c** Electropherogram of *PTENP1* genomic sequence, where the consequences of the cut by Cas9/sgRNA-*PTENP1* are shown. The electropherogram was obtained by PCR analysis of the genomic DNA extracted from DU145-Cas9/sgRNA-*PTENP1* double infected cells, 3 days after Cas9 induction using 2ug/ml doxycycline. The primers used for amplification were: Fw- attcgtcttctccccattcc; Rv-tctgcaggaaatcccatagc.

Abbreviations

ceRNA: competing endogenous RNA; CRISPR: Clustered regularly interspaced short palindromic repeats

Acknowledgements

The authors thank C. Baldanzi, A. Prantera, and L. Maresca for technical support.

Authors' contributions

MV, PPP, and LP conceived the project. MV, YZ, and LP designed the experiments. MV and ME performed the experiments. MV, LS, PPP, and LP analyzed the data. PPP and LP supervised the research. LP wrote the manuscript with the help of all authors. The manuscript was discussed and approved by all authors.

Funding

This work was supported by ISPRO-Istituto per lo Studio, la Prevenzione e la Rete Oncologica [institutional funding to LP]. It was also partially supported by AIRC-Associazione Italiana Ricerca sul Cancro [MFAG #17095 to LP] and by R35CA197529-01 grant to PPP. MV was supported by an AIRC fellowship.

Availability of data and materials

Not applicable

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

None to declare

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Received: 21 March 2020 Accepted: 5 May 2020
Published online: 09 June 2020

References

1. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature. 2010;465:1033–8.
2. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell. 2011;146:353–8.
3. Tay Y, Rinn J, Pandolfi PP. The multilayered complexity of ceRNA crosstalk and competition. Nature. 2014;505:344–52.
4. Chan JJ, Tay Y. Noncoding RNA:RNA Regulatory networks in cancer. Int J Mol Sci. 2018;19.
5. Anastasiadou E, Jacob LS, Slack FJ. Non-coding RNA networks in cancer. Nat Rev Cancer. 2018;18:5–18.
6. Ebert MS, Sharp PA. Roles for microRNAs in conferring robustness to biological processes. Cell. 2012;149:515–24.
7. Jens M, Rajewsky N. Competition between target sites of regulators shapes post-transcriptional gene regulation. Nat Rev Genet. 2015;16:113–26.
8. Denzler R, Agarwal V, Stefano J, Bartel DP, Stoffel M. Assessing the ceRNA hypothesis with quantitative measurements of miRNA and target abundance. Mol Cell. 2014;54:766–76.
9. Broderick JA, Zamore PD. Competitive endogenous RNAs cannot alter microRNA function in vivo. Mol Cell. 2014;54:711–3.
10. Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD. Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. Cell. 2018;173:665–76 e614.
11. Zheng Q, Cai X, Tan MH, Schaffert S, Arnold CP, Gong X, Chen CZ, Huang S. Precise gene deletion and replacement using the CRISPR/Cas9 system in human cells. Biotechniques. 2014;57:115–24.
12. Johnsson P, Ackley A, Vidarsdottir L, Lui WO, Corcoran M, Grander D, Morris KV. A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells. Nat Struct Mol Biol. 2013;20:440–6.
13. Kerwin J, Khan I. Reproducibility Project: Cancer B, Iorns E, Tsui R, Denis A, Perfito N, Errington TM: Replication study: a coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Elife. 2020;9.

Publisher's Note

SUPPLEMENTARY REFERENCES

Contexts in which *PTENP1* has been shown to act as sponge for *PTEN*:

-bladder cancer [1]

-breast cancer [2] [3] [4]

-clear cell renal cell carcinoma [5]

-endometrial carcinoma [6]

-gastric cancer [7]

-head and neck squamous cell carcinoma [8] [9]

-hepatocellular carcinoma [10] [11]

-prostate cancer [12] [13]

-other patho-physiological conditions [14] [15] [16] [17].

1. Zheng R, Du M, Wang X, Xu W, Liang J, Wang W, Lv Q, Qin C, Chu H, Wang M, et al: **Exosome-transmitted long non-coding RNA PTENP1 suppresses bladder cancer progression.** *Mol Cancer* 2018, **17:**143.
2. Li RK, Gao J, Guo LH, Huang GQ, Luo WH: **PTENP1 acts as a ceRNA to regulate PTEN by sponging miR-19b and explores the biological role of PTENP1 in breast cancer.** *Cancer Gene Ther* 2017, **24:**309-315.
3. Shi X, Tang X, Su L: **Over-expression of long non-coding RNA PTENP1 inhibits cell proliferation and migration via suppression of miR-19b in breast cancer cells.** *Oncol Res* 2017.
4. Gao X, Qin T, Mao J, Zhang J, Fan S, Lu Y, Sun Z, Zhang Q, Song B, Li L: **PTENP1/miR-20a/PTEN axis contributes to breast cancer progression by regulating PTEN via PI3K/AKT pathway.** *J Exp Clin Cancer Res* 2019, **38:**256.
5. Yu G, Yao W, Gumireddy K, Li A, Wang J, Xiao W, Chen K, Xiao H, Li H, Tang K, et al: **Pseudogene PTENP1 functions as a competing endogenous RNA to suppress clear cell renal cell carcinoma progression.** *Mol Cancer Ther* 2014.
6. Chen R, Zhang M, Liu W, Chen H, Cai T, Xiong H, Sheng X, Liu S, Peng J, Wang F, et al: **Estrogen affects the negative feedback loop of PTENP1-miR200c to inhibit PTEN expression in the development of endometrioid endometrial carcinoma.** *Cell Death Dis* 2018, **10:**4.
7. Zhang R, Guo Y, Ma Z, Ma G, Xue Q, Li F, Liu L: **Long non-coding RNA PTENP1 functions as a ceRNA to modulate PTEN level by decoying miR-106b and miR-93 in gastric cancer.** *Oncotarget* 2017, **8:**26079-26089.
8. Gao L, Ren W, Zhang L, Li S, Kong X, Zhang H, Dong J, Cai G, Jin C, Zheng D, Zhi K: **PTENp1, a natural sponge of miR-21, mediates PTEN expression to inhibit the proliferation of oral squamous cell carcinoma.** *Mol Carcinog* 2017, **56:**1322-1334.
9. Liu J, Xing Y, Xu L, Chen W, Cao W, Zhang C: **Decreased expression of pseudogene PTENP1 promotes malignant behaviours and is associated with the poor survival of patients with HNSCC.** *Sci Rep* 2017, **7:**41179.
10. Chen CL, Tseng YW, Wu JC, Chen GY, Lin KC, Hwang SM, Hu YC: **Suppression of hepatocellular carcinoma by baculovirus-mediated expression of long non-coding RNA PTENP1 and MicroRNA regulation.** *Biomaterials* 2015, **44:**71-81.
11. Qian YY, Li K, Liu QY, Liu ZS: **Long non-coding RNA PTENP1 interacts with miR-193a-3p to suppress cell migration and invasion through the PTEN pathway in hepatocellular carcinoma.** *Oncotarget* 2017, **8:**107859-107869.

12. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP: **A coding-independent function of gene and pseudogene mRNAs regulates tumour biology.** *Nature* 2010, **465:**1033-1038.
13. Johnson TS, Li S, Franz E, Huang Z, Dan Li S, Campbell MJ, Huang K, Zhang Y: **PseudoFuN: Deriving functional potentials of pseudogenes from integrative relationships with genes and microRNAs across 32 cancers.** *Gigascience* 2019, **8**.
14. Johnsson P, Ackley A, Vidarsdottir L, Lui WO, Corcoran M, Grander D, Morris KV: **A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells.** *Nat Struct Mol Biol* 2013, **20:**440-446.
15. Wang L, Zhang N, Wang Z, Ai DM, Cao ZY, Pan HP: **Pseudogene PTENP1 Functions as a Competing Endogenous RNA (ceRNA) to Regulate PTEN Expression by Sponging miR-499-5p.** *Biochemistry (Mosc)* 2016, **81:**739-747.
16. Lai Y, Li J, Zhong L, He X, Si X, Sun Y, Chen Y, Zhong J, Hu Y, Li B, et al: **The pseudogene PTENP1 regulates smooth muscle cells as a competing endogenous RNA.** *Clin Sci (Lond)* 2019, **133:**1439-1455.
17. Wang Z, Song Y, Han X, Qu P, Wang W: **Long noncoding RNA PTENP1 affects the recovery of spinal cord injury by regulating the expression of miR-19b and miR-21.** *J Cell Physiol* 2019.

SUPPLEMENTARY METHODS

Cells and culture. HEK293T cells were grown in DMEM low glucose medium plus 10% foetal bovine serum. DU145 were grown in RPMI medium plus 10% foetal bovine serum. Both cell lines were grown in 1% glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (Euroclone) containing medium, at 37°C in a humidified atmosphere with 5% CO_2.

Oligos. PCR and qRT-PCR oligos were purchased from Eurofins Genomics. The non-targeting siCT and the siRNAs for *PTEN* and *PTENP1* knock-down were purchased from Dharmacon. The siRNA for *PTENP1 AS α+β* knock-down was purchased from Eurofins Genomics. For sequences, see below:

Sequence of qRT-PCR primers

Gene	Forward primer	Reverse primer	ref
PTEN	GTTTACCGGCAGCATCAAAT	CCCCCACTTTAGTGCACAGT	PMID: 20577206
PTENP1	TCAGAACATGGCATACACCAA	TGATGACGTCCGATTTTTCA	PMID: 20577206
PTENP1 AS α+β	AAGGCTGCACTGTCAACTTCCCTA	TCCCACAATAGGTCTTCTGCAAGC	PMID: 23435381
CasRx	AGCTGACCAACTCCTTCTCC	AGCATCACTTCCCTGAGCTT	
GAPDH	CGCTCTCTGCTCCTCCTGTT	CCATGGTGTCTGAGCGATGT	PMID:28445987
PBGD	TCCAAGCGGAGCCATGTCTG	AGAATCTTGTCCCCTGTGGTGGA	PMID:28445987
SDHA	CCACTCGCTATTGCACACC	CACTCCCCATTCTCCATCA	PMID:28445987

Sequence of siRNAs

Gene	Sequence
siCT	#D 001210-02-20
siPTENP1 #1 *	TGAATAAAGGGTTCGAATA
siPTENP1 #2 *	GCCAGAATGATGATTATTA
siPTENP1 #3 *	CATCAGAGATCATATAGGA
siPTENP1 #4 *	CCTCACACATTGACGATAG
siPTEN #1 *	GGAAATTAGAGTTGCAGTA
siPTEN #2 *	ACTTATTGGTGCTGAAATT
siPTEN #3 *	GGCAAATAGATTACCCAGA
siPTEN #4 *	GATTCTACAGTAAGCGTTT
siPTEN/PTENP1 #1*	GAUCAGCAUACACAAAUUA
siPTEN/PTENP1 #2*	GACUUAGACUUGACCUAUA
siPTEN/PTENP1 #3*	GAUCUUGACCAAUGGCUAA
siPTEN/PTENP1 #4*	CGAUAGCAUUUGCAGUAUA
siPTENP1 AS α+β**	UGUACUGUCUGAUAUCUCC

* siRNA reported in PMID:20577206

** PTENpg1 asRNA Ex2 shRNA sequence reported in PMID:23435381

Plasmids.

pRL-TK. The pRL-TK plasmid expresses Renilla Luciferase and is cotransfected with pGLU plasmids as normalization control.

pGLU/ψ3'UTR. This plasmid expresses the R1 high homology and the R2 low homology regions of *PTENP1* 3'UTR downstream of Firefly Luciferase coding sequence. The empty pGLU plasmid is used as negative control [1].

pXR001:pEF1a-CasRx-2A-eGFP. This lentiviral vector, which expresses CasRx and eGFP from a bicistronic transcript, was purchased from Addgene (#109049).

pCasRx-gRNA-*PTENP1* #1,2,3,4. The 4 gRNAs against *PTENP1* (see **Fig.2b** for sequences) were cloned into pXR003:pCasRx-gRNA plasmid (Addgene, #109053), using BbSI restriction site. pCasRx-gRNA plasmid, which expresses a non-targeting gRNA, was used as control (SCR).

pCW-Cas9. This lentiviral vector, which expresses a doxycycline-inducible version of Cas9 and carries puromycin resistance, was purchased from Addgene (#50661).
pLX-sgRNA-*PTENP1*. In order to obtain pLX-sgRNA-*PTENP1* lentiviral vector, first of all the sequence of the sgRNA for *PTENP1* was chosen using CHOPCHOP online tool (https://chopchop.cbu.uib.no/) and double-checked for its inability to target *PTEN* gene **(Supplementary Fig.1a and b)**. Then, according to the protocol available at www.addgene.org, the PCR product corresponding to sgRNA-*PTENP1* was generated using as template pLX-AAVS1-sgRNA lentiviral vector (Addgene, #50662), Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) and the following primers: F2 5'-G**CGGCAGCAGCTGCTGGATGG**GTTTTAGAGCTAGAAATAGCAA-3', R2 5'-**CCATCCAGCAGCTGCTGCCG**CGG-3'. Finally, the PCR product was cloned into pLX-AAVS1-sgRNA lentiviral vector itself, using XhoI and NheI restriction sites, so that pLX-sgRNA-*PTENP1* lentiviral vector was obtained. Being derived from pLX-AAVS1-sgRNA, this vector carries blasticidine resistance. The electropherogram reported in **Supplementary Fig.1c** confirms the ability of pLX-sgRNA-*PTENP1* to cut the intended region within *PTENP1* gene.
pHR410PA-1-*PTENP1*. This plasmid was used as template for homologous recombination, after Cas9/sgRNA-*PTENP1*-mediated cleavage at PAM site. The genomic regions located upstream and downstream of the PAM were amplified using the following primers: *PTENP1* upstream Fw 5'-catgtcgacctgaagcccctctcagcaacccg-3'; *PTENP1* upstream Rv 5'-atgggatccctactgggcctgcttctcctcagc-3'; *PTENP1* downstream Fw 5'-catagatctgcagctgctgccgcagccattacc-3'; *PTENP1* upstream Rv 5'- atggaattctatgcataaatcattataccag-3'. The amplified regions were then cloned into pHR410PA-1 plasmid (kind gift from Dr. Landi, University of Pisa, Italy) using SalI/BamHI (*PTENP1* upstream region) and EcoRI/BglII (*PTENP1* downstream region) restriction sites. In between the upstream and downstream homology regions, pHR410PA-1-*PTENP1* plasmid contains a GFP expression cassette flanked by 2 insulators and 2 loxP sites.
pMD2.G and pSpax2. These two plasmids were used for lentiviral packaging and they were cotransfected with pCasRx, pCW-Cas9 or pLX-sgRNA-*PTENP1* for the production of specific lentiviruses. They were a kind gift from Dr. Hernando, New York University, New York, USA.

siRNA transfection. DU145 cells ($3.3x10^5$) were seeded in 6-well dishes. The following day they were transfected with 100nM siRNAs using Dharmafect I according to the manufacturer's protocol. Six hours later, cells were trypsinized and seeded for the specific assays.

Plasmid electroporation. For plasmid electroporation, DU145 cells ($1.5x10^6$) were seeded in 10cm dishes. The following day, 10^6 cells were electroporated with 2ug of plasmidic DNA in total, using Amaxa Nucleofector™ II (Lonza) and following the manufacturer's protocol.

Viral infection. HEK293T cells were plated in 10cm dishes ($3x10^6$/dish) and 24h later they were transfected with 12ug of pEF1a-CasRx-2A-eGFP, pCW-Cas9 or pLX-sgRNA-*PTENP1*, 4ug of pMD2.G and 8ug of pSpax2 using PEI. Six hours later, the medium was changed. Virus containing media was collected at 48h and 72h after transfection. Then, it was filtered and the virus was pelleted using LentiX Concentrator (Takara, #631231). Once quantified, the virus was added to DU145 cells using 4ug/ml polybrene.

Generation of GFP knock-in clones. DU145 cells were stably infected with pCW-Cas9 and pLX-sgRNA-*PTENP1*. Once DU145-Cas9/sgRNA-*PTENP1* cells were obtained, they were electroporated with pHR410PA-1-*PTENP1* and then Cas9 was induced using 2ug/ml doxycycline (Merck, #D9891). 10-14 days later, green DU145-GFP-KI cells (i.e. cells in which the GFP cassette is stably inserted within the *PTENP1* gene) were sorted using FACSJazz (BD) and seeded in a 96-well plate at 1 cell/well density, in order to isolate individual KI clones.

Genomic DNA extraction and analysis of *PTENP1* genomic status. Genomic DNA was extracted from parental DU145 cells (used as negative control C(-)), unsorted DU145-GFP-KI cells (used as positive control C(+)) and KI clones (10^6 cells), following the protocol reported in [2]. *PTENP1* status was evaluated using two couples of primers: *PTENP1* UP primers that amplify the junction region located upstream of the knock-in construct (Fw: TCTCCTCTCAGAAGCTGCAG; Rv: GTGTCTGCAGGCTCAAAGAG (purple arrows in **Fig.3a**)) and *PTENP1* DOWN primers that amplify the junction region located downstream of the knock-in construct (Fw: GTGTCTGCAGGCTCAAAGAG; Rv: CGCCTCTGACTGGGAATAGT (dark red arrows in **Fig.3a**)). *PTENP1* UP Fw and *PTENP1* DOWN Rv were used to detect non knocked-in copies of chromosome 9.

Luciferase reporter assay. HEK293T cells were seeded at a density of $7.5x10^4$ cells per 24-well dish. The following day, 50ng of pGLU/ψ3'UTR and 10ng of pRL-TK were co-transfected with 5nM of specific siRNAs or 300ng of pCasRx plus 500ng of pCasRx-gRNA-*PTENP1* #1,2,3,4 mix, using Lipofectamine 2000 (Life Technologies). Luciferase activity was measured 36 hours after transfection.

Real-time PCR (qRT-PCR). RNA was extracted using QIAzol reagent (Qiagen), following the manufacturer's instructions. RNA was subsequently quantified using Nanodrop Lite (Thermo Scientific). DNAse treatment and retrotranscription of the mRNA was performed using QuantiTect Reverse transcription Kit (Qiagen), according to the manufacturer's protocol and using a S1000 Thermal Cycler (Bio-Rad). qRT-PCR was performed with SsoAdvanced Universal Supermix (Bio-Rad) on a CFX96 Real-Time System (Bio-Rad).

Protein extraction and western blot. DU145 cells (10^6) were resuspended in 40ul of Lysis buffer (50mM Tris HCl, 1% TritonX100, 0.25% NaDeoxicholate, 1mM PMSF, 2mM Orthovanadate, proteinase inhibitors cocktail). Then proteins were extracted as described in [2]. The following antibodies were purchased from Cell Signaling and used according to the manufacturer's instructions: PTEN (#9559), pAKT-Ser^{473} (#4060) and GAPDH (#2118). Quantification of band intensities was performed using ImageJ software (http://rsb.info.nih.gov/ij/).

Growth curve. DU145 cells ($7x10^3$) were seeded in 12-well plates (3 wells per time point). Each time point was fixed with 4% PFA and stained using a crystal violet solution (0.1% crystal violet, 20% methanol, in water) as reported in [2]. Each sample was normalized on the time 1 (T1) sample and the data were graphed as cell percentage compared to time 1.

Statistical analyses. Data were analysed using unpaired t test (GraphPad Prism, GraphPad Software Inc.). The mean ± SEM of 3 independent experiments is reported. Values of $p<0.05$ were considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

REFERENCES

1. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP: **A coding-independent function of gene and pseudogene mRNAs regulates tumour biology.** *Nature* 2010, **465:**1033-1038.
2. Vitiello M, Tuccoli A, D'Aurizio R, Sarti S, Giannecchini L, Lubrano S, Marranci A, Evangelista M, Peppicelli S, Ippolito C, et al: **Context-dependent miR-204 and miR-211 affect the biological properties of amelanotic and melanotic melanoma cells.** *Oncotarget* 2017, **8:**25395-25417.

A

sgRNA-*PTENP1* CGGCAGCAGCTGCTGGATGG

B

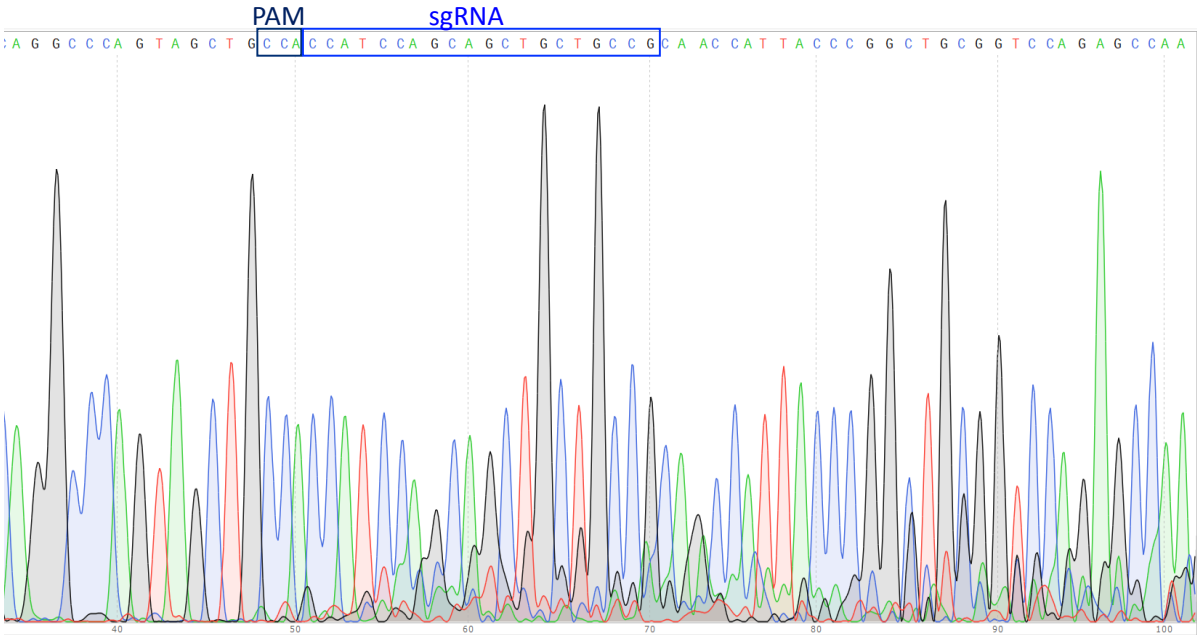
PTENP1 gene

5’-CCA**CCATCCAGCAGCTGCTGCCG**-3’
3’-GGTGGTAGGTCGTCGACGACGGC-5’

PTEN gene

5’-CA**ACCATCCAGCAGCCGCCGCAG**-3’
3’-GTTGGTAGGTCGTCGGCGGCGTC-5’

C



Supplementary Figure 1.
a sgRNA-*PTENP1* sequence. **b** (left) *PTENP1* genomic sequence recognized by sgRNA-*PTENP1* (bold), and PAM sequence (5’-TGG-3’, underlined). (right) Orthologous *PTEN* genomic sequence. sgRNA-*PTENP1* cannot mediate the cleavage of *PTEN* because of 4 mismatches (red), one of which falls in the PAM sequence. **c** Electropherogram of *PTENP1* genomic sequence, where the consequences of the cut by Cas9/sgRNA-*PTENP1* are shown. The electropherogram was obtained by PCR analysis of the genomic DNA extracted from DU145-Cas9/sgRNA-*PTENP1* double infected cells, 3 days after Cas9 induction using 2ug/ml doxycycline. The primers used for amplification were: Fw-attcgtcttctccccattcc; Rv-tctgcaggaaatcccatagc.