PTENβ is an alternatively translated isoform of PTEN that regulates rDNA transcription

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PTEN is a critical tumour suppressor that is frequently mutated in human cancer. We have previously identified a CUG initiated PTEN isoform designated PTENα, which functions in mitochondrial bioenergetics. Here we report the identification of another N-terminal extended PTEN isoform, designated PTENβ. PTENβ translation is initiated from an AUU codon upstream of and in-frame with the AUG initiation sequence for canonical PTEN. We show that the Kozak context and a downstream hairpin structure are critical for this alternative initiation. PTENβ localizes predominantly in the nucleolus, and physically associates with and dephosphorylates nucleolin, which is a multifunctional nucleolar phosphoprotein. Disruption of PTENβ alters rDNA transcription and promotes ribosomal biogenesis, and this effect can be reversed by re-introduction of PTENβ. Our data show that PTENβ regulates pre-rRNA synthesis and cellular proliferation. These results demonstrate the complexity of the PTEN protein family and the diversity of its functions.
The PTEN gene was first identified and cloned in 1997, and PTEN protein has proved to be a powerful tumour suppressor. It is one of the most frequently mutated or deleted genes in cancer, including cancer of the brain, breast, prostate, pancreas and ovary. Germline mutations of the PTEN gene lead to inherited autosomal dominant hamartoma tumour syndromes, including Bannayan–Zonana syndrome and Cowden disease. Heterozygous Ptne deletion leads to multiple tumours in mice, while the homozygote is embryonically lethal. In the cytoplasm, PTEN antagonizes the PI3K-akt-mTOR pathway by dephosphorylation of phosphoinositide-3,4,5-triphosphate (PIP3). Loss of PTEN function leads to uncontrolled activation of the PI3K/akt pathway, which stimulates cell growth, cell proliferation and cell survival, and inhibits apoptosis. PTEN also accumulates in the cell nucleus through passive diffusion, Ran-MVP-mediated transport and mono-ubiquitination-regulated mechanisms. In the nucleus, PTEN maintains Ran-MVP-mediated transport and mono-ubiquitination-proliferation and cell survival, and inhibits apoptosis. PTEN also regulates mechanisms similar to or identical with canonical PTEN. In order to confirm this, we examined cancer cell lines with an anti-PTEN antibody raised in our laboratory by immunization with purified recombinant protein composed of the 173 N-terminal amino acids of PTEN (ref. 25). This unidentified protein was recognized by this anti-PTEN antibody (Fig. 1c), indicating that it was most likely another N-terminally extended PTEN variant generated by initiation at an in-frame initiation codon within the PTEN mRNA 5’ leader. We designated this PTEN-like protein PTENb.

**Results**

An unidentified protein is recognized by a PTENx antibody. The protein PTENx functions in mitochondrial metabolism, and structurally distinct compounds such as acrilavin and aurin tricarboxylic acid (ATA) yield different results in regulation of PTENx synthesis initiated by a CUG start codon. The CUG inhibitor acrilavin reduces PTENx expression in a dose-dependent manner without affecting canonical PTEN expression. We observed that a full length PTEN antibody reacted with an unidentified protein of molecular weight lower than PTENx (around 70 kDa), and expression of this protein showed dose dependent variation under acrilavin treatment parallel to that of PTENx (Fig. 1a). A panel of cancer cell lines was examined to determine whether differences in PTEN status affect this unidentified PTEN-like protein, and the protein was undetectable in PTEN-null PC3 cells (Fig. 1b). This protein was also recognized by a monoclonal antibody against the PTEN C-terminal domain, suggesting its C-terminal region is similar to or identical with canonical PTEN. In order to confirm this, we examined cancer cell lines with an anti-PTEN antibody raised in our laboratory by immunization with purified recombinant protein composed of the 173 N-terminal amino acids of PTENx (ref. 25). This unidentified protein was recognized by this anti-PTEN antibody (Fig. 1c), indicating that it was most likely another N-terminally extended PTEN variant generated by initiation at an in-frame initiation codon within the PTEN mRNA 5’ leader. We designated this PTEN-like protein PTENb.

**Alternative initiation of PTENb translation from AUU**

Evaluation of the 5’ UTR of human PTEN mRNA for alternative translation start sites in frame with the AUG1032 start codon revealed a total of four non-AUG alternative initiation codons in favourable Kozak contexts (Fig. 2a). Translation from AUU594 or UUG621 is expected to encode larger forms of PTEN of 549 or 540 amino acids respectively, with predicted molecular weights of 61.6–62.9 kDa. A protein of this size should migrate under gel electrophoresis at around 70 kDa, matching the observed PTENb band.

To determine whether one of these non-AUG codons initiates translation of this putative PTENb protein with upstream extension of the open reading frame (ORF), the coding sequence of PTENx was cloned into a plasmid with a C-terminal GFP tag (Fig. 2b, upper panel). As expected, the CUG513 initiated PTENx ORF was translated into three distinct proteins with masses of 120 ± 1 DA (PTENx-GFP), 120 kDa (PTENb-GFP) and 90 kDa (PTEN-GFP) (Fig. 2b, lower panel). This suggested that PTENb is translated from a new ORF in the 5’ UTR of PTEN mRNA upstream of the canonical AUG start codon.

To identify PTENb translation initiation sites, a plasmid was constructed to express CUG513-initiated PTENx with a C-terminal GFP tag, and mutations were created in this plasmid to determine which non-AUG codon is critical for PTENb expression (Fig. 2c). Mutation of CUG513 into a non-initiating CUC triplet led only to disappearance of the most slowly migrating proteoform PTENx, thus excluding the possibility that PTENb is a cleavage product (Fig. 2d, lane 4). On the basis of CUG513 mutation, potential non-AUG codons (AUU594, UUG621) were mutated separately to a non-initiating CUC triplet. Anti-GFP immunoblots from these transfected lysates revealed that mutation of AUU594 abolishes PTENb, whereas mutation of UUG621 has no such effect (Fig. 2d, lane 6 versus lane 5). These results indicate that AUU594 but not UUG621 is essential for PTENb expression, and that AUU594 is likely the translation initiation site for PTENb.

AUU594 resides in a favourable Kozak context as noted (GUCACCAU594) (Fig. 2a). In order to determine whether this context influences translation initiation at AUU594, we constructed mutants to disrupt it and examined PTENb expression (Fig. 2e). Disruption of this Kozak context markedly
reduced PTENβ expression (Fig. 2f), strongly suggesting that translation initiation of PTENβ is reliant on the Kozak context.

Detailed evaluation of the 5′ UTR of human PTEN mRNA revealed a 12 bp perfect palindromic sequence starting 18 bp downstream of AUU594, and phylogenetic analysis suggests that this secondary structure is evolutionarily conserved (Fig. 2g). As shown in Fig. 2h, disruption of this palindromic motif by mutation led to a marked reduction of PTENβ without affecting canonical PTEN expression (lower panel, lane 4 versus lane 3), indicating this sequence is critical for translation initiation of PTENβ.

These data demonstrate that PTENβ synthesis relies on an alternative translation initiation at the AUU594 codon, and expression of PTENβ is influenced both by the Kozak context and by the secondary structure downstream of AUU594.

Mass spectrometry analysis of the PTENβ sequence. Mass spectrometry was used for peptide sequencing to validate the PTENβ translation initiation site. Purified human PTENβ with a C-terminal His tag (Fig. 3a) was expressed in S9 insect cells for mass spectrometry (Fig. 3b). Mascot reports revealed three peptide fragments covering 39.04% of the N-terminal region of PTENβ (from AUU594 to AUG1032) (Fig. 3b). LC-MS/MS captured MSRGNAGE, which is the most proximal N-terminal peptide of PTENβ (9 aa, MS/MS spectrum shown in Fig. 3c). Additional analysis showed that the first N-terminal amino acid of PTENβ is acetylated. These mass spectrum data therefore verified AUU594 is the PTENβ translation initiation site.

The 5′ UTR of Homo sapiens PTEN and Mus musculus Pten are highly homologous as shown in our recent study25, suggesting that PTENβ expression in mice is conserved and has a similar alternative translation initiation mechanism. We previously generated a FLAG-knockin mouse model (PtenFLAG)25, in which the C-terminus of the Pten gene was targeted for insertion of a FLAG-coding sequence 25. To verify PTENβ resides at the Pten gene locus, tissue samples were extracted from heterozygous PtenFLAG mice and wild-type control mice for FLAG pull-down followed by immunoblotting with an anti-PTENα antibody raised in our laboratory25. A protein band with a molecular weight lower than PTENα which was similar to the PTENβ band identified in cancer cell lines was detected in the FLAG elute from PtenFLAG tissues, while no band was detected in wild-type tissues (Fig. 3d). The existence of in vivo PTENβ was further confirmed by mass spectrometric analysis of a FLAG-purified 70+ kDa band (molecular weight...
lower than PTENα) from PtenFLAG tissues. Mascot reports revealed four peptide fragments that cover 68.5% of the N-terminal region of PTENβ (Fig. 3e). These results verified the existence of PTENβ in vivo, and confirmed that PTENβ and PTENx are translated in vivo from identical mRNA at the same gene locus. This PtenFLAG knockin animal model demonstrates the natural occurrence of alternative initiation that results in translation of PTENβ.
PTENβ is localized predominantly in the nucleolus. Longer protein isoforms resulting from alternative translation initiation frequently contain a signal for subcellular localization that is absent in shorter forms26,27. The length of the PTENβ protein differs from PTENα and PTEN due to its N-terminal extension, which raised the possibility that PTENβ has unique subcellular localization and special function. Immunofluorescence examination of the subcellular localization of PTENβ was compared with PTENα and PTEN. Separate plasmids expressing C-terminal GFP-tagged PTEN, PTENα or PTENβ were constructed, and to ensure expression of only a single isoform in each instance, we created an ATG1652→ATA mutation in GFP-tagged PTENα and PTENβ (Fig. 4a). The subcellular distribution of PTENβ was completely different from that of PTEN and PTENα as shown in Fig. 4b. PTEN was distributed ubiquitously in both the nucleus and cytoplasm, while PTENα predominately showed cytoplasmic localization. In contrast, strong PTENβ fluorescence signals were distributed mainly in the relatively shallow DAPI staining areas of the nucleus which have been suggested to be nucleolar regions28, and little cytoplasmic PTENβ localization was detected. In order to exclude the possibility protein localization was influenced by the GFP tag, PTEN, PTENα and PTENβ expression plasmids were constructed without a tag (Supplementary Fig. 1a), and immunofluorescence with PTEN monoclonal antibody in PTEN null cells revealed that these untagged isoforms have subcellular distribution patterns identical to the GFP-tagged molecules (Supplementary Fig. 1b,c). UBF that is a well-known nucleolar marker was used to evaluate for co-localization of C-terminal GFP tagged PTENα, PTENβ or PTEN within the nucleolus. There was prominent co-localization of PTENβ with this marker in the nucleolus, but considerably less nucleolar co-localization was detected with PTEN or PTENα (Fig. 4c). This extensive distribution of PTENβ in the nucleolus was confirmed through cell fractionation (Fig. 4d). Moreover, we demonstrated that the N-terminal extended sequence was solely responsible for PTENβ accumulation in the nucleolus, and this was also true for PTENα localization on mitochondria as we reported previously (Supplementary Fig. 2).

The nucleolar-localization signals (NoLSs) that govern nucleolar localization and retention are usually rich in arginine and lysine residues29,30. We noted a series of poly-arginine residues in the N-terminal extended domain of PTENβ (aa 20–25), which is evolutionarily conserved (Supplementary Fig. 3). To determine whether this sequence acts as an NoLS and directs PTENβ nucleolar localization, we established a PTENβΔR6 construct in which these six arginines were deleted. This ΔR6 mutation was remarkable in that it completely abolished PTENβ nucleolar accumulation (Fig. 4e), indicating that this poly-arginine sequence is critical for PTENβ nucleolar localization, and showing that it functions as an NoLS. These results indicated that the distinctive N-terminal extension sequence of PTENβ confers unique cellular localization, and raised the possibility that PTENβ is involved in nucleolar function.

Nucleolin is a novel downstream target of PTENβ. In order to identify potential PTENβ targets and explore PTENβ function in the nucleolus, mass spectrometry was used to analyse the results of S-tag-PTENβ pull down. Results with S-tag-PTEN and S-tag-PTENα were analysed separately as controls. Mass spectrometry revealed that PTENβ specifically interacts with a large number of nucleolar proteins (Supplementary Fig. 4a), including nucleolin that is the most abundant multifunctional phosphoprotein of the nucleolus. Nucleolin is comprehensively involved in ribosome biogenesis31,32, and was thus considered to be a potential PTENβ target (Fig. 5a). Interaction of PTENβ and nucleolin was demonstrated by reciprocal immunoprecipitation (Fig. 5b,c). In addition, immunofluorescence showed extensive co-localization of PTENβ with nucleolin in the nucleolus, whereas PTEN or PTENα did not (Fig. 5d), further confirming the specificity of the interaction of PTENβ and nucleolin. To identify the functional domains by which PTENβ binds to nucleolin, two truncated forms of PTENβ were constructed with C-terminal S-tags (Supplementary Fig. 5a). Immunoprecipitation demonstrated that the N-terminal extension domain of PTENβ is essential for interaction with nucleolin (Supplementary Fig. 5b). These data show PTENβ and nucleolin co-localize and interact each other.

Like canonical PTEN, PTENβ contains an intact phosphatase domain, and we found that exogenous expression of PTENβ, PTENα or PTEN in null cells all efficiently reduce p-AKT to a similar level (Supplementary Fig. 6a). Moreover, missense mutations in the phosphatase domain of PTENβ (G275E, analogous to PTEN (G129E)) and (C270S, analogous to PTEN (C124S)) both eliminated the lipid phosphatase activity of the protein (Supplementary Fig. 6b). PTENβ thus has phosphatase activity that is presumably similar to PTENα and canonical PTEN. In addition, we found that affinity for nucleolin binding was largely diminished in protein phosphatase activity deficient PTENβ mutants (Y284L and C270S) (Fig. 5e, lane 3 and lane 5 versus lane 2 and lane 4), suggesting that PTENβ protein phosphatase activity is necessary for interaction with nucleolin. This further indicated PTENβ acts as a nucleolin phosphatase.

In order to clarify the relationship of PTENβ with nucleolin and evaluate PTENβ function in the nucleolus, somatic PTENβ knockout was established with CRISPR-Cas9. CRISPR-Cas9-mediated gene targeting eliminated PTENβ and PTENβ without affecting canonical PTEN expression, and we also designed a specific target sequence for sole knockout of PTENβ as a control.

Figure 2 | Identification and validation of PTENβ. (a) The potential non-AUG initiation codons in favourable Kozak contexts in the 5′ UTR region of Homo sapiens PTEN mRNA are highlighted in red. The initiation codons of PTENα and PTEN are separately highlighted in yellow or green. (b) A pEGFP-N1 plasmid containing PTENα with a C-terminal GFP tag was used for detection of PTENα, PTEN and unknown PTEN isoforms (upper panel); the indicated plasmids were introduced into HEK 293T cells followed by western blotting analysis using GFP antibody. β-tubulin was used as a control (lower panel). (c) pEGFP-N1 plasmids containing PTEN or PTENα with a C-terminal GFP tag, in which one of the two potential initiation codons (AUU594 or UUG621) was mutated to CUC combined with mutation of CUG13, (d) Mutation of AUU594 but not UUG621 eliminates PTENβ expression. Plasmids as indicated in c were introduced into HEK 293T cells separately, followed by immunoblotting with GFP antibody. (e) The Kozak context of AUU594 and disruption of the Kozak context by site directed mutagenesis. (f) The Kozak context disruption abolishes PTENβ expression. HEK 293T cells were transfected with indicated constructs in e, followed by immunoblotting with GFP antibody. (g) A 12 bp AUU594 downstream palindromic motif in the 5′ UTR of PTEN is evolutionarily conserved. Phylogenetic analysis of the 5′ UTR of PTEN mRNA in bonobo (Pan paniscus), killer whale (Orcaena orca) and mouse (Mus musculus). The alternative CUG13 and AUU594 codons are highlighted in blue boxes, and the 12 bp palindromic sequence is highlighted in a red box. (h) Disruption of the palindromic motif abolishes PTENβ expression. Upper panel, site directed mutagenesis was used to disrupt the AUU594 downstream palindromic motif; lower panel, C-terminal GFP-tagged PTENβ expression plasmids with or without disruption of the palindromic motif were introduced into HEK 293T cells, followed by immunoblotting with GFP antibody.
Figure 3 | The PTEN\(\beta\) translation initiation codon was identified by LC-MS/MS. (a) A pFastBac1 plasmid containing PTEN\(\alpha\) with a C-terminal His-tag was used for in vitro purification of PTEN\(\beta\). The PTEN AUG start codon was mutated to AUA and the PTEN\(\alpha\) CUG start codon was mutated to CUC to avoid co-purification of PTEN and PTEN\(\alpha\) with PTEN\(\beta\). (b) Mass spectrometry analysis of purified PTEN\(\beta\). Sf9-expressed PTEN\(\beta\) with a C-terminal His-tag was purified using nickel affinity chromatography. The bound proteins were separated with SDS–PAGE, and gel slices at around 70 kDa (red box) were analysed by mass spectrometry. Three segments of peptide that match the 5′ UTR region of PTEN were identified, including the most proximal N-terminal peptide of PTEN\(\beta\) MSRAGNAGE. (c) The MS/MS spectrum of the most proximal N-terminal peptide of PTEN\(\beta\) MSRAGNAGE. (d) Verification of endogenous PTEN\(\beta\) expression in Pten\(-\)FLAG knockin mice. Various tissues from Pten\(-\)FLAG knockin mice were lysed for immunoprecipitation with FLAG antibody, followed by immunoblotting with PTEN\(\alpha\) antibody raised in our laboratory. Wild-type mice tissues were used as control. (e) Brain tissues from Pten\(-\)FLAG knockin mice or control wild-type mice were lysed for immunoprecipitation with anti-FLAG M2 agarose. The bound proteins were separated with SDS–PAGE. Mass spectrometry analysis of gel slices at around 70 kDa (molecular weight lower than PTEN\(\alpha\)) revealed four peptides in Pten\(-\)FLAG knockin tissues that matched the PTEN\(\beta\) N-terminal extended region.
(Fig. 5f,g). We found that PTENα and PTENβ double knockout led to elevation of nucleolin phosphorylation levels at Thr84, which is one of the XTPXKKXX motifs located in the nucleolin N-terminal region important for nucleolin function (Fig. 5h). Moreover, exogenous expression of wild-type PTENβ but not protein phosphatase activity abolished PTENβ efficiently.

(a) 

(b) 

(c) 

(d) 

(e)
downregulated nucleolin phosphorylation levels at Thr84 (Fig. 5i). These results raised the possibility that PTENβ acts as a nucleolin phosphatase. In vitro dephosphorylation assays further verified Cdk1/CyclinB1-phosphorylated nucleolin Thr84 is a target of PTENβ for dephosphorylation (Fig. 5j, lane 5 versus lane 4). In addition, we found that the poly-arginine sequence deleted mutant that eliminated PTENβ nucleolar localization also effectively abolishes its phosphatase activity on nucleolin. This indicates that PTENβ nucleolin phosphatase activity is linked to its localization in the nucleolus (Supplementary Fig. 7). These results argue that PTENβ acts as a nucleolin phosphatase, and show that the PTENβ–nucleolin interaction results in dephosphorylation modification of nucleolin, which influences its function.

PTENβ inhibits rDNA transcription and cell proliferation. Nucleolin is involved in almost every step of ribosome biogenesis33–35, and a recent study showed the most prominent effect of nucleolin is regulation of rDNA transcription by promotion of the rDNA transcription complex assembly, in which the phosphorylation status of nucleolin is rigorously regulated36,37. The fact that PTENβ interacts with nucleolin and dephosphorylates nucleolin at Thr84 raised a question as to whether nucleolin mediates PTENβ regulation of rDNA transcription, and thus represents a direct mechanism by which PTENβ controls ribosomal biogenesis.

To determine the influence of PTENβ on rRNA synthesis, total rRNA levels were evaluated by gel electrophoresis. Knockout of PTENz and PTENβ without affecting PTEN expression led to elevation of 28S and 18S total rRNA levels, whereas knockout of PTENz alone had no such effect (Fig. 6a, lane 3 versus lane 2 and lane 1). Moreover, exogenous expression of PTENβ but not PTENz in PTENz and PTENβ null cells efficiently downregulated 28S and 18S rRNA levels (Fig. 6b, lane 3 versus lane 2 and Supplementary Fig. 8a). These results argue that PTENβ plays a role in regulation of RNA, and in particular is involved in regulation of pre-rRNA synthesis from the outset of ribosome biogenesis. Transcription levels of rRNA genes were therefore evaluated by analysing pre-rRNA with qPCR in PTENβ wild-type and knockout cells. As expected, 45S pre-rRNA was increased in PTENβ and PTENz double knockout cells as compared with PTENz only knockout or wild-type cells (Fig. 6c). To determine whether rDNA transcription is regulated by PTENβ, a pHrD-IRES-Luc or pGL3-basic control vector was transfected into PTENβ and PTENz double knockout cells, and the luciferase reporter activity of the rDNA promoter was measured. Introduction of PTENβ, but not PTENz, efficiently downregulated luciferase activity (Fig. 6d and Supplementary Fig. 8b). These results indicate that PTENβ negatively regulates rDNA transcription, which may act at least in part through PTENβ dephosphorylation of nucleolin.

Nucleolar morphology is closely related to ribosome biogenesis, and enlarged nucleoli are typically found in association with activation of ribosome biogenesis. To evaluate alterations in nucleolar morphology, silver staining was used for the identification of argyrophilic nucleolar organizer regions (AgNOR). PTENβ and PTENz double knockout resulted in greater average areas of AgNOR staining, while PTENz only knockout did not have this effect (Fig. 6e). This suggested that elimination of PTENβ leads to upregulation of ribosomal biogenesis. Stimulation of cell proliferation is a characteristic biologic feature of nucleolin, and is typically associated with elevated ribosomal biogenesis. We therefore investigated biologic changes resulting from regulation of ribosomal biogenesis by PTENβ. Growth curves showed significant increases in cell growth rate resulting from PTENβ knockout (Fig. 6f). This activation of cell proliferation resulting from PTENβ deprivation was further confirmed by S phase analysis (Fig. 6g). These results argue that PTENβ controls cellular proliferation and may act through a mechanism involving negative regulation of pre-rRNA synthesis.

Discussion

In eukaryotic cells, the ribosome typically initiates protein synthesis at the AUG codon closest to the 5′ end of the mRNA38. However, there is now evidence including observations from our previous study25 that demonstrates initiation of protein translation can occur at most codons which differ from AUG by no more than a single nucleotide (non-AUG initiation)20. More than 50 instances of non-AUG-initiated N-terminal extensions have been predicted or verified experimentally in mammals39,40. In this study, we report identification of a translational variant of PTEN, designated PTENβ. This protein is synthesized from an alternative translation start site 438 bp upstream of the AUG initiation sequence, which adds 146 N-terminal amino acids to the canonical PTEN ORF. We found that PTENβ specifically localizes in the nucleolus and inhibits pre-rRNA synthesis, and our experimental evidence strongly suggests this inhibition acts through the mechanism of nucleolin dephosphorylation.

In previous studies both our group and Hopkins et al.41 independently identified a translational variant of PTEN with N-terminal extension, designated ‘PTENz’ in our study, and ‘PTEN-long’ by Hopkins et al.41. These have proven to be the same isoform that is translated from a CUG codon 519 bp upstream of the AUG initiation sequence of canonical PTEN (refs 24,25). In addition to the initiation sites for PTENz and PTENβ (CUG213, AUU594), we found another two alternative non-AUG codons in favourable Kozak contexts, which may be sites for initiation of other as yet unidentified isoforms. As multiple additional forms of PTEN may exist, we consider it to be prudent to designate PTEN isoforms as a sequential series, using α, β, γ, δ as labels.

In eukaryotes, the efficiency of initiation depends on the Kozak context41 which encompasses the AUG codon. Our data show that initiation of PTENβ relies on the Kozak context surrounding AUU594, which suggests this context is also essential for efficient initiation of other non-AUG codons. We identified a 12 bp perfect palindromic sequence starting 18 bp downstream of AUU594, and showed that disruption of this conserved
palindrome sharply downregulates expression of PTENβ. The crucial role this downstream palindromic sequence plays in AUU-mediated initiation, together with discovery of the fact in our previous study that the CUG centred palindromic sequence may be a signature motif for alternative Leu-tRNA initiation\textsuperscript{25}, underscores the importance of secondary structure for non-AUG initiation. This is consistent with the concept that a strong secondary structure downstream of the non-AUG codon may significantly increase initiation efficiency as reported by Kozak\textsuperscript{42}.
We previously showed that PTEN\(\text{a}\) localizes in mitochondria and functions in mitochondrial metabolism\(\text{25}\). Hopkins \textit{et al.} reported that PTEN-long (designated PTEN-L) is secreted into adjacent cells, which relies on an N-terminal secretion sequence\(\text{24}\). These findings indicate that the N-terminal extended PTEN\(\text{a}\) isoform is endowed with capability for subcellular localization differing from canonical PTEN. Data in the current study show that there is partial co-localization of PTEN and PTEN\(\text{a}\) in the cytoplasm, and this is consistent with findings in our previous study that PTEN interacts and co-localizes with PTEN\(\text{a}\) in mitochondria\(\text{25}\). However, PTEN isoforms localize for the most part in a mutually exclusive manner (Supplementary Fig. 9). PTEN\(\text{a}\) specifically localizes in the nucleolus, whereas PTEN and PTEN\(\text{a}\) do not, and an evolutionarily conserved poly-arginine sequence has proven to be an NoLS of PTEN\(\text{a}\). The PTEN\(\text{b}\) N-terminus is 27 amino acids shorter than PTEN\(\text{a}\), and PTEN\(\text{b}\) therefore does not contain the N-terminal secretion signal sequence, which may in part account for the differences in cellular distribution of PTEN\(\text{a}\) and PTEN\(\text{b}\). Moreover, protein conformation may be altered by differences in the length of the N-terminal domain, leading to differing affinities for interacting proteins or nucleotide sequences\(\text{27,43}\).

This may in some measure explain the diversity of PTEN isoform distribution.

Our data demonstrate that PTEN\(\text{b}\) is involved in regulation of pre-rRNA synthesis, and disruption of PTEN\(\text{b}\) results in elevated ribosomal biogenesis and cellular proliferation. Our findings suggest that this PTEN\(\text{b}\) regulatory mechanism may act through dephosphorylation of nucleolar protein nucleolin. It has been reported that PTEN acts on ribosomal biogenesis through the P3K-mTOR pathway by downregulation of 45S pre-rRNA and that PTEN localizes to the nucleolus to regulate nucleolar homeostasis\(\text{44-46}\). However, our results indicate there is extensive distribution of PTEN\(\text{b}\) in the nucleolus while little nucleolar PTEN can be detected. The PTEN\(\text{b}\) coding sequence overlaps the sequence of full-length canonical PTEN and PTEN\(\text{b}\) can be efficiently recognized by PTEN antibodies. It is therefore possible that the nucleolar immunofluorescence signals detected by the PTEN monoclonal antibody in this study are derived from recognition of this previously unidentified PTEN\(\text{b}\) isoform instead of from canonical PTEN. Furthermore, it must be noted that most of the observations from the studies cited above were made in PTEN knockout cells or with PTEN\(\text{a}\) deficient mice. As PTEN and its isoforms PTEN\(\text{a}\) and PTEN\(\text{b}\) are coded by the same gene, current PTEN knockout mouse models are essentially equivalent to at least triple deletion of PTEN and its isoforms. The phenotypic deficiencies of PTEN knockout models may thus be partially attributed to loss of PTEN or one of its isoforms. By use of specific disruption of PTEN\(\text{b}\), our study reveals that PTEN\(\text{b}\) plays an essential role in rRNA transcription and regulation of cellular proliferation.

Several recent studies have described a diversity of nucleolar functions in addition to ribosome biogenesis, such as modification of small RNAs, regulation of the cell cycle and control of aging\(\text{47,48}\), and nucleolar perturbations have been observed in various diseases ranging from auto-immunity to cancer\(\text{49,50}\). Our results show that in addition to nucleolin, PTEN\(\text{b}\) may physically interact with a number of other nucleolar proteins (Supplementary Fig. 4b), which raises the possibility that it participates in nucleolar functions apart from ribosome biogenesis.

In this study, we identify PTEN\(\text{b}\) as a new isoform of PTEN, which negatively regulates rRNA transcription and cellular proliferation. Based on previous studies that show imbalance of rRNA transcription closely correlates with tumour progression\(\text{51,52}\), identification of PTEN\(\text{b}\) may provide a new track for therapeutic drug design. These results together with our previous observations regarding PTEN\(\text{b}\) illustrate the complexity of PTEN function and enrich our understanding of the PTEN family.

**Methods**

**Cell lines.** All human cell lines used in this study (HepG2, H1299, HCT116, PC3, 786-O, Hela, U2OS, HEK 293T, SMCC-7721, DU145 and LoVo) were from the American Type Culture Collection. These cell lines were authenticated by STR analysis and were tested for mycoplasma contamination. PC3 and 786-O cells were maintained in RPMI 1640 (CORNING), and all other cells were maintained in DMEM (CORNING), supplemented with 10% FBS (Hyclone) in a 37°C incubator with 5% (v/v) CO\(_2\). The insect cell line S9 was obtained from Invitrogen and cultured in Grace’s insect medium (Gibco).

**Antibodies and reagents.** Primary antibodies used for western blot, immunoprecipitation and immunofluorescence are listed in Supplementary Table 1. The anti-PTEN\(\text{b}\) (ref. 25) and anti-nucleolin antibodies were raised in our laboratory (Supplementary Fig. 10). The reagent Acriflavins was from Sigma, A8126.

**Plasmids and cloning strategies.** The plasmids pcMV-tag-2h-pS\(_{\text{a}}\), pEGFP-N1, pDsRed2-N1 and pFastBac1 were purchased from Addgene. pX330 was purchased from BEIJING IDMO CO., LTD. pcDNA3.1-Cas9 was a gift from Dr Jianzhong Xi in the College of Engineering, Peking University. PTEN, PTEN\(\text{a}\), PTEN\(\text{b}\), or nucleolin or truncations and mutations of these molecules were inserted into these plasmids. pBcDT-IRE-S-Luc was a gift from Dr Xiaojian Du in the Department of Cell Biology, Peking University Health Science Center\(\text{53}\).

**Figure 5 | PTEN\(\text{b}\) interacts with and dephosphorylates nucleolin.** (a) In vivo S-tag pull-down analysis. Whole cell extracts from HEK 293T cells transfected with S-tagged PTEN, S-tagged PTEN\(\text{a}\), S-tagged PTEN\(\text{b}\) or mock constructs were immunoprecipitated with S-protein beads followed by mass spectrometric peptide sequencing. Nucleolin was found in the PTEN\(\text{b}\) pull-down list. (b,c) Reciprocal immunoprecipitation of PTEN\(\text{b}\) and nucleolin. (b) C-terminal GFP-tagged PTEN\(\text{b}\) or GFP-tagged mock was transfect, followed by immunoprecipitation with GFP antibody and immunoblotting with an anti-nucleolin antibody. (c) FLAG-tagged nucleolin or mock plasmid was co-transfected with GFP-tagged PTEN\(\text{b}\), followed by immunoblotting with FLAG antibody and immunoblotting with GFP antibody. (d) Images of immunofluorescence staining for PTEN\(\text{b}\) and nucleolin. Hela PTEN null cells were transfected as in Fig. 4b, followed by staining with an anti-nucleolin antibody, and were imaged by confocal microscopy. The scale bars represent 5 \(\mu\)m. (e) PTEN\(\text{b}\) protein phosphatase activity is required for its interaction with nucleolin. Wild-type PTEN\(\text{b}\) or PTEN\(\text{b}\) mutants as indicated in Supplementary Fig. 6b was transfected separately, followed by immunoprecipitation with GFP antibody and immunoblotting with an anti-nucleolin antibody. (f) Schematic strategy of PTEN\(\text{b}\) CRISPR-Cas9 knockout. The initiation codons of PTEN isoforms were labelled. (g) Immunoblot confirming abolition of PTEN\(\text{b}\) only (lane 2), or PTEN\(\text{a}\) and PTEN\(\text{b}\) double knockout (lane 3) with a PTEN antibody. (h) Phospho-nucleolin level in PTEN\(\text{b}\) knockout cells. PTEN\(\text{b}\) knockout cells as indicated in (g) were lysed, followed by immunoblotting with p-nucleolin (Thr 84), nucleolin or \(\beta\)-actin antibody. Experiments were carried out in three biological replicates and quantification of indicated protein levels relative to \(\beta\)-actin is shown. Bars represent mean \pm s.e.m. (i) C-terminal GFP-tagged wild-type PTEN\(\text{b}\) or PTEN\(\text{b}\) mutants as indicated in (e) were introduced into PTEN\(\text{b}\) knockout cells as indicated in (g), followed by immunoblotting with p-nucleolin (Thr 84), nucleolin or GFP antibody. (j) Purified nucleolin was phosphorylated in vitro by Cdk1/CyclinB1 and subsequently used in a phosphatase assay with FLAG-PTEN\(\text{b}\) or FLAG peptide alone as a control, followed by immunoblotting with p-nucleolin (Thr 84).
Figure 6 | PTENβ negatively regulates rDNA transcription and cell proliferation. (a,b) 28S and 18S rRNA levels in PTENβ knockout cells. (a) Total RNA was extracted from wild-type Hela cells and knockout cells as indicated in Fig. 5g, followed by resolution on a 1% formaldehyde-agarose gel for comparison. (b) Exogenous PTENα or PTENβ was transfected into PTENα and PTENβ double knockout cells as indicated in Fig. 5g, followed by resolution on a 1% formaldehyde-agarose gel for comparison. Grey scale quantification results of total 28S rRNA and 18S rRNA levels are shown. (c) 45S pre-rRNA level in PTENβ knockout cells as indicated in Fig. 5g. (d) rDNA promoter luciferase reporter activity in HeLa cells. PTENα and PTENβ double knockout cells as indicated in Fig. 5g were co-transfected with pHD-IRES-Luc or pGL3-basic plasmid and exogenous PTENα or PTENβ. Luciferase activity was measured 24 h after transfection. (e) Average area of nucleolar organizing regions in cells as indicated in Fig. 5g. Left panel, images of silver staining. Right panel, quantitative analysis of AgNOR indices. AgNOR from 50 cells were measured in each group. The scale bars represent 10 μm. (f) Cell growth curves in cells as indicated in Fig. 5g. One thousand cells were plated per well in 96-well plates in triplicate. Quantification was performed every 24 h for 5 days following the instructions for the Cell Titer 96 AQueous One Solution Cell Proliferation Assay Kit. The significance of the difference in PTENα and PTENβ double knockout and wild-type Hela cells is shown with red asterisks, and the significance of the difference in PTENα only knockout and wild-type cells is shown with green asterisks. (g) Detection of S-phase cells using the APC BrdU Flow Kit. Wild-type, PTENα and PTENβ double knockout or PTENα knockout only cells were treated with 1 mM BrdU, followed by staining with fluorochrome-conjugated anti-BrdU antibody and evaluation with flow cytometry. Data are presented as mean ± s.e.m. of three independent experiments and were analysed with the paired t-test. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
**Mass spectrometry.** For *in vitro* PTENβ identification experiments, the PTENβ coding sequence (CUG513-UGA2240, PTENβ initiation codon CUG2240 mutated to CUC and canonical PTEN initiation codon AGU3224 to AUA) was subcloned with a C-terminal His-tag into a pFastBac1 vector for expression in *S.9* insect cells. His affinity chromatography was used for purification of PTENβ-His before SDS–PAGE resolution of a 70-kDa band. Immunoprecipitated proteins were digested in gel by endoproteinase Glu-C (Promega) following the manufacturer's instructions. Peptides were separated by online reversed-phase nanoscale capillary liquid chromatography (Easy-nLC 1,000, Thermo Scientific). The data dependent mass spectra were acquired with the LTQ-Orbitrap Elite mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray ion source (Thermo Scientific). Raw files were searched by Proteome Discoverer (Version 1.4.1.14, Thermo Scientific) against the UniProt Mouse database supplemented with the N-terminal extended PTEN sequence (Supplementary Fig. 11a). Search parameters were set as follows: enzyme with Glu-C; up to two missed cleavages; carbamidomethyl cysteine as fixed modification; methionine oxidation and peptide N-terminal acetylation as variable modifications. The false discovery rates were set at 0.01 (ref. 25). For endogenous PTENβ identification experiments, a FLAG-purified 70-kDa band (molecular weight lower than PTENs) from PtetFLAG44 tissues was treated in the same way as described above. Raw files were searched with Proteome Discoverer (Version 1.4.1.14, Thermo Scientific) against the UniProt Mouse database supplemented with the N-terminal extended PTEN sequence (Supplementary Fig. 11b). For peptide analysis of pull down experiments, proteins were digested by endoproteinase Trypsin (Promega) in gel following the manufacturer's instructions. The following process was the same as described above. Raw files were searched by Proteome Discoverer (Version 1.4.1.14, Thermo Scientific) against the UniProt Human database. Search parameters were set as set by Proteome Trypsin; up to two missed cleavages; carbamidomethyl cysteine as fixed modification; methionine oxidation as variable modifications. The false discovery rates were set at 0.01.

**CRISPR-CAS9-mediated somatic PTENβ knockout.** Sequences (ACCTCCGCGCTCTGAGGAGG or AGTCGCCTGTCACCATTTCC) of the PTENb gene in exon1 were targeted separately. Oligos were purchased from Tsingke and ligated into the U6-sgRNA plasmid. Hela cells were seeded onto 24-well plates containing 500 μM DMEM medium at a density of 1,000,000 cells per ml of cell suspension. 200 ng of U6-sgRNA plasmid and 200 ng of Cas9 plasmid in total were transfected into cells using PEI according to the manufacturer's instructions. Medium was replaced at around 12 h post transfection. Cells were maintained for another 72 h to allow sufficient time for genomic engineering mediated by the CRISPR-Cas9 system. Transfected cells were then treated with 500 μg/ml of G418 for 3 days. After G418 selection, cell clones were selected and amplified for mutation sequencing.

**Co-immunoprecipitation.** Immunoprecipitation was performed as previously described55. In brief, cells were extracted and lysed in lysis buffer (50 mM Tris-pH 7.5, 150 mM NaCl, 0.5% NP40) with sodium dodecyl sulphate (SDS) protein sample buffer (1:1, v/v) under reducing conditions and boiled for 10 min. After centrifugation of the cell supernatant at 14,000 g for 10 min, the supernatant was added to fresh lysis buffer with protease and phosphatase inhibitors. Cell lysate (500 μg) was incubated with antibodies against GFP-tag or FLAG-tag for 8 h, followed by incubation with protein A/g agarose for 1 h. The protein-bead complex mixture was washed in washing buffer containing 0.1% NP40 and subjected to western blot to evaluate protein interaction.

**Immunofluorescence and confocal microscopy.** HeLa cells were seeded on cover glass in DMEM medium followed by transfection with C-terminal GFP-tagged PTEN, PTEN2 or PTENβ. After transfection for 24 h, cells were fixed with 4% PFA, permeabilized with 0.2% Triton X-100 and blocked for at least 30 min using 4% BSA dissolved in PBS + 0.1% Tween-20 (PBS-T). Primary antibody (diluted in blocking solution) was then applied for 1 h, followed by five washes in PBS-T. and fluorophore-conjugated secondary antibody was applied for 45 min. Coverslips were mounted and evaluated with fluorescence microscopy. A Nikon A1 microscope was used for confocal microscopy.

**Cell fractionation.** Nucleoli were isolated in Hela cells as previously described33. Isolated nucleoli were lysed with RIPA buffer. Equal amounts of whole cell lysate, cytosolic fraction, nuclear plasma fraction and nucleolar fraction were evaluated with western blot analysis. Briefly, Hela cells (one 15-cm dish) were scraped into ice-cold PBS and washed three times with ice-cold PBS. Cell pellets were then re-suspended in hypotonic buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTI), allowed to swell and subjected to Dounce homogenization. The crude nuclear fraction was recovered by centrifugation at 218g for 5 min, and the cytoplasmic supernatant was retained. Nuclei were re-suspended in 0.35 M sucrose plus 0.5 mM magnesium acetate and then subjected to brief sonication to release the nucleoli. Pellets were recovered by two cycles of centrifugation through 88% and 95% Percoll, followed by one cycle of centrifugation through the supernatant from the first sucrose gradient was recovered as a crude nucleoplasmic fraction. The pellets consisted of nucleoli.

**S-tag pull down assay.** HEK 293T cells were transfected with pSA, pSA-PTEN, pSA-PTENβ, pSA-PTENb or pSA-NCL plasmids and harvested 36 h after transfection. Cells were lysed with lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40 and 1 mM NaF). Equal amounts of protein were incubated with S protein agarose (Novagen) for 4 h. The protein-bead complexes were washed four times with washing buffer containing 0.1% NP40. After boiling at 100 °C in loading buffer, proteins were loaded onto NuPAGE 4–12% gels (Invitrogen) and visualized with silver staining (Pierce Silver Stain Kit) or were subjected to western blot. The potential interacting proteins in specific bands were evaluated with mass spectrum analysis.

**In vitro kinase and phosphatase assay.** His-nucleolin was expressed in S9 cells and purified using Ni-NTA agarose (Qagen). Control FLAG peptides, FLG-CDK1 and FLAG-PTENb were purified with ANTI-FLAG M2 Affinity Gel (F2426) and 3 × FLAG Peptide (F4799) from HEK 293T cells after transfection with pCMV vector, pCMV-CDK1 or pCMV-PTENb for 30 h. For kinase assay, purified His-nucleolin on Ni-NTA agarose was incubated with CDK1 in kinase buffer (50 mM Tris-HCl, 1 mM MgCl2, 2 mM DTT, 1 mM EGTA and 0.2 mM ATP) for 30 min at 30 °C. For the following phosphatase assay, phosphorylated His-nucleolin was incubated with control FLAG peptide or purified FLAG-PTENb in dephosphorylation buffer (20 mM HEPES, 1 mM MgCl2, 1 mM EDTA, 1 mM DTT and 0.1 mg/ml 1/1 BSA) for 1 h at 37 °C. The phosphorylation levels of nucleolin sites with potential for alteration were evaluated with western blot.

**Functional pathway enrichment analysis and visualization.** Gene Ontology (GO) and KEGG enrichment analysis were performed using ClueGo (ref. 55), which is a Cytoscape plug-in. During analysis, over-represented terms in KEGG and the GO Biological Process and Cellular Component were enriched, and the GO level was set at three to five. The GO term fusion option was selected to reduce redundancy. In order to account for multiple testing, P values were corrected with Benjamini-Hochberg correction. Only terms of P < 0.05 were displayed. The selection criteria for the terms that have associated genes from the uploaded pull down list was set as a minimum of 50 genes per term, and a minimum of 4% of total genes associated with the term for the analysis of the PTENb pull down network. The Kappa score was set at 0.4.

**Luciferase reporter assay and real-time PCR.** For luciferase reporter assays, after transfection of various combinations of control, reporter or protein expression vectors for 24 h, cells were lysed with a passive lysis buffer (Promega). Luciferase activity was measured with the Dual Luciferase Assay System (Promega) following the manufacturer’s protocol. For quantification of 45S pre-rRNA, total RNA was extracted from cells followed by reverse transcription and evaluated with qPCR. The qPCR primers used are listed in Supplementary Table 4.

**Silver staining assay.** Silver staining assays were performed as previously described56. Briefly, cells were seeded on glass coverslips overnight and were then fixed in 2% glutaraldehyde, followed by postfixation in a 3:1 ethanol–acetic acid solution. Cells were stained with a 0.33% formic acid–33.3% silver nitrate solution in 0.66% gelatin and mounted on slides with VectaMount (Vector Labs). Nucleolar area was measured in cells with cellSens software provided by Olympus.

**BrdU incorporation and cell cycle analysis.** For BrdU incorporation, cells were stained using the APC Brdu Flow Kit (BD Bioscience) according to the manufacturer's instructions and subjected to analysis using FACSVerse (BD Biosciences). Experiments were each performed three times, and at least 10 000 cells were analysed per sample.

**Statistical analysis.** Prism GraphPad software v5.0 was used for analysis. The statistical significance of differences between different groups was calculated with the two-tailed paired t-test. P values of 0.05 or less were considered significant.

**Data availability.** Nucleotide sequence data reported in this work are available in the Genbank databases under the accession numbers KX399836 (*Homo sapiens*) and KX421108 (*Mus musculus*). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://protemecentral.proteomeexchange.org/cgi/GetDataset) via the PRIDE (ref. 57) partner repository with the data set identifier PXD003850. The original immunoblots, polycrylamide gels and agarose gels are provided as Supplementary Figs 12–15. Primers used in this study are listed in Supplementary Tables 2–4. All other data supporting the findings of this study are available within the article and its Supplementary Information or from the corresponding author on reasonable request.
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Author contributions
Y.Y. and H.L. conceived the study. H.L. and Q.Y. wrote the article. H.L., X.C. and Q.Y. designed and performed the experiments and analysed the data. D.R. performed several experiments. X.Z. performed mass spectrometric analysis. C.Z. performed functional pathway enrichment analysis. Y.Y. and M.A.M. revised the manuscript.

Additional information
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