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A pseudogene long noncoding RNA network regulates PTEN transcription and translation in human cells

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Abstract

PTEN is a tumor suppressor gene that has been shown to be under the regulatory control of a *PTEN* pseudogene expressed noncoding RNA, *PTENpg1*. Here, we characterize a previously unidentified *PTENpg1* encoded antisense RNA (asRNA), which regulates *PTEN* transcription and *PTEN* mRNA stability. We find two *PTENpg1* asRNA isoforms, alpha and beta. The alpha isoform functions *in trans*, localizes to the *PTEN* promoter, and epigenetically modulates *PTEN* transcription by the recruitment of DNMT3a and EZH2. In contrast, the beta isoform interacts with *PTENpg1* through an RNA:RNA pairing interaction, which affects PTEN protein output via changes of *PTENpg1* stability and microRNA sponge activity. Disruption of this asRNA-regulated network induces cell cycle arrest and sensitizes cells to doxorubicin, suggesting a biological function for the respective *PTENpg1* expressed asRNAs.

Keywords

Pseudogene; PTEN; PTENp1; PTENpg1; antisense RNA; noncoding RNA; Epigenetics; Transcriptional regulation; DNMT3a; EZH2

Introduction

The tumor suppressor gene phosphatase and tensin homolog (*PTEN*) is a negative regulator of the PI3K-Akt pathway and is epigenetically silenced in several cancers[1]. The dosage of *PTEN* expression has been found to correlate with the severity of epithelial cancers[2], indicating that a fine-tuned regulation of the *PTEN* gene is critical for maintaining cellular

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P.J., and K.V.M., designed, performed and supervised the experiments. A.A., and L.V., performed experiments. M.C., provided helpful discussions. W.-O.L., supervised ChIP-sequencing analysis. D.G., supervised the experiments. P.J., D.G., and K.V.M., wrote the paper.

homeostasis. *PTEN* expression has been found to be post transcriptionally regulated by the action of a *PTEN* pseudogene (*PTENpg1*, also known as *PTENp1*, *PTEN2* and *PTENΨ1*)[3]. The *PTENpg1* is a long noncoding RNA (lncRNA), which was found to sequester numerous *PTEN*-targeting microRNAs (miRNAs) by acting as a miRNA sponge. The observed miRNA sponge effect from over-expressing the 3'UTR of the *PTENpg1* lncRNA resulted in increased *PTEN* mRNA stability and increased amounts of PTEN protein, presumably due to miRNA sequestration away from the *PTEN* protein coding transcripts. In contrast, suppression of the *PTENpg1* lncRNA released miRNAs targeting *PTEN*, which instead lead to destabilization of *PTEN*.

Expressed pseudogenes are conserved across millions of years of primate evolution[4] and have traditionally been considered "non-functional DNA" that litters the human genome. Pseudogenes are almost as numerous as protein-coding genes[5] and recent evidence show some of these to be transcribed into RNA[3,6]. An emerging paradigm suggests that some pseudogenes may be functional in directly regulating their protein-coding counterparts at either the transcriptional or post-transcriptional level[3,7–9]. In contrast to *PTENpg1* mediated post-transcriptional regulation of *PTEN*, the pluripotency-associated transcription factor *OCT4* was reported to be under the epigenetic regulation of an antisense RNA (asRNA) to the *OCT4* pseudogene 5[7]. Taken together, these previous observations encouraged us to investigate whether there was an asRNA also encoded from the *PTENpg1* locus and to what extent such an asRNA was involved in epigenetically regulating the tumor suppressor gene *PTEN*.

Results

The *PTEN* pseudogene, *PTENpg1*, encodes asRNA

To investigate the genomic landscape and the presence of asRNA transcription at the *PTENpg1* locus, we assessed Expressed Sequence Tags (EST) in the UCSC genome browser and also carried out an independent analysis of ENCODE Chromatin Immunoprecipitation (ChIP) sequencing data. The assessment of EST reads indicated asRNA transcription from the *PTENpg1* locus (Supplementary Fig. 1a). In addition, our analysis of ENCODE ChIP sequencing data for the presence of the active transcriptional histone mark H3K4me3 indicated differential patterns among different cell lines (Supplementary Fig. 1b). Moreover, analysis of H3K4me3 and RNA Polymerase II (RNAPII) localization in human embryonic stem cells (H1-hESC) and K562 cells, showed overlap and binding at two different loci, indicating promoter activity and two different transcriptional start sites (TSS) at the *PTENpg1* locus (Supplementary Fig. 1b).

Next, we set out to investigate whether the ChIP sequencing peaks for H3K4me3 and RNAPII corresponded to the TSS for the indicated *PTENpg1* asRNA transcripts (Supplementary Fig. 1a–b). To this end we carried out 5' RACE (Supplementary Table 1a) and primer walk (Supplementary Fig. 2a–b) analysis. These analyses indicated two different TSSs at the *PTENpg1* locus that initiate asRNA transcription. In total, three dominant *PTENpg1* asRNA isoforms were identified (unspliced, α, and β) (Fig. 1a, Supplementary Fig. 2a–e, and Supplementary Table 1a) as well as alternative splicing of the *PTENpg1* asRNA exon 3 (Supplementary Fig. 2c and Supplementary Table 1b). Cellular fractionation

showed that the spliced α and β isoforms were expressed at high levels in the cytoplasm, whereas the unspliced *PTENpg1* asRNA α isoform was exclusively found in the nuclear fraction (Supplementary Fig. 2d–e). Depletion of polyadenylated (poly(A)) RNA from total cellular RNA confirmed the spliced α and β isoforms to be poly(A) positive in contrast to the *PTENpg1* sense and unspliced *PTENpg1* asRNA α isoforms, which were mainly poly(A) negative transcripts (Fig. 1b and Supplementary Fig. 2f).

Characterization of the *PTENpg1* asRNA

The dominantly spliced α and β asRNA transcripts appeared to emanate from two different TSSs that curiously overlap with the *PTENpg1* sense promoter (Fig. 1a, Supplementary Fig. 2a–b and Supplementary Table 1a). These regions also appeared functional as divergently transcribed promoters, as determined in luciferase expression assay (Supplementary Fig. 3a–c). In a screen among different human cell lines, highly expressed *PTENpg1* asRNA significantly correlated with low expression of *PTEN* mRNA based on a Spearman rank correlation analysis (Fig. 1c **and** Supplementary Fig. 3d). Surprisingly, the opposite correlation was observed for *PTENpg1* sense and asRNA, which appeared to be co-expressed (Fig. 1d and Supplementary Fig. 3d). The discordant expression between *PTEN* and *PTENpg1* asRNA was further supported by qRTPCR analysis on a subset of cell lines (Fig. 1e–f) and also by western blot analysis of PTEN protein levels, with the only exception being the *PTEN* deleted PC3 prostate cancer cell line (Supplementary Fig. 3e). Furthermore, the absolute expression of *PTENpg1* sense and asRNA transcripts was also measured by cloning the cDNA of each transcript. Defined amounts of these cDNA clones were used for standard curve analysis on qRTPCR and the *PTENpg1* asRNA was in general expressed at higher copy numbers as compared to the sense counterpart (Supplementary Fig. 3f).

PTENpg1 asRNA α is a negative regulator of *PTEN* expression

The observed discordant expression across disparate cell lines was suggestive of a negative regulatory association between the *PTENpg1* asRNA and *PTEN* mRNA expression. To investigate this notion further, HEK293T cells were transfected with siRNAs (*pg1as α* siRNA) or shRNAs (*pg1as α* shRNA) targeting the *PTENpg1* asRNA α transcript (Supplementary Fig. 4a). Suppression of *PTENpg1* asRNA α with these si- or shRNAs, resulted in a significant increase in *PTEN* mRNA expression in HEK293T cells (Fig. 2a and Supplementary Fig. 4b–c). The activation of *PTEN* did not appear to involve any changes in the levels of the previously reported miRNA sponge acting *PTENpg1* (Fig. 2b). The discordant regulation between *PTEN* and *PTENpg1* asRNA α was further confirmed in the U2OS cell line (Fig. 2c) and the HeLa cell line (Fig. 2d). Notably, HeLa cells lack detectable levels of *PTENpg1* sense (Fig. 1f and Supplementary Fig. 3d), suggesting that *PTENpg1* asRNA α functions independently of *PTENpg1* sense in all investigated cell lines. The observed induction of *PTEN* after targeting of the *PTENpg1* asRNA α was transcriptional in nature based on changes in the expression of *PTEN* pre-mRNA (Fig. 2a). The transcriptional activation of *PTEN* was further supported by Run-On analysis (Fig. 2e) and ChIP for RNAPII binding at the *PTEN* promoter (Fig. 2f). Moreover, the activation of *PTEN* did not appear to involve any off target effects mediated by the siRNAs (Supplementary Fig. 4d–e), or shRNAs (Supplementary Fig. f). The si- or shRNA-constructs

targeting the *PTENpg1* asRNA α did not affect *PTEN* expression in HCT116 or MCF7 cells, which do not express appreciable levels of the *PTENpg1* asRNA (Supplementary Fig. 4d–f), suggesting the *PTEN* activation to be mediated by specific targeting of the *PTENpg1* asRNA α. Taken together, these observations suggest that *PTEN* is susceptible to *PTENpg1* asRNA mediated transcriptional regulation, possibly by similar mechanisms as previously observed for other genes where noncoding RNAs (ncRNA), in particular asRNAs[10,11], have been found to play a role in the recruitment of repressive chromatin remodelers such as Enhancer of Zeste (EZH2)[7,12], DNA methyltransferase 3A (DNMT3a)[13] and the methyl transferase G9A[7].

PTENpg1 asRNA α localizes to the *PTEN* promoter

Previous observations implied a regulatory role for asRNAs in epigenetic-based regulation of gene transcription in human cells, but direct evidence supporting this notion has remained enigmatic[7,10,11]. To explore whether the induced expression of *PTENpg1* asRNA α could suppress *PTEN*, we cloned the *PTENpg1* asRNA α into GFP encoding lentiviral vectors with a U6 promoter driving its expression[14]. Stable Jurkat cell lines with the induced expression of *PTENpg1* asRNA α were generated and followed over 40 days (Supplementary Fig. 5a). Expression of the *PTENpg1* asRNA α was verified and a corresponding suppression of *PTEN* was observed, supporting the notion that the *PTENpg1* asRNA α acts *in trans* as a negative regulator of *PTEN* (Fig. 3a–b). To further investigate the interaction between *PTEN* and *PTENpg1* asRNA, *PTENpg1* asRNA was *in vitro* transcribed using fluorinated and biotin linked nucleotides (Supplementary Fig. 5b). This modified *in vitro* transcript was successfully transfected into the target cells and had a half-life greater than 48 hours (Supplementary Fig. 5C). The fluorinated and biotin linked *PTENpg1* asRNA was also found to suppress *PTEN* expression (Fig. 3c) and to localize to the *PTEN* promoter in the MCF7 cells (Fig. 3d). Overexpression of *PTENpg1* asRNA did not induce any detectable levels of the IFNα-induced genes *OAS1* and *IL6*, which potentially could be triggered by double-stranded RNA formation (Supplementary Fig. 5d). Altogether, these data show that *PTENpg1* asRNA α localizes to the *PTEN* promoter and suppresses *PTEN* mRNA expression.

PTENpg1 asRNA α induces chromatin remodeling

Next, we set out to investigate the molecular mechanisms whereby *PTENpg1* asRNA α exerts its regulatory function on *PTEN* transcription. Based on previous work on transcriptional regulation mediated by ncRNAs, we investigated the involvement of a subset of previous identified proteins, such as Histone deacetylase 1 (HDAC1), Argonaute 1 (AGO1), Argonaute 2 (AGO2), DNMT3a, EZH2 and G9A[7,12,13,15–17]. SiRNA induced depletion showed that both *EZH2* and *DNMT3a* appeared to be involved in the regulation of *PTEN*. The suppression of *EZH2* and *DNMT3a* in cell lines with expression of *PTENpg1* asRNA resulted in activation of *PTEN* (Fig. 3e and Supplementary Fig. 6a–c). In contrast, depletion of *EZH2* and *DNMT3a* in the HCT116 cell line, which lack *PTENpg1* asRNA expression (Supplementary Fig. 4d), had only a modest effect on *PTEN* levels (Supplementary Fig. 6b–d). In addition, simultaneous depletion of both *EZH2* and *PTENpg1* asRNA α did not prove additive in two different cell lines with regards to activating *PTEN*, further supporting the notion that these two factors function in concert (Supplementary Fig.

6e–f). These observations intrigued us to investigate whether EZH2 and DNMT3a may directly interact within an protein:RNA complex together with *PTENpg1* asRNA α. RNA immunoprecipitation (IP) of DNMT3a showed DNMT3a and *PTENpg1* asRNA α to co-IP and thus interact within the same protein:RNA complex (Fig. 3f and Supplementary Table 1c). Taken together, these data indicate EZH2 and DNMT3a are involved in the regulation of *PTEN* expression and importantly, that DNMT3a interacts directly with the with *PTENpg1* asRNA α transcript.

Previous reports have suggested DNMT3a and EZH2 interact[16] with one another and regulate levels of transcription by catalyzing the addition of methyl groups to the histone 3 lysine 27 (H3K27), which forms a negative repressive chromatin mark[18,19]. Therefore, we decided to investigate the presence of DNMT3a and EZH2 at the *PTEN* promoter and whether the *PTENpg1* asRNA α actively recruits these factors. To explore this notion, we induced siRNA depletion of *PTENpg1* asRNA α and investigated possible changes in the levels of H3K27me3, DNMT3a and EZH2 at the *PTEN* promoter. The suppression of the *PTENpg1* asRNA α variant correlated with a loss of EZH2 and H3K27me3 at the *PTEN* promoter (Fig. 3g–h). In addition, we also set out to target the *PTENpg1* asRNA α variant using single stranded phosphorothioate oligonucleotides (ODNs) (Supplementary Fig. 4a). In contrast to siRNAs, ODNs function by the RNase H pathway, which induces strand specific targeting of RNA followed by degradation of the RNA-ODN duplex[20]. Notably, the suppression of the *PTENpg1* asRNA α variant using these ODNs demonstrated a substantial loss of DNMT3a at the *PTEN* promoter in two different cell lines (Fig. 3i).

Collectively, these data demonstrate that the *PTENpg1* asRNA α binds and recruits the chromatin remodelers DNMT3a and EZH2 to the *PTEN* promoter and catalyzes the formation of H3K27me3. This intrigued us to investigate whether the endogenous *PTENpg1* asRNA α localizes to the *PTEN* promoter, and more specifically to the H3K27me3 chromatin mark. We therefore carried out a modified ChIP protocol for H3K27me3 and analyzed whether the nuclear localized *PTENpg1* asRNA associated directly with this epigenetic mark. Indeed, endogenous unspliced *PTENpg1* asRNA was found to localize to this histone mark (Fig. 3j).

PTENpg1 asRNA β interacts with *PTENpg1* sense

The observation that the *PTENpg1* sense lacks a robust poly(A) tail (Fig. 1b and Supplementary Fig. 2f) was further validated by 3’ RACE. The cloned 3’ RACE end of *PTENpg1* sense was sequenced, revealing the lack of a poly(A) tail (Supplementary Table 1d). Interestingly, poly(A) tailed transcripts have been implicated in RNA stability[21] and transport of RNA from the nucleus to the cytoplasm[22,23]. Collectively, the lack of poly(A) tail suggested that the *PTENpg1* sense might be an unstable transcript that is retained in the nucleus. However, contrary to this notion, blocking transcription by Actinomycin D treatment demonstrated similar stability of all three transcripts analyzed; *PTENpg1* sense and the poly(A) positive transcripts *PTEN* and *PTENpg1* asRNA (Fig. 4a). Therefore, we surmised that *PTENpg1* sense RNA is stabilized via alternative means in the cell. One possible mechanism could involve sense:asRNA interactions, which have previously been reported to modulate RNA stability[24]. The notion that *PTENpg1* sense and asRNA are co-

expressed among different cell lines (Fig. 1d and Supplementary Fig. 3d) and that they also share a divergently transcribed promoter (Supplementary Fig. S3a–c), suggested that they might be co-regulated and possibly interacting with one another through a double-stranded RNA intermediate. *In vitro* RNAse A analysis reaffirmed this notion and indicated that the *PTENpg1* sense and asRNA transcripts interact with one another in an RNA:RNA dependent manner (Fig. 4b).

To explore this notion further, we first developed U6 expressed single stranded RNAs (ssRNAs) targeting the *PTENpg1* sense and asRNA β overlap (Supplementary Fig. 4a). Interestingly, interfering with this interaction resulted in a decreased half-life of *PTENpg1* sense (Fig. 4c). Next, single stranded ODNs, which specifically target the *PTENpg1* sense and asRNA β overlap, and shRNAs targeting the *PTENpg1* asRNA exon 2 and 3 were designed (Supplementary Fig. 4a). Both of these approaches resulted in a concordant reduction of *PTENpg1* sense, and interestingly also suppressed *PTEN* mRNA (Fig. 4d–e) and protein levels (Fig. 4f). This observed suppression of PTEN is in line with previous reports, where *PTENpg1* sense has been described to bind and sequester miRNAs[3]. More precisely, the RNA:RNA interaction is lost upon depletion of *PTENpg1* asRNA β, whereby *PTENpg1* sense is destabilized and its expression decreased. Consequently, miRNAs, which are normally bound and sequestered by *PTENpg1* sense, may be released and free to bind the *PTEN* mRNA instead. Taken together, these data suggest that the RNA:RNA interaction between *PTENpg1* sense and asRNA β are required for *PTENpg1* sense to function as a miRNA sponge. Interestingly, this also shows that the *PTENpg1* asRNA α and β isoforms have different functions; the α isoform regulates *PTEN* transcription while the β isoform appears to be involved as a miRNA sponge, ultimately affecting post-transcriptional regulation of *PTEN*.

We further set out to investigate whether this RNA:RNA interaction occurred in the nucleus or in the cytoplasm. To this end *PTENpg1* asRNA β was knocked down using shRNA targeting followed by fractionation of cytoplasm and nuclei. The *PTENpg1* asRNA β shRNA targeting resulted in a reduction in *PTENpg1* sense levels exclusively in the cytoplasm, while the levels were unaffected in the nucleus (Fig. 4g). In summary, these data suggest that the *PTENpg1* sense:asRNA β interaction is required for maintaining stable levels of *PTENpg1* sense in the cytoplasm, and ultimately *PTENpg1* sense sponge activity.

PTENpg1 asRNA α regulates the cell cycle and apoptosis

The mechanistic studies presented here suggest a regulatory interaction between *PTEN*, *PTENpg1* sense and *PTENpg1* asRNA. Since perturbations of *PTENpg1* asRNA α levels markedly affected *PTEN* mRNA expression, we decided to investigate the effects of disrupting this lncRNA network on PTEN protein expression. Western blot analysis following siRNA depletion of *PTENpg1* asRNA α resulted in a clear induction of PTEN protein levels at several time points in various cell lines (Fig. 5a). Furthermore, the elevated PTEN protein levels lead to a concomitant downregulation of the PTEN downstream target pAKT (Fig. 5b). Since PTEN is a negative regulator of the cell cycle, we studied the effect on the CDK inhibitor p21 following depletion of *PTENpg1* asRNA α. Depletion of *PTENpg1* asRNA α resulted in a marked induction of p21, both 48 and 96h post transfection

(Fig. 5b)[25]. Notably, p21 was not induced upon *PTENpg1* asRNA α depletion in cell lines lacking expression of *PTENpg1* asRNA α, arguing against off target effects of the siRNA (Supplementary Fig. 6g). More specifically, PTEN dependent induction of p21 was verified by simultaneous siRNA depletion of *PTEN* and *PTENpg1* asRNA α, which did not result in an induction of p21 protein levels (Fig. 5c). This negative effect on the cell cycle was further supported by FACS analysis, (Fig. 5d), demonstrating a significant G0/G1-arrest in the *PTENpg1* asRNA α suppressed U2OS cells. In contrast, simultaneous depletion of *PTEN* and *PTENpg1* asRNA α partially rescued the G0/G1 cell cycle arrest (Fig. 5e).

Apart from causing a negative cell cycle effect, PTEN levels are also important in regulating apoptosis sensitivity[26]. Suppression of *PTENpg1* asRNA α caused a modest effect on the basal levels of cell death but clearly sensitized cells to the DNA-damaging agent doxorubicin (Fig. 5f). Together, these data suggest that the regulatory impact of *PTENpg1* asRNA α on *PTEN* expression is physiologically relevant and may function in the sensitization of tumor cells to chemotherapeutic treatments, such as doxorubicin.

Discussion

The data presented here argue for a more complex role for lncRNAs in human cellular gene regulation than had previously been appreciated. We find here that lncRNAs can form higher ordered biologically relevant functional structures through RNA:protein as well as RNA:RNA interactions. Earlier studies have reported that *PTENpg1* sense lncRNA functions as a miRNA sponge, an interaction that has typically been thought to occur in the cytoplasm[3,27]. Mechanistically, it was suggested that *PTENpg1* sense lncRNA binds to the *PTEN* targeted miRNAs whereby the miRNA targeting of *PTEN* is diminished, ultimately resulting in increased PTEN mRNA and protein expression. These previous studies however overlooked the presence of *PTENpg1* associated asRNA transcripts.

We report here that the *PTENpg1* locus expresses mechanistically functional asRNAs and that these transcripts appear to function as major regulators of *PTEN* expression, both at the transcriptional and translational levels.

Within this body of work, we investigate three *PTEN* regulatory lncRNAs; *PTENpg1* sense, *PTENpg1* asRNA α and *PTENpg1* asRNA β and observe that these transcripts are expressed from two divergently transcribed promoters (Supplementary Fig. 3a–c). We find that a complex regulatory network of lncRNAs is operative whereby *PTENpg1* asRNA α physically localizes to and directs epigenetic remodeling proteins to the *PTEN* promoter to control *PTEN* transcription. In contrast, the *PTENpg1* asRNA β transcript forms an RNA:RNA interaction with *PTENpg1* sense, altering cellular compartmental localization of the *PTENpg1* sense transcripts, miRNA sequestration, and ultimately PTEN protein levels (Fig. 6). While more complex than previously appreciated it still remains unknown as to how the transcriptional regulation of the *PTENpg1* locus is controlled and if any regulatory switch or interplay is functional between *PTENpg1* asRNA α and *PTENpg1* asRNA β. In addition, even though we find the *PTENpg1* asRNA α to directly localize at the *PTEN* promoter and to co-IP with DNMT3a, the details of these interactions remain to be explored.

These observations presented here suggest a model whereby a pseudogene, *PTENpg1*, contains bimodal functionality that directly and simultaneously affects *PTEN* transcription and translation (Fig. 6). This model, built on RNA:RNA and RNA:protein interactions, might highlight a mechanism whereby organisms lacking an RNA dependent RNA polymerase (RdRP), such as primates, can utilize a different paradigm such as pseudogene expressed lncRNAs to regulate gene expression. A functional RdRP would be expected to result in amplification of these RNA:RNA complexes and ultimately RNAi based mechanisms of regulation. However, the lack of a viable RdRP offers increased functional complexities in both the nucleus and cytoplasm, as the lncRNA complexes would not be processed by RdRP but rather functional in a different context, such as is demonstrated here whereby lncRNA complexes simultaneously govern both gene transcription and translation. Such observations add a significant layer of complexity to our current understanding of gene regulation and highlight a biological role for pseudogene-expressed lncRNAs in human cells. Such a system, as is determined here (Fig. 6), provides for a useful cellular adaptation strategy that allows for fine control and balance of the output of PTEN proteins, ultimately resulting in control of cell growth.

It is also interesting to speculate whether the *PTENpg1* asRNA α could act as an ncRNA oncogene due to its negative regulatory effect on *PTEN*. In future studies, it would be of great interest to profile human cancer tumors with diminished *PTEN* expression, and investigate whether increased expression of *PTENpg1* asRNA could promote epigenetic silencing of *PTEN* and tumor development. If so, knowledge of this *PTENpg1* and other pseudogene ncRNA regulatory pathways may prove useful in therapeutically relevant approaches to controlling gene expression as well as informative with regards to tumor biology.

Online methods

Cell cultures

HEK293, HEK293T and U2OS were cultured in 5% CO_2 at 37 °C in Dulbecco modified MEM medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 50 μg/ml of streptomycin and 50 μg/ml of penicillin.

Bioinformatics

ChIP sequencing data were aligned to *PTEN* and *PTENpg1* using Bowtie 0.12.8[1] with the parameter options -v0 and -m1. The alignments were visualized using IGV 2.1[2]. Data for H3K4me3 (K562 and H1-hESC cells): http://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeBroadHistone/ Data for RNAPII (K562 and H1-hESC cells): http://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeHaibTfbs/ Data for H3K4me3 (HCT116 and HEK293 cells): http://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeUwHistone/

ShRNAs, ssRNAs and siRNAs

ShRNA and ssRNA expressing constructs were generated and cloned into the U6M2 construct using the Bgl II and Kpn I restriction sites (Supplementary Table 2) as previously

described[3]. ShRNA and ssRNA constructs were transfected using Lipofectamine 2000 (Life Technologies). SiRNAs were ordered from the respective manufactures (Supplementary Table 2) and transfected using Lipofectamine 2000 (Life Technologies) or HiPerFect (Qiagen) at 10-20 nM unless stated otherwise.

RNA extraction and cDNA

RNA was extracted using Qiagen RNeasy mini kit (Qiagen) and DNase treated (Ambion Turbo DNA-free, Life Technologies). RNA (~500ng) was used for the generation of cDNAs using MuMLV (Life Technologies) and a mixture of oligo$(dT)_{18}$ with nanomers.

PCR and qRTPCR

PCR was performed using the KAPA2G FAST mix (Kapa Biosystems) with the corresponding oligos (Supplementary Table 2). QRTPCR quantification was carried out using the KAPA 2G SyberGreen (Kapa Biosystems) on the Eppendorf RealPlex or Applied Biosystem 7900HT platform with the following cycling conditions: 95°C for 3min, 95°C for 3sec, 60°C for 30sec.

Nuclear Run-On

HEK293T cells were transfected with 50nM of siRNA using HiPerFect (Qiagen). Pellets were collected 48h later and lysed (10mM Tris-HCl, pH 7.4, 10 mM NaCl, 3mM $MgCl_2$, 0.5% NP-40). The nuclei were resuspended in Glycerol storage buffer (50mM Tris-HCl, pH 8.3, 0.1 mM EDTA, 40% glycerol, 5 mM $MgCl_2$) and flash frozen in liquid nitrogen. Glycerol storage buffer (50 μL) was defrosted, mixed with 60 μL transcription buffer (20mM Tris-HCl, pH8, 5mM $MgCl_2$, 300mM KCl, 4mM DTT, 2mM ATP, 2mM CTP, 2mM GTP, 1mM biotin-16-AA-5'-UTP, 100U RNase out), incubated at 30°C for 45min followed by DNase treatment and nuclear lysis (50mM Tris-HCL pH 7.4, 5% SDS, 0.125M EDTA) with proteinase K treatment. The RNA was extracted and biotinylated RNA captured using Dynabeads MyOne Streptavidin beads (Life Technologies). The beads were resuspended in H_2O and cDNA prepared directly on beads.

Protein analysis

Samples were lysed in 50 mM Tris–HCl, pH 7.4, 1% NP40, 150 mM NaCl, 1mM EDTA, 1% glycerol, 100 μM vanadate, protease inhibitor cocktail and PhosSTOP (Roche Diagnostics GmbH). Lysates were subjected to SDS-PAGE and transferred to PVDF membranes. The proteins were detected by Western blot analysis using an enhanced chemiluminescence system (Western Lightning–ECL, PerkinElmer). Antibodies used: PTEN (Cell signaling, Cat# 9552, 1:1000), AKT (Cell signaling, Cat# 9272, 1:1000), phospho-AKT (Cell signaling, Cat# 4060S, 1:1000), p21 (BD Biosciences Sparks, Cat# 610234, 1:1000), β-actin (Sigma-Aldrich, Cat# A5441, 1:5000).

Fractionation

Fractionation of HEK293 cells was performed using the PARIS kit according to the manufacturer's recommendations (Life Technologies).

Promoter activity

HEK293T cells were co-transfected with the Renilla/Firefly bidirectional promoter construct[4] and GFP using lipofectamine 2000 (Life Technologies). The expression of GFP and Luminescence was measured 24h post transfection using the Dual-Glo Luciferase Assay System (Promega) and detected by the The GloMax®-Multi+ Detection System, (Promega). The expression of Luminescence was normalized to GFP.

ChIP; RNAPII and H3K27me3

The ChIP was carried out using the ChIP Assay Kit (Upstate/Millipore). Cells were crosslinked in 1% formaldehyde, quenched and lysed according to the manufacturer's recommendations. The samples were sonicated with a Bioruptor Sonicator (Diagenode) and incubated over night at 4°C with the appropriate antibody. Salmon Sperm DNA/Protein A Agarose (Upstate/Millipore) was used to pulldown the antibody. DNA was eluted in Elution buffer (1% SDS, 100mM $NaHCO_3$), followed by reverse crosslinking, RNaseA and protease K treatment. The DNA was eluted using Qiagen PCR purification kit. The following antibodies were used (4 μg/sample); Polymerase II (Santa Cruz biotechnologies, Cat# sc899x) and H3K27me3 (Upstate/Millipore, Cat# 17-622).

PTENpg1 asRNA binding to H3K27me3

ChIP protocol, as described above, was carried out without LiCl wash and RNaseA treatment. The beads were resuspended in 95μL proteinase K buffer (100 mM NaCl, 10mM Tris-HCl, 1mM EDTA, 0.5% SDS) with 5 μL proteinase K (20mg/ml), incubated for 60min at 50°C and heated to 95°C for 10min. The RNA was eluted using the miRNeasy mini kit (Qiagen) and DNase treated and cDNA was prepared as previously.

ChIP and IP-RT-sequencing- DNMT3a

ChIP analysis of ODN treated cells were carried out in an automated fashion using the EpiMotion (Eppendorf) and previously published protocols[5,6] adapted to this system. The resultant ChIPs were carried out with one modification, no RNAse A was added. The resultant elutes were DNAse treated and cDNA prepared as described above. PCR was carried out on the IP-RTs using *PTENpg1* sense or antisense specific primer sets 3 and 2, respectively. Only the sequencing results for primer set 2 (Supplementary Table 2), *PTENpg1* asRNA, are shown as all primer set 3 sampling proved negative. The antibodies used were; DNMT3a (ProSci, Cat# xw-7148), AGO-2 (Cell signaling, Cat# C34C6), Nucleolin (Abcam, Cat# ab50279), AGO-1 (Upstate/Millipore, Cat# 07-599), G9a (Upstate/Millipore, Cat# 07-55) and EZH2 (Upstate/Millipore, Cat# 07-689).

RNAse A

RNA from HEK293 cells was exposed to RNAse A (0.1μg) for 30 min at 37°C. The samples were heat inactivated (95°C for 10min) and cDNA prepared as described above. The resultant cDNAs were PCR amplified with *PTENpg1* sense and *PTENpg1* asRNA primers (Sets 1–2, Supplementary Table 2).

5´ RACE

RNA from several human cell lines was pooled and cDNA prepared according to 5´ RACE system kit (Life Technologies) using primer PTENpg1 asRNA R1 (Supplementary Table 2). PCR was carried out using forward primer AAP with the reverse primer PTENpg1 asRNA R5. Nested PCR was followed using forward primer AUAP with reverse primer PTENpg1 asRNA R3 (Supplementary Table 2). PCR products were gel purified and sequenced.

Poly(G) tailing

RNA was 3´ poly(G) tailed using yeast poly(A) polymerase (Affymetrix) and guanosine 5´-triphosphate (GE Healthcare). Poly(G) tailed RNA was used for first strand cDNA synthesis, followed by nested PCR (Supplementary Table 2). The PCR product was cloned into StrataClone PCR cloning Kit (Agilent Technologies) and sequenced.

Poly(A) depletion

Dynabeads MyOne Streptavidin beads (Life Technologies) were pre-loaded with 5'-biotinylated oligo$(Dt)_{18}$ or control (biotin-362as)[7]. DNase treated RNA from HEK293T cells was depleted of poly(A) transcripts and used to generate cDNA.

Generation of promoter constructs

The *PTENpg1* promoter sequences were PCR amplified using primers containing Kpn1 restriction sites (Supplementary Table 2). The resultant PCR products were cloned into the pLucRLuc Bidirectional vector[4].

Generation of U6 expressed *PTENpg1* asRNA α lentiviral constructs

The U6 promoter was amplified from the U6M2 cloning plasmid[3] and ligated into the Not1 restriction site of the pHIV7-IMPDH2 vector[8]. *PTENpg1* asRNA α was PCR amplified and subsequent cloned into the Nhe1 and Pac1 restriction sites in the pHIV7-IMPDH2-U6 plasmid (Supplementary Table 2).

Lentiviral production, transduction and selection

Lentiviral particles were produced and titrated as previously described[8]. Jurkat cells were infected at a multiplicity of infection = 1 by spinoculation for 30min. Transduced cells were placed under selection with 1–2 μM mycophenolic acid (Sigma-Aldrich) for 20 days.

Generation of biotin linked *PTENpg1* asRNAs

PTENpg1 asRNA α was PCR amplified using primers with Nhe1 or Kpn1 restriction sites (Supplementary Table 2) and cloned into pcDNA 3.1. T7 transcripts were generated by linearization of pcDNA 3.1 with Kpn1 and T7 transcribed with the Durascribe T7 kit (Epicenter, Madison WI, USA) using fluorinated CTPs and UTPs (TriLink). Biotin UTPs were incorporated at a ratio of 1:10. The resultant transcripts were DNase treated and dephosporylation using Antartic Phosphatase (New England Biolabs). Transcripts were precipitated using phenol-chloroform and EtOH precipitation. The T7 transcripts were transfected into target cells at 25-50nM using Lipofectamine 2000 (Life Technologies). For

in vivo pulldown, MCF7 cells were transfected, 30h later collected and avidin IPs performed as described[5,6].

PI-annexin V staining

Treated cells were harvested, washed in PBS, pelleted, and resuspended in 100μL Annexin V incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) containing 1% annexin V FLOUS (Annexin V FLUOS, Roche Molecular Biochemicals) and 500 μg/μl PI staining. The samples were incubated for 15min followed by the addition of 400 µL of Annexin V incubation buffer and subsequent analysis on a cytometry machine using the Cell Quest software.

Cell cycle distribution

Cells were washed in PBS and fixed in 4% PFA at room temperature over night. PFA was removed and cells were resuspended in 95% EtOH. The samples were then rehydrated in distilled water, stained with DAPI and analyzed by flow cytometry on a FACS Calibur (Becton Dickinson) using the cell Quest software.

ODN targeting of *PTENpg1* asRNA transcripts

The ODNs were produced (IDT) and transfected at 100nM using RNAiMax (Life Technologies).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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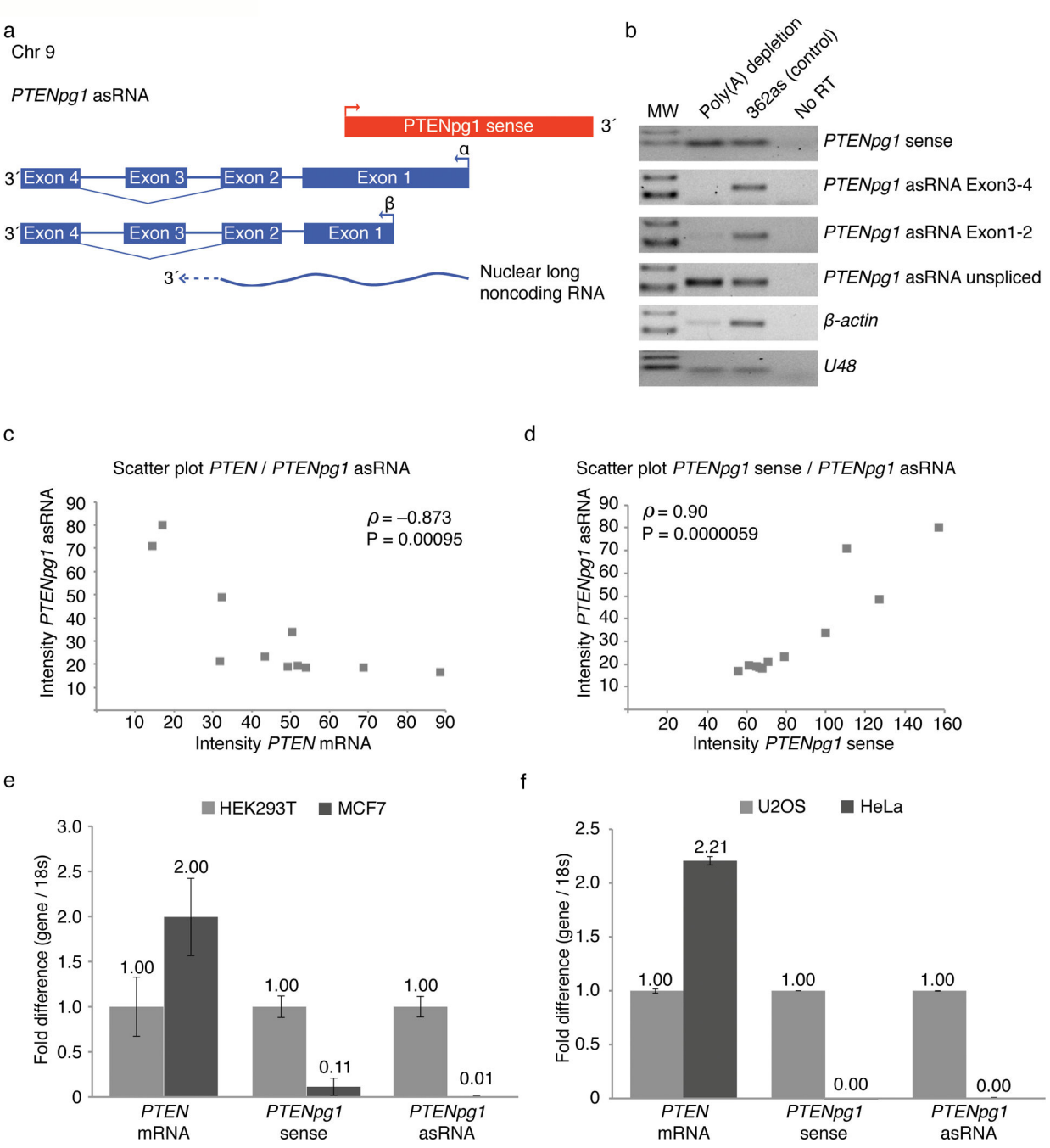

Figure 1. High expression of *PTENpg1* asRNA correlates with low *PTEN* mRNA expression

(a) A schematic depicting the *PTENpg1* sense and asRNA divergently transcribed locus. **(b)** Semi-qRTPCR of poly(A) depleted fractions was assessed for *PTENpg1* sense and different isoforms of the *PTENpg1* asRNA. **(c–d)** Semi-qRTPCR (Supplementary Fig. 3d) was quantified using ImageJ software. A Spearman rank correlation (n=11) and scatter plot was carried out on the quantified PCR products. **(c)** Scatter plot contrasting *PTEN* and *PTENpg1* asRNA. **(d)** Scatter plot contrasting *PTENpg1* sense and *PTENpg1* asRNA. **(e)** QRTPCR assay on HEK293T cells (high *PTENpg1* asRNA) contrasted the MCF7 cell line (low

PTENpg1 asRNA) (n=2). (**f**) QRTPCR assay on U2OS cells (high *PTENpg1* asRNA) contrasted the HeLa cell line (low *PTENpg1* asRNA) (n=2).

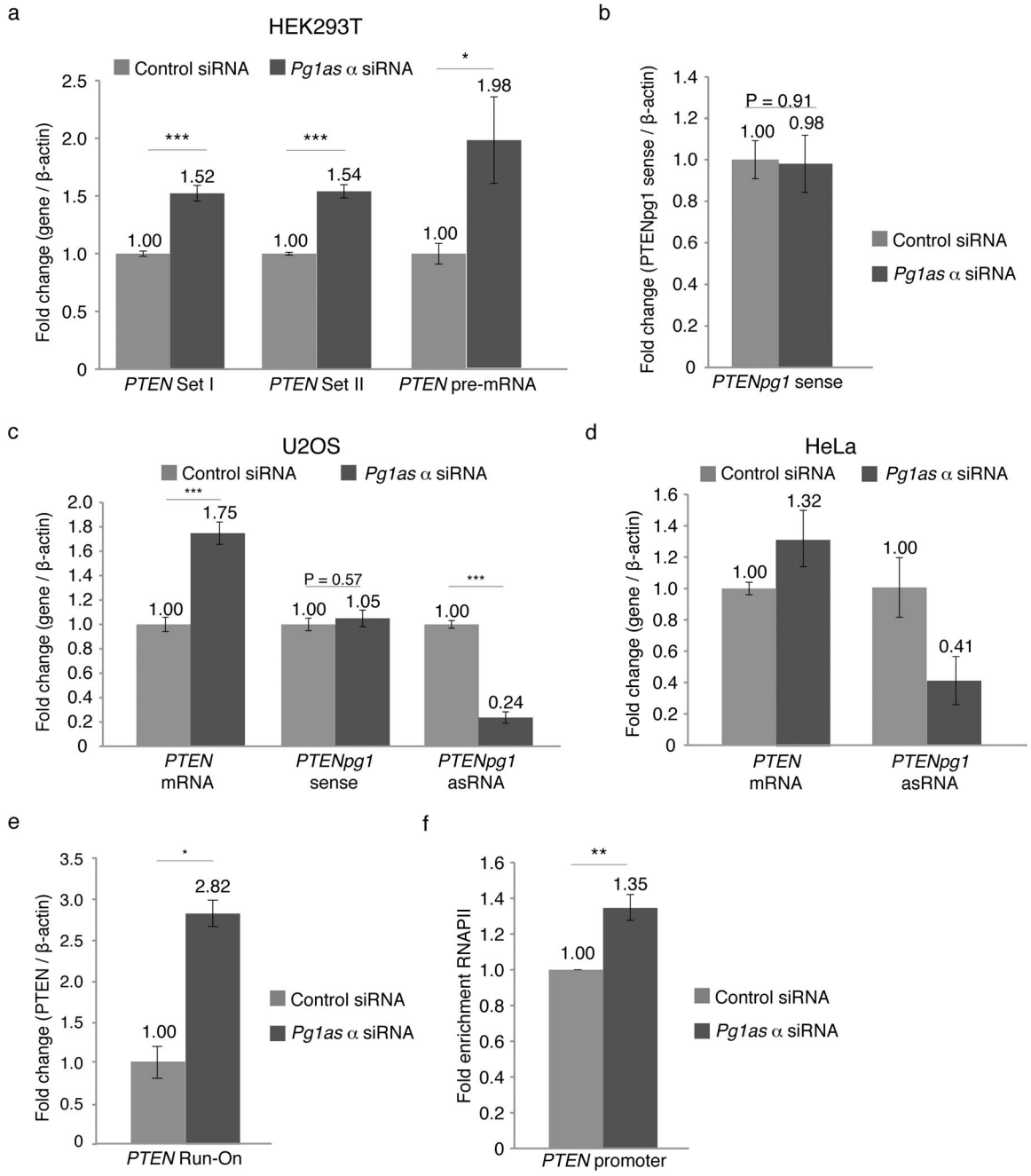

Figure 2. Functional characterization of *PTENpg1* asRNA α

(a) QRTPCR analysis of *PTEN* transcripts in HEK293T cells 72h post-transfection with siRNAs targeting the *PTENpg1* asRNA α isoform (n=3). (**b**) QRTPCR analysis of *PTENpg1* sense in cultures treated as described above (n=3). **(c)** QRTPCR analysis of *PTEN* and *PTENpg1* transcripts in U2OS cells treated as described above (n=3). **(d)** QRTPCR analysis of *PTEN* transcripts in HeLa cells treated as described above (n=2). **(e)** Nuclear Run-On analysis of *PTEN* 48h post-transfection with siRNAs targeting the *PTENpg1* asRNA α isoform (n=3). **(f)** ChIP analysis of RNAPII enrichment at the *PTEN* promoter in cultures

treated as described (a–d above) (n=3). The (*) indicates the significance $p<0.05$, (**) $p<0.01$ and (***) $p<0.005$ using a Student's T-test. Error bars represent the standard errors of the mean.

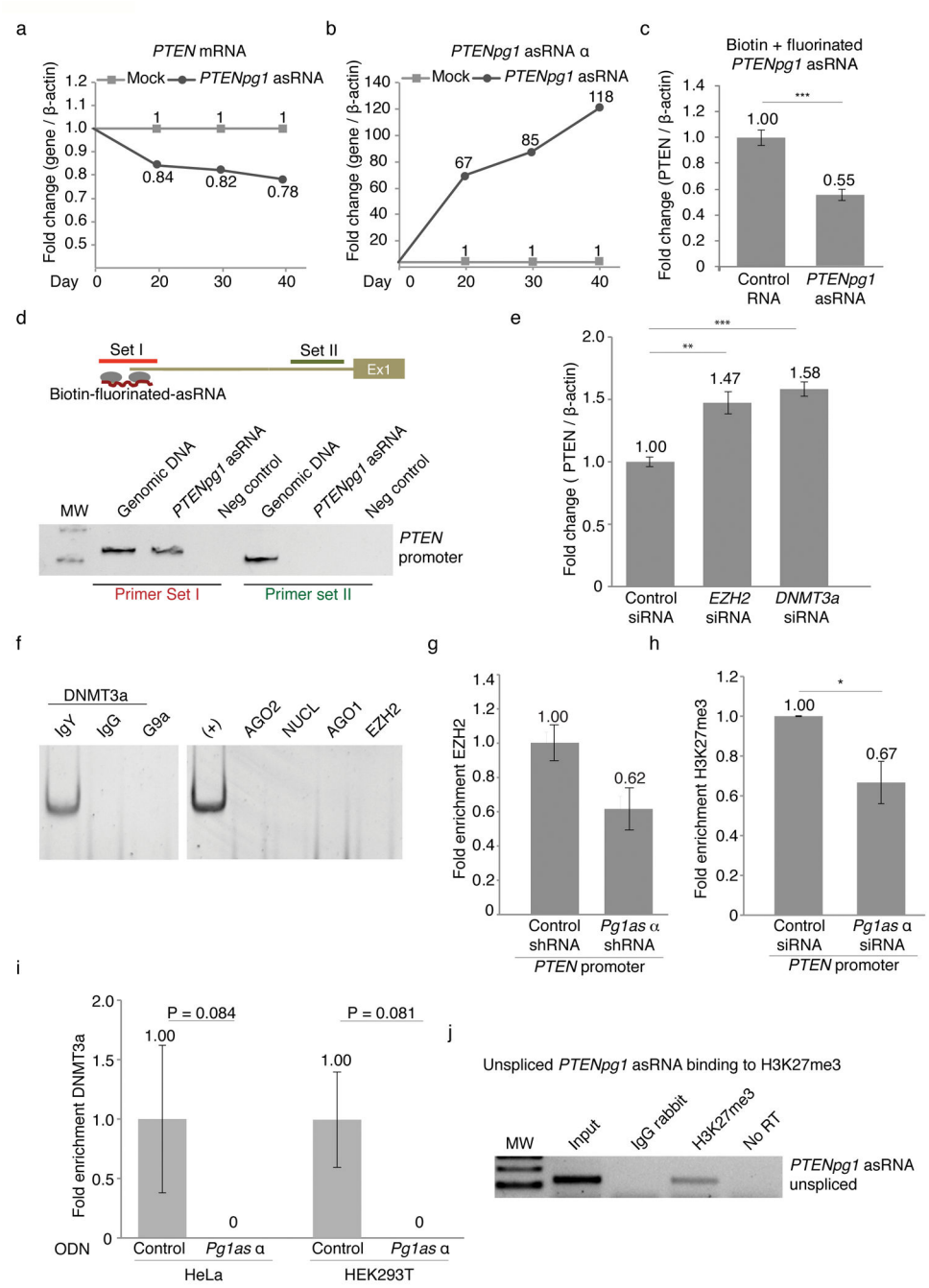

Figure 3. Mechanistic insights into *PTENpg1* asRNA α regulation of *PTEN*

(a–b) QRTPCR analysis of stable Jurkat cell lines. **(a)** *PTEN* mRNA expression and **(b)** *PTENpg1* asRNA expression. **(c)** QRTPCR analysis of MCF7 cells transfected biotinylated-fluorinated *PTENpg1* asRNA (n=3). **(d)** *In vitro* transcribed biotinylated-fluorinated *PTENpg1* asRNA was transfected into MCF7 cells followed by a biotin-ChIP and semi-qRTPCR targeting different regions of the *PTEN* promoter. **(e)** QRTPCR analysis of siRNA depletion of *DNMT3a* and *EZH2* in HEK293T cells (n=3). **(f)** RNA-IP followed by subsequent semi-qRTPCR for *PTENpg1* asRNA α. Only the chicken (IgY) anti-DNMT3a

antibody was found to IP with *PTENpg1* asRNA α. The pulldown was contrasted to the non-specific protein G antibody.**(g)** QRTPCR analysis of ChIP-EZH2 samples after shRNA induced targeting of *PTENpg1* asRNA α in HEK293T cells (n=2). **(h)** QRTPCR analysis of ChIP-H3K27me3 samples after siRNA induced targeting of *PTENpg1* asRNA α in HEK293T cells (n=3). **(i)** QRTPCR analysis of ChIP-DNMT3a samples following single stranded ODN targeting of *PTENpg1* asRNA α in HeLa and HEK293T cells (n=3). **(j)** Semi-qRTCPR of nuclear expressed and unspliced *PTENpg1* asRNA α following a modified ChIP protocol for RNA bound to H3K27me3. The (*) indicates the significance $p<0.05$, (**) $p<0.01$ and (***) $p<0.005$ using a Student's T-test. Error bars represent the standard errors of the mean.

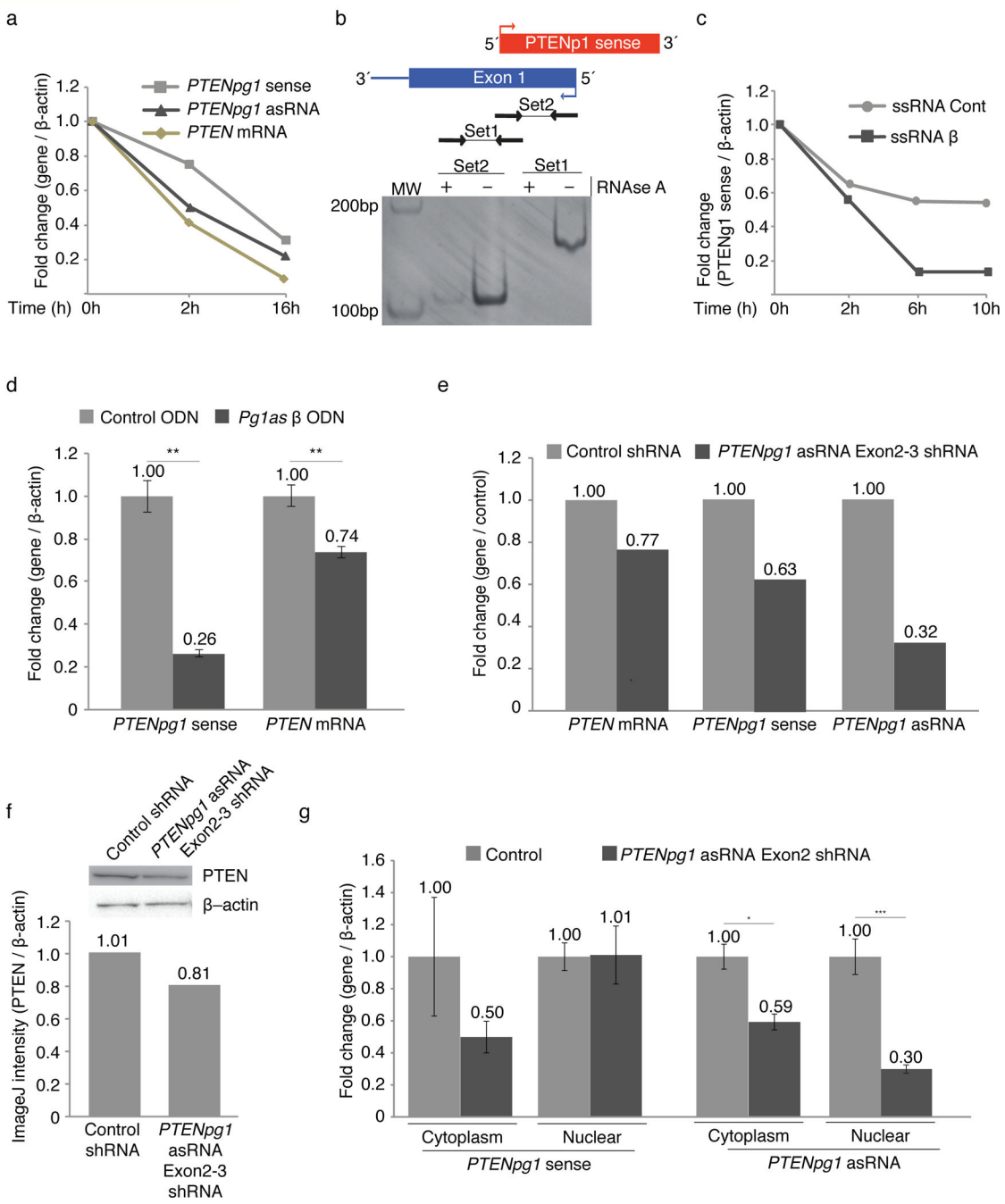

Figure 4. Mechanistic insights into *PTENpg1* asRNA β regulation of *PTENpg1*

(a) QRTPCR analysis of the stability of *PTENpg1* sense, *PTENpg1* asRNA and *PTEN* after blocking transcription in HEK293T cells using Actinomysin D. The data were normalized to T=0h. **(b)** Semi-qRTPCR of RNase A analysis for the detection of RNA:RNA pairing. RNAse A treatment followed by RT and PCR with primers spanning the *PTENpg1* sense and asRNA divergently transcribed locus (shown schematically). **(c)** QRTPCR analysis of the stability of *PTENpg1* sense in HEK293T cells after interfering with the *PTENpg1* sense:asRNA β interaction using U6 encoded ssRNAs. Transcription was blocked using

Actinomysin D and the data were normalized to T=0h. **(d)** QRTPCR analysis of *PTEN* and *PTENpg1* sense after interfering with the *PTENpg1* sense:asRNA β interaction using single stranded ODNs (n=3). **(e)** QRTPCR analysis after shRNA targeting of the *PTENpg1* asRNA transcripts. (**f**) Western blot showing the expression of PTEN after *PTENpg1* asRNA depletion. The intensities were quantified using the ImageJ software. **(g)** QRTPCR analysis on fractionated HEK293T cells after depletion of *PTENpg1* asRNA. The (*) indicates the significance $p<0.05$, (**) $p<0.01$ and (***) $p<0.005$ using a Student's T-test. Error bars represent the standard errors of the mean.

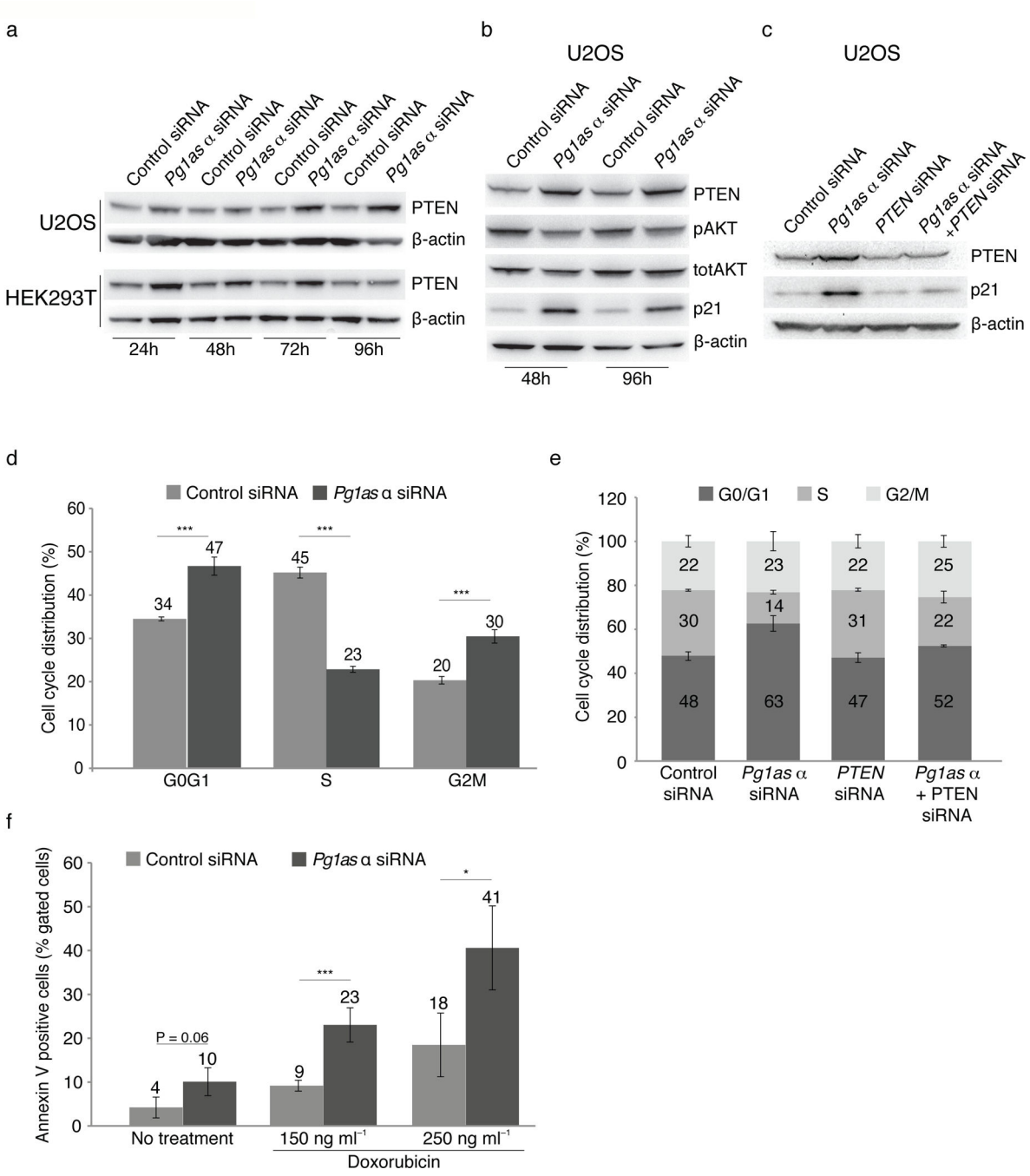

Figure 5. Functional impact of *PTENpg1* asRNA α

(a) Western blot showing PTEN expression after knockdown of *PTENpg1* asRNA α in two different cell lines at different time points. The data were standardized to β-actin.**(b)** Western blot at two different time points after knockdown of *PTENpg1* asRNA α. **(c)** Western blot showing the effect on PTEN and p21 after simultaneous knockdown of the *PTENpg1* asRNA α and *PTEN*. **(d)** Cell cycle distribution in U2OS cells following knockdown of *PTENpg1* asRNA α (n=3). **(e)** Cell cycle distribution after simultaneous knockdown of *PTENpg1* asRNA α and *PTEN* in U2OS cells (n=3) **(f)** Cell death analysis by

AnnexinV / PI staining after 72h knockdown of *PTENpg1* asRNA α and 48h treatment with doxorubicin (n=3). The (*) indicates the significance $p<0.05$, (**) $p<0.01$ and (***) $p<0.005$ using a Student's T-test. Error bars represent the standard errors of the mean.

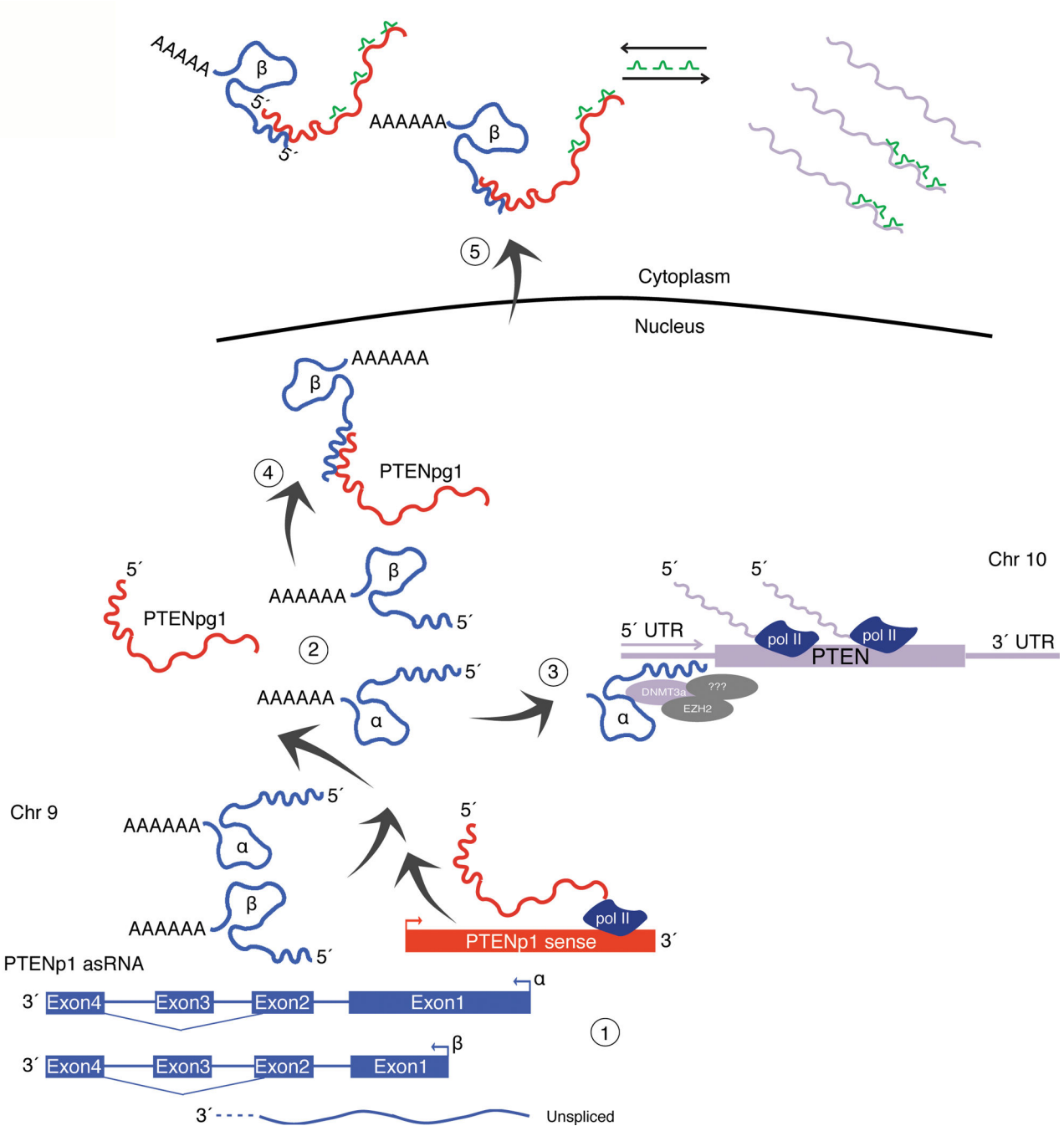

Figure 6. Model of *PTENpg1* asRNA based regulation of *PTEN*

(1) The *PTENpg1* locus is transcribed in both sense (red) and antisense orientation (blue). **(1–2)** Three different isoforms of the *PTENpg1* asRNA are transcribed; *PTENpg1* asRNA α, β and the unspliced nuclear localized *PTENpg1* asRNA α. **(3)** *PTEN* transcription is suppressed by the *PTENpg1* asRNA α and unspliced isoforms, which act *in trans* by recruiting the chromatin repressor proteins EZH2 and DNMT3a to the *PTEN* promoter. **(4)** The *PTENpg1* sense lacks a poly(A) tail and the stability and export of *PTENpg1* sense to the cytoplasm is facilitated by the interaction with the spliced *PTENpg1* asRNA β

transcripts. **(5)** Such interactions lead to an increase of *PTENpg1* sense sponge activity and thus increased *PTEN* mRNA stability and translation. This mechanism **(1–5)** results in both transcriptional and post-transcriptional regulation of *PTEN* by its pseudogene. The model indicates a level of synchronization between the pseudogene and its protein-coding counterpart.