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# PTEN $\beta$ 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 PTEN $\alpha$ , which functions in mitochondrial bioenergetics. Here we report the identification of another N-terminal extended PTEN isoform, designated PTEN $\beta$ . PTEN $\beta$  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. PTEN $\beta$  localizes predominantly in the nucleolus, and physically associates with and dephosphorylates nucleolin, which is a multifunctional nucleolar phosphoprotein. Disruption of PTEN $\beta$  alters rDNA transcription and promotes ribosomal biogenesis, and this effect can be reversed by re-introduction of PTEN $\beta$ . Our data show that PTEN $\beta$  regulates pre-rRNA synthesis and cellular proliferation. These results demonstrate the complexity of the PTEN protein family and the diversity of its functions.

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The *PTEN* gene was first identified and cloned in 1997, and PTEN protein has proved to be a powerful tumour suppressor<sup>1,2</sup>. It is one of the most frequently mutated or deleted genes in cancer, including cancer of the brain, breast, prostate, pancreas and ovary<sup>3</sup>. Germline mutations of the *PTEN* gene lead to inherited autosomal dominant hamartoma tumour syndromes, including Bannayan–Zonana syndrome<sup>4</sup> and Cowden disease<sup>5</sup>. Heterozygous *Pten* deletion leads to multiple tumours in mice, while the homozygote is embryonically lethal<sup>6,7</sup>. In the cytoplasm, PTEN antagonizes the PI3K-AKT-mTOR pathway by dephosphorylation of phosphoinositide-3,4,5-triphosphate (PIP3)<sup>8</sup>. 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<sup>9</sup>. In the nucleus, PTEN maintains genome integrity and regulates the process of DNA replication through regulation of RPA1, MCM2 and TOP2A (refs 10–13). In addition to its tumour suppressor function, PTEN is also involved in embryonic development, lipid metabolism<sup>14,15</sup>, Alzheimer's disease<sup>16</sup> and antiviral innate immunity<sup>17</sup>. However, the current concepts of PTEN function do not fully explain the multifarious activity of this protein.

In eukaryotes, the translation initiation codon is generally recognized and selected by the 5' cap structure dependent ribosomal scanning mechanism, as well as by 5' cap structure independent internal ribosome entry sites. This scanning complex rigorously controls the fidelity of initiation through recognition of the correct AUG triplet in the optimum context GCC(A/G)CCAUGG, referred to as the Kozak sequence, which includes a purine at the  $-3$  and a G at the  $+4$  position (relative to the A of the AUG codon, which is designated  $+1$ ) (refs 18,19).

Triplets that differ from AUG by only one nucleotide can also direct initiation of polypeptide chain synthesis in mammalian cells in an optimum context<sup>20</sup>. It is also reported that recognition of non-AUG codons is strongly stimulated by a downstream stem-loop (hairpin) structure that is separated from the preceding initiation codon by about 14 nucleotides<sup>21</sup>. Isoforms initiated from non-AUG codons are frequently endowed with functions additional to and differing from the canonical form of a given protein<sup>22,23</sup>.

Previously, we and others reported that a CUG codon in the 5' untranslated region (5' UTR) of PTEN mRNA initiates an N-terminal extended isoform of PTEN now known as PTEN $\alpha$  (refs 24,25). In the current study we describe another PTEN isoform designated PTEN $\beta$ , which comprises an extended N-terminal extension of 146 amino acids (*Homo sapiens*) as compared with canonical PTEN protein. Our data show PTEN $\beta$  localizes specifically in the nucleolus and negatively regulates rDNA transcription. Disruption of PTEN $\beta$  leads to an abnormal increase in pre-rRNA synthesis and results in promotion of cellular proliferation. These findings characterize an N-terminal extended PTEN isoform with distinct localization and function, which results from alternative AUU translation initiation. Identification of PTEN $\beta$  further reveals the diversity of PTEN protein family function, and the correspondence of protein function with isoform subtype.

## Results

**An unidentified protein is recognized by a PTEN $\alpha$  antibody.** The protein PTEN $\alpha$  functions in mitochondrial metabolism, and structurally distinct compounds such as acriflavin and aurin tricarboxylic acid (ATA) yield different results in regulation of

PTEN $\alpha$  synthesis initiated by a CUG start codon<sup>25</sup>. The CUG inhibitor acriflavin reduces PTEN $\alpha$  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 PTEN $\alpha$  (around 70 kDa), and expression of this protein showed dose dependent variation under acriflavin treatment parallel to that of PTEN $\alpha$  (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 $\alpha$  antibody raised in our laboratory by immunization with purified recombinant protein composed of the 173 N-terminal amino acids of PTEN $\alpha$  (ref. 25). This unidentified protein was recognized by this anti-PTEN $\alpha$  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 PTEN $\beta$ .

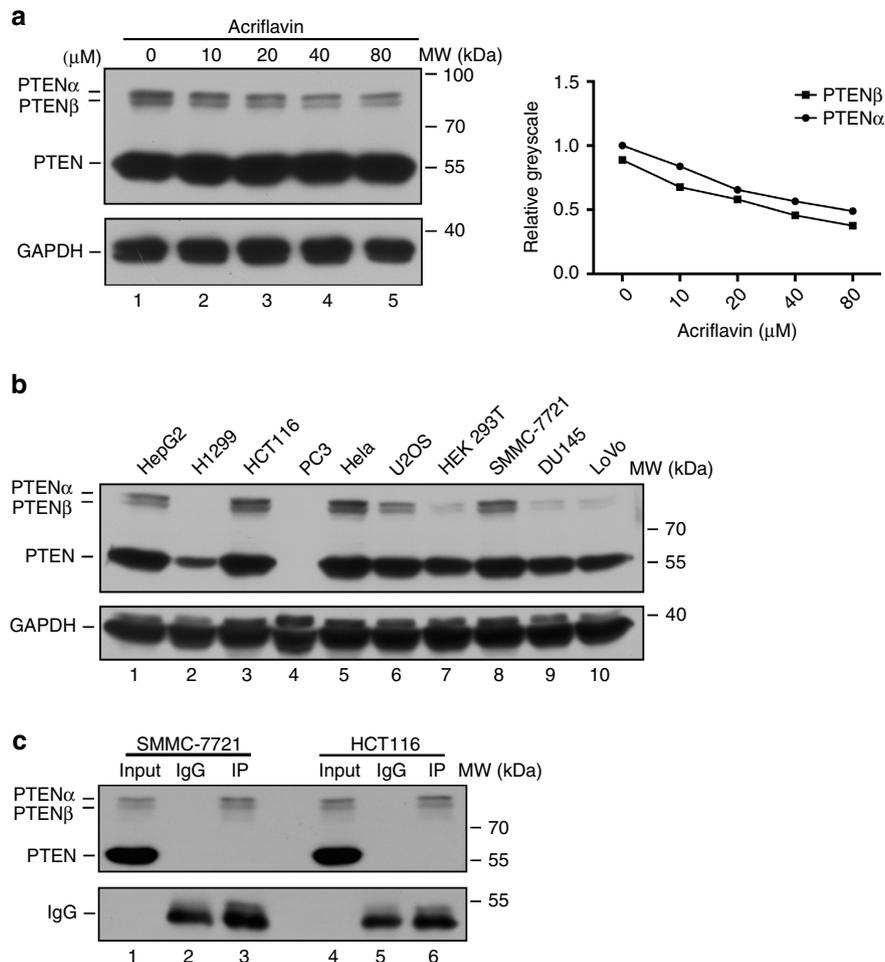
## Alternative initiation of PTEN $\beta$ translation from AUU<sup>594</sup>.

Evaluation of the 5' UTR of human PTEN mRNA for alternative translation start sites in frame with the AUG<sup>1032</sup> start codon revealed a total of four non-AUG alternative initiation codons in favourable Kozak contexts (Fig. 2a). Translation from AUU<sup>594</sup> or UUG<sup>621</sup> 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 PTEN $\beta$  band.

To determine whether one of these non-AUG codons initiates translation of this putative PTEN $\beta$  protein with upstream extension of the open reading frame (ORF), the coding sequence of PTEN $\alpha$  was cloned into a plasmid with a C-terminal GFP tag (Fig. 2b, upper panel). As expected, the CUG<sup>513</sup> initiated PTEN $\alpha$  ORF was translated into three distinct proteins with masses of 120<sup>+</sup> kDa (PTEN $\alpha$ -GFP), 120 kDa (PTEN $\beta$ -GFP) and 90 kDa (PTEN-GFP) (Fig. 2b, lower panel). This suggested that PTEN $\beta$  is translated from a new ORF in the 5' UTR of PTEN mRNA upstream of the canonical AUG start codon.

To identify PTEN $\beta$  translation initiation sites, a plasmid was constructed to express CUG<sup>513</sup>-initiated PTEN $\alpha$  with a C-terminal GFP tag, and mutations were created in this plasmid to determine which non-AUG codon is critical for PTEN $\beta$  expression (Fig. 2c). Mutation of CUG<sup>513</sup> into a non-initiating CUC triplet led only to disappearance of the most slowly migrating proteoform PTEN $\alpha$ , thus excluding the possibility that PTEN $\beta$  is a cleavage product (Fig. 2d, lane 4). On the basis of CUG<sup>513</sup> mutation, potential non-AUG codons (AUU<sup>594</sup>, UUG<sup>621</sup>) were mutated separately to a non-initiating CUC triplet. Anti-GFP immunoblots from these transfected lysates revealed that mutation of AUU<sup>594</sup> abolishes PTEN $\beta$ , whereas mutation of UUG<sup>621</sup> has no such effect (Fig. 2d, lane 6 versus lane 5). These results indicate that AUU<sup>594</sup> but not UUG<sup>621</sup> is essential for PTEN $\beta$  expression, and that AUU<sup>594</sup> is likely the translation initiation site for PTEN $\beta$ .

AUU<sup>594</sup> resides in a favourable Kozak context as noted (GUCACCAUU<sup>594</sup>) (Fig. 2a). In order to determine whether this context influences translation initiation at AUU<sup>594</sup>, we constructed mutants to disrupt it and examined PTEN $\beta$  expression (Fig. 2e). Disruption of this Kozak context markedly



**Figure 1 | Discovery of an unknown protein that is recognized by PTEN $\alpha$  antibody.** (a) Western blot of PTEN $\alpha$  and PTEN in HeLa cells after acriflavin treatment at different doses with a polyclonal antibody against full-length PTEN. PTEN immunoblotting revealed an unknown band with a molecular weight lower than PTEN $\alpha$ . Quantification of indicated protein levels relative to GAPDH is shown (right panel). (b) Western blot of PTEN $\alpha$  and PTEN in a panel of human cancer cell lines using a rabbit monoclonal antibody against the C-terminal region of PTEN (Cell Signaling, 138G6). The PTEN null prostate cancer cell line PC3 was used as a negative control. (c) Immunoprecipitation of PTEN $\alpha$  with a homemade PTEN $\alpha$  antibody before PTEN immunoblotting of SMMC-7721 and HCT116 cells of known PTEN status.

reduced PTEN $\beta$  expression (Fig. 2f), strongly suggesting that translation initiation of PTEN $\beta$  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 AUU<sup>594</sup>, 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 PTEN $\beta$  without affecting canonical PTEN expression (lower panel, lane 4 versus lane 3), indicating this sequence is critical for translation initiation of PTEN $\beta$ .

These data demonstrate that PTEN $\beta$  synthesis relies on an alternative translation initiation at the AUU<sup>594</sup> codon, and expression of PTEN $\beta$  is influenced both by the Kozak context and by the secondary structure downstream of AUU<sup>594</sup>.

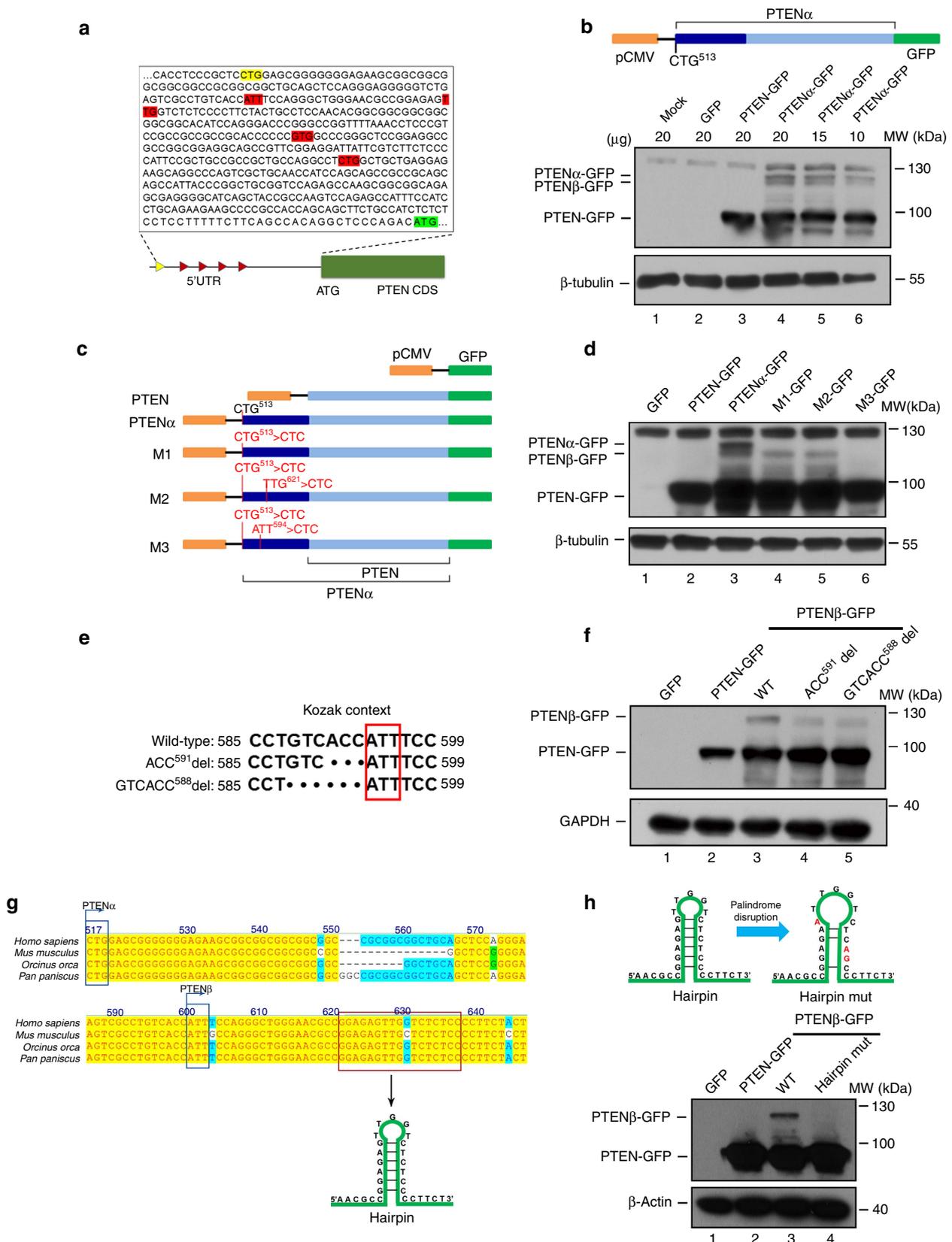
**Mass spectrometry analysis of the PTEN $\beta$  sequence.** Mass spectrometry was used for peptide sequencing to validate the PTEN $\beta$  translation initiation site. Purified human PTEN $\beta$  with a C-terminal His tag (Fig. 3a) was expressed in Sf9 insect cells for mass spectrometry (Fig. 3b). Mascot reports revealed three peptide fragments covering 39.04% of the N-terminal region of

PTEN $\beta$  (from AUU<sup>594</sup> to AUG<sup>1032</sup>) (Fig. 3b). LC-MS/MS captured MSRAGNAGE, which is the most proximal N-terminal peptide of PTEN $\beta$  (9 aa, MS/MS spectrum shown in Fig. 3c). Additional analysis showed that the first N-terminal amino acid of PTEN $\beta$  is acetylated. These mass spectrum data therefore verified AUU<sup>594</sup> is the PTEN $\beta$  translation initiation site.

The 5' UTR of *Homo sapiens* PTEN and *Mus musculus* Pten are highly homologous as shown in our recent study<sup>25</sup>, suggesting that PTEN $\beta$  expression in mice is conserved and has a similar alternative translation initiation mechanism. We previously generated a FLAG-knockin mouse model (*Pten*<sup>FLAG</sup>), in which the C-terminus of the *Pten* gene was targeted for insertion of a FLAG-coding sequence<sup>25</sup>. To verify PTEN $\beta$  resides at the *Pten* gene locus, tissue samples were extracted from heterozygous *Pten*<sup>FLAG</sup> mice and wild-type control mice for FLAG pull-down followed by immunoblotting with an anti-PTEN $\alpha$  antibody raised in our laboratory<sup>25</sup>. A protein band with a molecular weight lower than PTEN $\alpha$  which was similar to the PTEN $\beta$  band identified in cancer cell lines was detected in the FLAG elute from *Pten*<sup>FLAG</sup> tissues, while no band was detected in wild-type tissues (Fig. 3d). The existence of *in vivo* PTEN $\beta$  was further confirmed by mass spectrometric analysis of a FLAG-purified 70<sup>+</sup> kDa band (molecular weight

lower than PTEN $\alpha$ ) from *Pten*<sup>FLAG</sup> tissues. Mascot reports revealed four peptide fragments that cover 68.5% of the N-terminal region of PTEN $\beta$  (Fig. 3e). These results verified the existence of PTEN $\beta$  *in vivo*, and confirmed that PTEN $\beta$  and

PTEN $\alpha$  are translated *in vivo* from identical mRNA at the same gene locus. This *Pten*<sup>FLAG</sup> knockin animal model demonstrates the natural occurrence of alternative initiation that results in translation of PTEN $\beta$ .



**PTEN $\beta$  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 forms<sup>26,27</sup>. The length of the PTEN $\beta$  protein differs from PTEN $\alpha$  and PTEN due to its N-terminal extension, which raised the possibility that PTEN $\beta$  has unique subcellular localization and special function. Immunofluorescence examination of the subcellular localization of PTEN $\beta$  was compared with PTEN $\alpha$  and PTEN. Separate plasmids expressing C-terminal GFP-tagged PTEN, PTEN $\alpha$  or PTEN $\beta$  were constructed, and to ensure expression of only a single isoform in each instance, we created an ATG<sup>1032</sup>>ATA mutation in GFP-tagged PTEN $\alpha$  and PTEN $\beta$  (Fig. 4a). The subcellular distribution of PTEN $\beta$  was completely different from that of PTEN and PTEN $\alpha$  as shown in Fig. 4b. PTEN was distributed ubiquitously in both the nucleus and cytoplasm, while PTEN $\alpha$  predominately showed cytoplasmic localization. In contrast, strong PTEN $\beta$  fluorescence signals were distributed mainly in the relatively shallow DAPI staining areas of the nucleus which have been suggested to be nucleolar regions<sup>28</sup>, and little cytoplasmic PTEN $\beta$  localization was detected. In order to exclude the possibility protein localization was influenced by the GFP tag, PTEN, PTEN $\alpha$  and PTEN $\beta$  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 PTEN $\alpha$ , PTEN $\beta$  or PTEN within the nucleolus. There was prominent co-localization of PTEN $\beta$  with this marker in the nucleolus, but considerably less nucleolar co-localization was detected with PTEN or PTEN $\alpha$  (Fig. 4c). This extensive distribution of PTEN $\beta$  in the nucleolus was confirmed through cell fractionation (Fig. 4d). Moreover, we demonstrated that the N-terminal extended sequence was solely responsible for PTEN $\beta$  accumulation in the nucleolus, and this was also true for PTEN $\alpha$  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 residues<sup>29,30</sup>. We noted a series of poly-arginine residues in the N-terminal extended domain of PTEN $\beta$  (aa 20–25), which is evolutionarily conserved (Supplementary Fig. 3). To determine whether this sequence acts as an NoLS and directs PTEN $\beta$  nucleolar localization, we established a PTEN $\beta\Delta R^6$  construct in which these six arginines were deleted. This  $\Delta R^6$  mutation was remarkable in that it completely abolished PTEN $\beta$  nucleolar accumulation (Fig. 4e), indicating that this poly-arginine sequence is critical for PTEN $\beta$  nucleolar localization, and

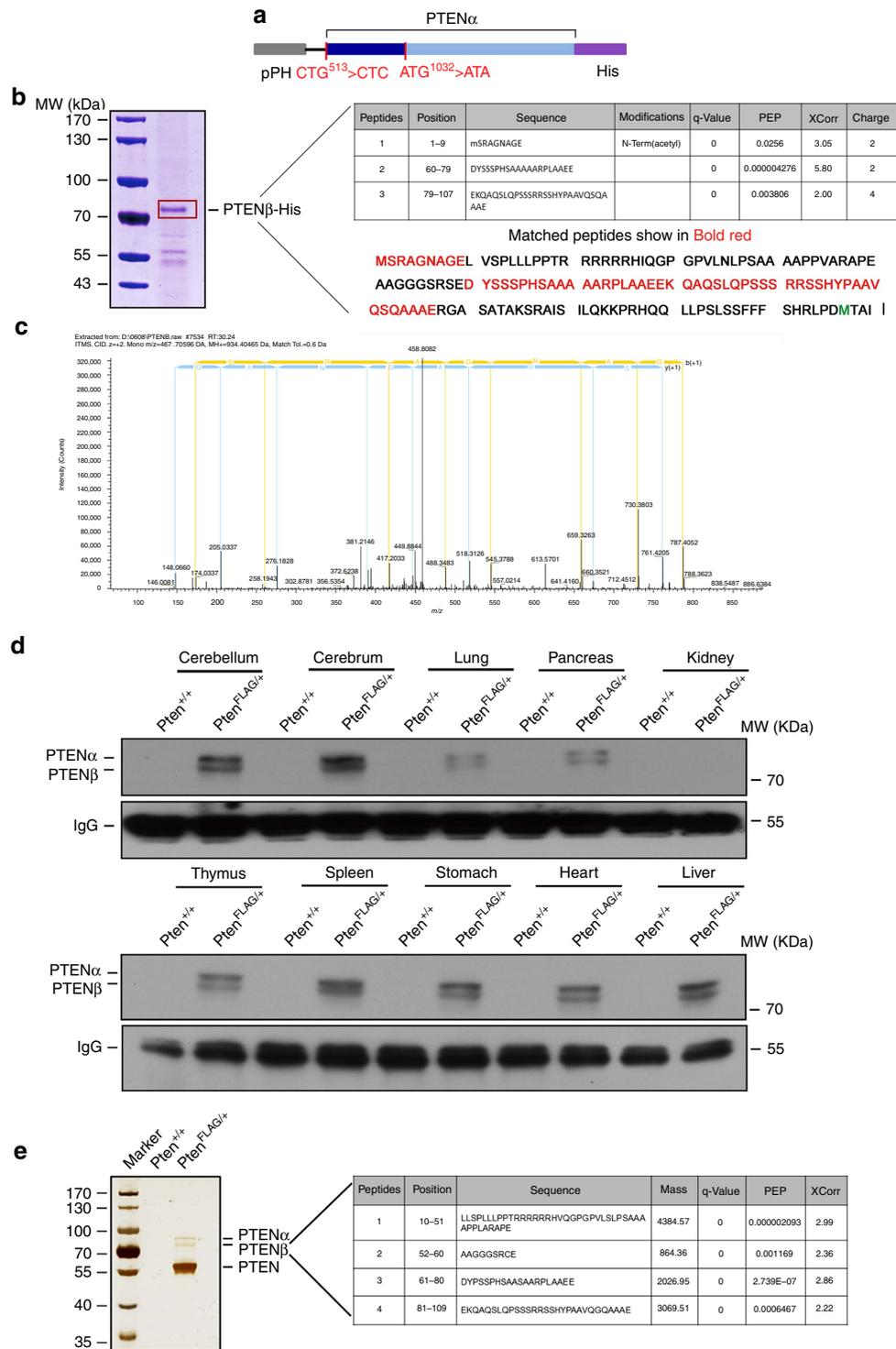
showing that it functions as an NoLS. These results indicated that the distinctive N-terminal extension sequence of PTEN $\beta$  confers unique cellular localization, and raised the possibility that PTEN $\beta$  is involved in nucleolar function.

**Nucleolin is a novel downstream target of PTEN $\beta$ .** In order to identify potential PTEN $\beta$  targets and explore PTEN $\beta$  function in the nucleolus, mass spectrometry was used to analyse the results of S-tag-PTEN $\beta$  pull down. Results with S-tag-PTEN and S-tag-PTEN $\alpha$  were analysed separately as controls. Mass spectrometry revealed that PTEN $\beta$  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 biogenesis<sup>31,32</sup>, and was thus considered to be a potential PTEN $\beta$  target (Fig. 5a). Interaction of PTEN $\beta$  and nucleolin was demonstrated by reciprocal immunoprecipitation (Fig. 5b,c). In addition, immunofluorescence showed extensive co-localization of PTEN $\beta$  with nucleolin in the nucleolus, whereas PTEN or PTEN $\alpha$  did not (Fig. 5d), further confirming the specificity of the interaction of PTEN $\beta$  and nucleolin. To identify the functional domains by which PTEN $\beta$  binds to nucleolin, two truncated forms of PTEN $\beta$  were constructed with C-terminal S-tags (Supplementary Fig. 5a). Immunoprecipitation demonstrated that the N-terminal extension domain of PTEN $\beta$  is essential for interaction with nucleolin (Supplementary Fig. 5b). These data show PTEN $\beta$  and nucleolin co-localize and interact each other.

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

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

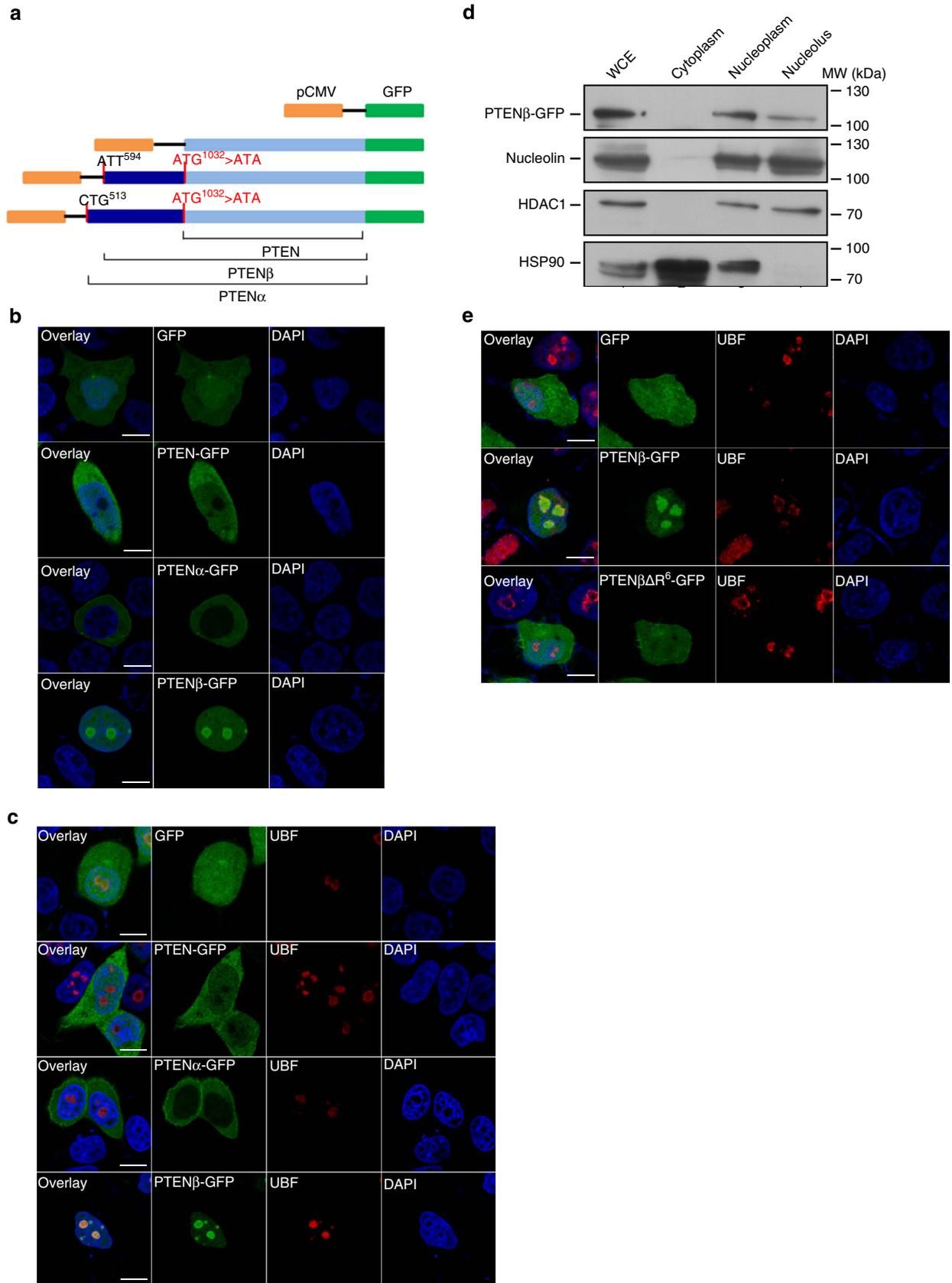
**Figure 2 | Identification and validation of PTEN $\beta$ .** (a) The potential non-AUG initiation codons in favourable Kozak contexts in the 5' UTR region of *Homo sapiens* PTEN mRNA are highlighted in red. The initiation codons of PTEN $\alpha$  and PTEN are separately highlighted in yellow or green. (b) A pEGFP-N1 plasmid containing PTEN $\alpha$  with a C-terminal GFP tag was used for detection of PTEN $\alpha$ , PTEN and unknown PTEN isoforms (upper panel); the indicated plasmids were introduced in HEK 293T cells followed by western blotting analysis using GFP antibody.  $\beta$ -tubulin was used as a control (lower panel). (c) pEGFP-N1 plasmids containing PTEN or PTEN $\alpha$  with a C-terminal GFP tag, in which one of the two potential initiation codons (AUU<sup>594</sup> or UUG<sup>621</sup>) was mutated to CUC combined with mutation of CUG<sup>513</sup>. (d) Mutation of AUU<sup>594</sup> but not UUG<sup>621</sup> eliminates PTEN $\beta$  expression. Plasmids as indicated in c were introduced into HEK 293T cells separately, followed by immunoblotting with GFP antibody. (e) The Kozak context of AUU<sup>594</sup> and disruption of the Kozak context by site directed mutagenesis. (f) The Kozak context disruption abolishes PTEN $\beta$  expression. HEK 293T cells were transfected with indicated constructs in e, followed by immunoblotting with GFP antibody. (g) A 12 bp AUU<sup>594</sup> 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 (*Orcinus orca*) and mouse (*Mus musculus*). The alternative CUG<sup>513</sup> and AUU<sup>594</sup> 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 PTEN $\beta$  expression. Upper panel, site directed mutagenesis was used to disrupt the AUU<sup>594</sup> downstream palindromic motif; lower panel, C-terminal GFP-tagged PTEN $\beta$  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 PTENβ translation initiation codon was identified by LC-MS/MS.** (a) A pFastBac1 plasmid containing PTENα with a C-terminal His-tag was used for *in vitro* purification of PTENβ. The PTEN AUG start codon was mutated to AUA and the PTENα CUG start codon was mutated to CUC to avoid co-purification of PTEN and PTENα with PTENβ. (b) Mass spectrometry analysis of purified PTENβ. Sf9-expressed PTENβ 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 PTENβ MSRAGNAGE. (c) The MS/MS spectrum of the most proximal N-terminal peptide of PTENβ MSRAGNAGE. (d) Verification of endogenous PTENβ expression in *Pten*-FLAG knockin mice. Various tissues from *Pten*-FLAG knockin mice were lysed for immunoprecipitation with FLAG antibody, followed by immunoblotting with PTENα 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 PTENα) revealed four peptides in *Pten*-FLAG knockin tissues that matched the PTENβ N-terminal extended region.

(Fig. 5f,g). We found that PTEN $\alpha$  and PTEN $\beta$  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 PTEN $\beta$  but not protein phosphatase activity abolished PTEN $\beta$  efficiently



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

### PTEN $\beta$ inhibits rDNA transcription and cell proliferation.

Nucleolin is involved in almost every step of ribosome biogenesis<sup>33–35</sup>, 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 regulated<sup>36,37</sup>. The fact that PTEN $\beta$  interacts with nucleolin and dephosphorylates nucleolin at Thr84 raised a question as to whether nucleolin mediates PTEN $\beta$  regulation of rDNA transcription, and thus represents a direct mechanism by which PTEN $\beta$  controls ribosomal biogenesis.

To determine the influence of PTEN $\beta$  on rRNA synthesis, total rRNA levels were evaluated by gel electrophoresis. Knockout of PTEN $\alpha$  and PTEN $\beta$  without affecting PTEN expression led to elevation of 28S and 18S total rRNA levels, whereas knockout of PTEN $\alpha$  alone had no such effect (Fig. 6a, lane 3 versus lane 2 and lane 1). Moreover, exogenous expression of PTEN $\beta$  but not PTEN $\alpha$  in PTEN $\alpha$  and PTEN $\beta$  null cells efficiently downregulated 28S and 18S rRNA levels (Fig. 6b, lane 3 versus lane 2 and Supplementary Fig. 8a). These results argue that PTEN $\beta$  plays a role in regulation of rRNA, 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 PTEN $\beta$  wild-type and knockout cells. As expected, 45S pre-rRNA was increased in PTEN $\beta$  and PTEN $\alpha$  double knockout cells as compared with PTEN $\alpha$  only knockout or wild-type cells (Fig. 6c). To determine whether rDNA transcription is regulated by PTEN $\beta$ , a pHrD-IRES-Luc or pGL3-basic control vector was transfected into PTEN $\beta$  and PTEN $\alpha$  double knockout cells, and the luciferase reporter activity of the rDNA promoter was measured. Introduction of PTEN $\beta$ , but not PTEN $\alpha$ , efficiently downregulated luciferase activity (Fig. 6d and Supplementary Fig. 8b). These results indicate that PTEN $\beta$  negatively regulates rDNA transcription, which may act at least in part through PTEN $\beta$  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). PTEN $\beta$  and PTEN $\alpha$  double knockout resulted in greater average areas of AgNOR staining, while PTEN $\alpha$  only knockout did not have this effect (Fig. 6e). This suggested that elimination of PTEN $\beta$  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 PTEN $\beta$ . Growth curves showed significant increases in cell growth rate resulting from PTEN $\beta$  knockout (Fig. 6f). This activation of cell proliferation resulting from PTEN $\beta$  deprivation was further confirmed by S phase analysis (Fig. 6g). These results argue that PTEN $\beta$  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 mRNA<sup>38</sup>. However, there is now evidence including observations from our previous study<sup>25</sup> 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)<sup>20</sup>. More than 50 instances of non-AUG-initiated N-terminal extensions have been predicted or verified experimentally in mammals<sup>39,40</sup>. In this study, we report identification of a translational variant of PTEN, designated PTEN $\beta$ . 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 PTEN $\beta$  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.

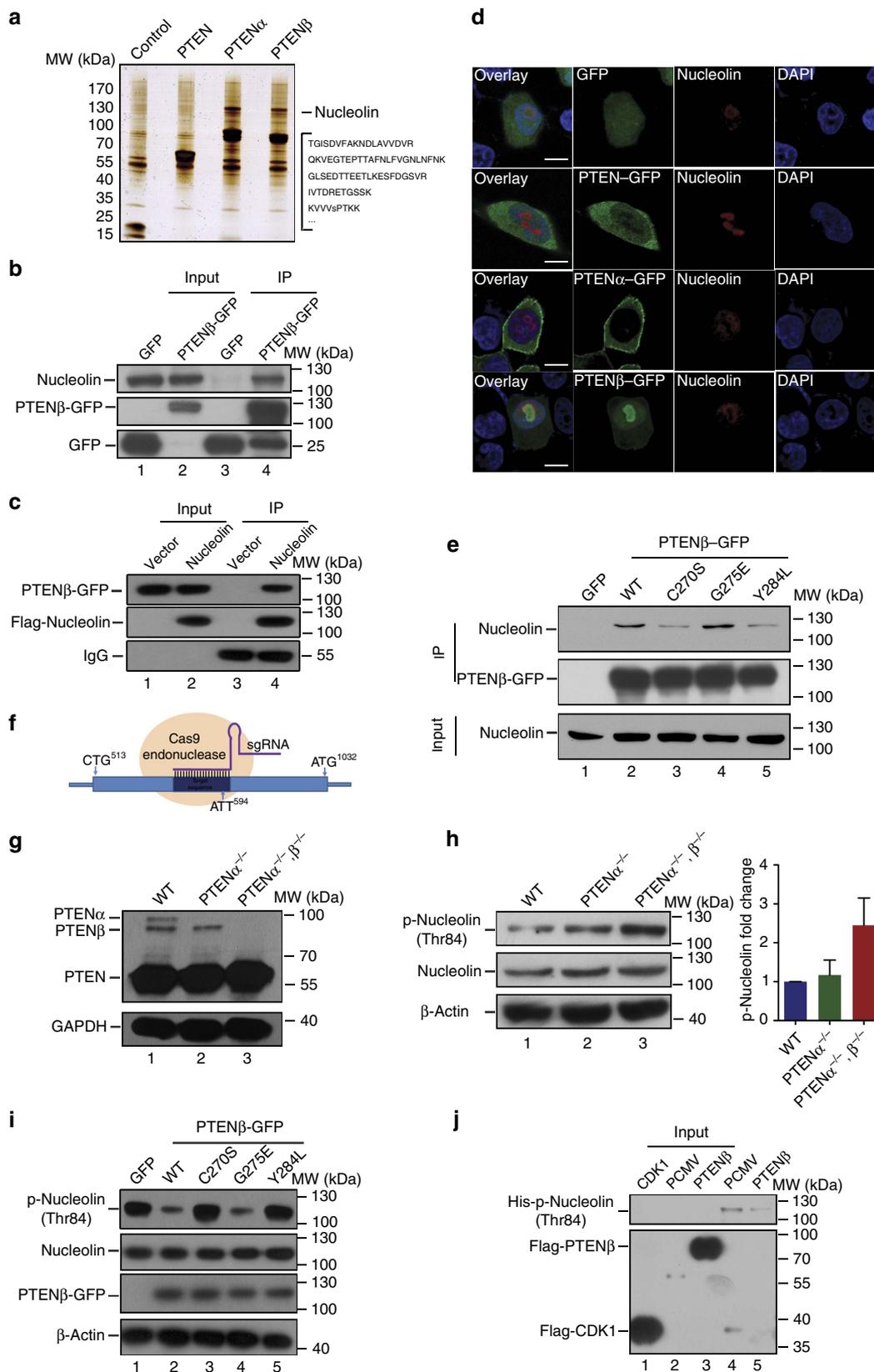
In previous studies both our group and Hopkins *et al.* independently identified a translational variant of PTEN with N-terminal extension, designated 'PTEN $\alpha$ ' in our study, and 'PTEN-long' by Hopkins *et al.*<sup>24</sup>. These have proven to be the same isoform that is translated from a CUG codon 519 bp upstream of the AUG initiation sequence of canonical PTEN (refs 24,25). In addition to the initiation sites for PTEN $\alpha$  and PTEN $\beta$  (CUG<sup>513</sup>, AUU<sup>594</sup>), 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  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  as labels.

In eukaryotes, the efficiency of initiation depends on the Kozak context<sup>41</sup> which encompasses the AUG codon. Our data show that initiation of PTEN $\beta$  relies on the Kozak context surrounding AUU<sup>594</sup>, 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 AUU<sup>594</sup>, and showed that disruption of this conserved

**Figure 4 | PTEN $\beta$  is localized predominantly in the nucleolus.** (a) A set of different constructs of PTEN, PTEN $\alpha$  and PTEN $\beta$  with a C-terminal GFP tag. The AUG start codon of canonical PTEN was mutated to AUA in PTEN $\alpha$  and PTEN $\beta$  constructs in order to abolish expression of canonical PTEN. (b) Subcellular localization of C-terminal GFP-tagged PTEN $\alpha$ , PTEN $\beta$  and PTEN. The constructs indicated in a were introduced into HeLa PTEN null cells. Twenty-four hours after transfection, cells were stained with DAPI, followed by imaging with confocal microscopy. The scale bars represent 5  $\mu$ m. (c) HeLa PTEN null cells were transfected as in b, then stained with an anti-UBF antibody and DAPI, and imaged with confocal microscopy. The scale bars represent 5  $\mu$ m. (d) HeLa cells were transfected with C-terminal GFP-tagged PTEN $\beta$ , and subjected to cell fractionation, followed by western blotting with GFP, nucleolin, HDAC1 and HSP90 antibodies. WCE: whole cell lysate. (e) HeLa PTEN null cells were transfected with C-terminal GFP-tagged PTEN $\beta$  or C-terminal GFP-tagged PTEN $\beta$  $\Delta$ R<sup>6</sup>. Twenty-four hours after transfection, cells were stained with anti-UBF antibody and DAPI, followed by imaging with confocal microscopy. The scale bars represent 5  $\mu$ m.

palindrome sharply downregulates expression of PTEN $\beta$ . 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<sup>25</sup>,

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<sup>42</sup>.



We previously showed that PTEN $\alpha$  localizes in mitochondria and functions in mitochondrial metabolism<sup>25</sup>. Hopkins *et al.* reported that PTEN-long (designated PTEN-L) is secreted into adjacent cells, which relies on an N-terminal secretion sequence<sup>24</sup>. These findings indicate that the N-terminal extended PTEN $\alpha$  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 $\alpha$  in the cytoplasm, and this is consistent with findings in our previous study that PTEN interacts and co-localizes with PTEN $\alpha$  in mitochondria<sup>25</sup>. However, PTEN isoforms localize for the most part in a mutually exclusive manner (Supplementary Fig. 9). PTEN $\beta$  specifically localizes in the nucleolus, whereas PTEN and PTEN $\alpha$  do not, and an evolutionarily conserved poly-arginine sequence has proven to be an NoLS of PTEN $\beta$ . The PTEN $\beta$  N-terminus is 27 amino acids shorter than PTEN $\alpha$ , and PTEN $\beta$  therefore does not contain the N-terminal secretion signal sequence, which may in part account for the differences in cellular distribution of PTEN $\beta$  and PTEN $\alpha$ . 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<sup>27,43</sup>. This may in some measure explain the diversity of PTEN isoform distribution.

Our data demonstrate that PTEN $\beta$  is involved in regulation of pre-rRNA synthesis, and disruption of PTEN $\beta$  results in elevated ribosomal biogenesis and cellular proliferation. Our findings suggest that this PTEN $\beta$  regulatory mechanism may act through dephosphorylation of nucleolar protein nucleolin. It has been reported that PTEN acts on ribosomal biogenesis through the PI3K-mTOR pathway by downregulation of 45S pre-rRNA and that PTEN localizes to the nucleolus to regulate nucleolar homeostasis<sup>44–46</sup>. However, our results indicate there is extensive distribution of PTEN $\beta$  in the nucleolus while little nucleolar PTEN can be detected. The PTEN $\beta$  coding sequence overlaps the sequence of full-length canonical PTEN and PTEN $\beta$  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 $\beta$  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* deficient mice. As PTEN and its isoforms PTEN $\alpha$  and PTEN $\beta$  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 $\beta$ , our study reveals that PTEN $\beta$  plays an essential role in rDNA 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<sup>47,48</sup>, and nucleolar perturbations have been observed in various diseases ranging from auto-immunity to cancer<sup>49,50</sup>. Our results show that in addition to nucleolin, PTEN $\beta$  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 $\beta$  as a new isoform of PTEN, which negatively regulates rDNA transcription and cellular proliferation. Based on previous studies that show imbalance of rDNA transcription closely correlates with tumour progression<sup>51,52</sup>, identification of PTEN $\beta$  may provide a new track for therapeutic drug design. These results together with our previous observations regarding PTEN $\alpha$  illustrate the complexity of PTEN function and enrich our understanding of the PTEN family.

## Methods

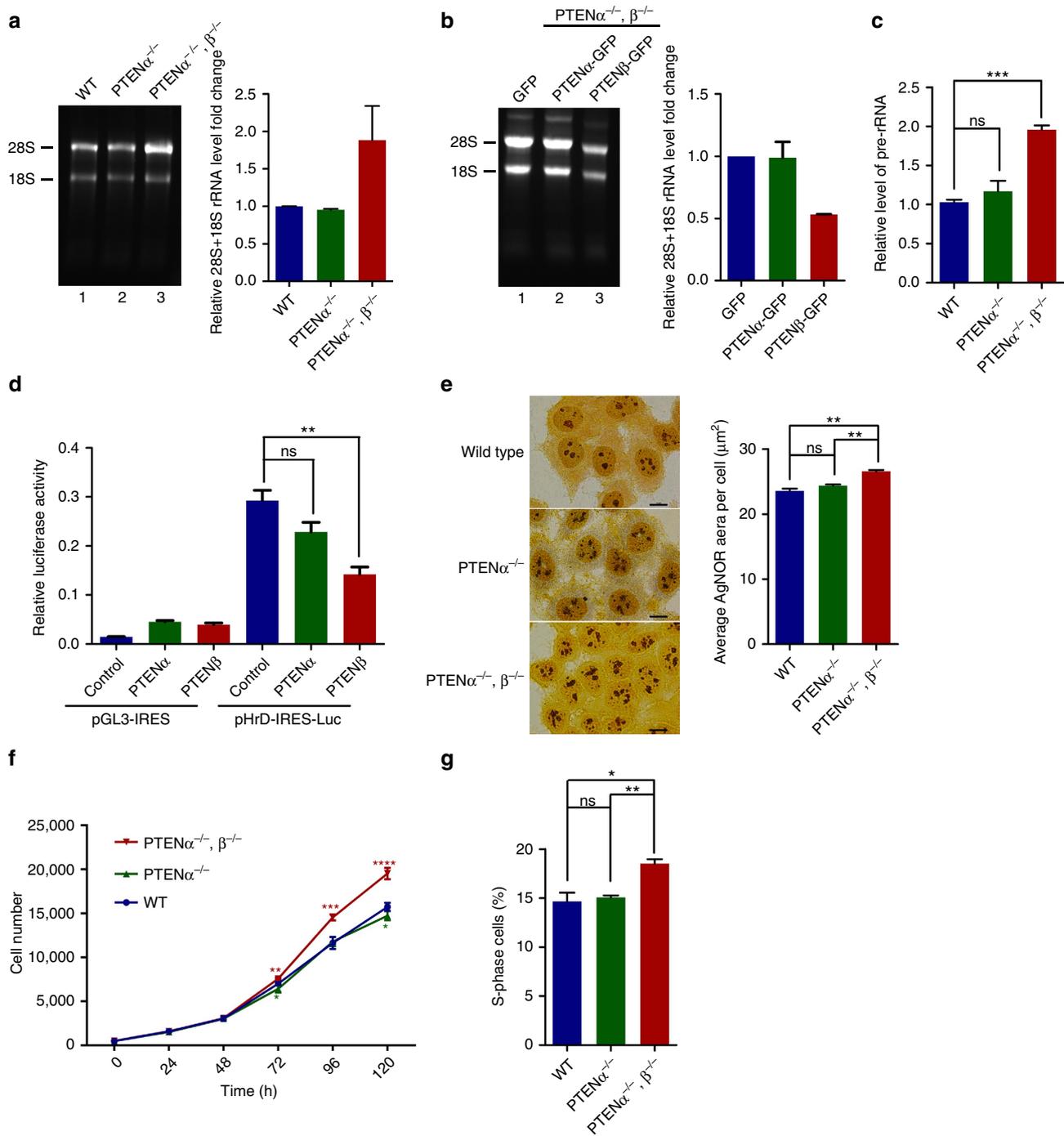
**Cell lines.** All human cell lines used in this study (HepG2, H1299, HCT116, PC3, 786-O, HeLa, U2OS, HEK 293T, SMMC-7721, DU145 and LoVo) were from the American Type Culture Collection. These cell lines were authenticated by STR locus analysis and were tested for mycoplasma contamination. PC3 and 786-O 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<sub>2</sub>. The insect cell line Sf9 was obtained from Invitrogen and cultured in Grace's insect medium (Gibco).

**Mice.** Tissues of C57BL/6 mice<sup>25</sup> were lysed in lysis buffer (50 mM Tris-pH 7.5, 150 mM NaCl, 1% NP40) and subjected to immunoblotting or immunoprecipitation. All animals were maintained in a special pathogen-free facility, and the animal study protocols used were approved by the ethics committee of Peking University Health Science Center (approval number bjmu20110301).

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

**Plasmids and cloning strategies.** The plasmids pCMV-tag-2b, pSA, 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 $\alpha$ , PTEN $\beta$ , or nucleolin or truncations and mutations of these molecules were inserted into these plasmids. pHrD-IRES-Luc was a gift from Dr Xiaojuan Du in the Department of Cell Biology, Peking University Health Science Center<sup>53</sup>.

**Figure 5 | PTEN $\beta$  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 $\alpha$ , S-tagged-PTEN $\beta$  or mock constructs were immunoprecipitated with S-protein beads followed by mass spectrometric peptide sequencing. Nucleolin was found in the PTEN $\beta$  pull-down list. (b,c) Reciprocal immunoprecipitation of PTEN $\beta$  and nucleolin. (b) C-terminal GFP-tagged PTEN $\beta$  or GFP-tagged mock was transfected, 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 $\beta$ , followed by immunoprecipitation with FLAG antibody and immunoblotting with GFP antibody. (d) Images of immunofluorescence staining for PTEN $\beta$  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 $\beta$  protein phosphatase activity is required for its interaction with nucleolin. Wild-type PTEN $\beta$  or PTEN $\beta$  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 $\beta$  CRISPR-Cas9 knockout. The initiation codons of PTEN isoforms were labelled. (g) Immunoblot confirming abolition of PTEN $\alpha$  only (lane 2), or PTEN $\alpha$  and PTEN $\beta$  double knockout (lane 3) with a PTEN antibody. (h) Phospho-nucleolin level in PTEN $\beta$  knockout cells. PTEN $\beta$  knockout cells as indicated in f,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 $\beta$  or PTEN $\beta$  mutants as indicated in e were introduced into PTEN $\alpha$  and PTEN $\beta$  double 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 $\beta$  or FLAG peptide alone as a control, followed by immunoblotting with p-nucleolin (Thr 84).



**Figure 6 | PTEN $\beta$  negatively regulates rDNA transcription and cell proliferation.** (a,b) 28S and 18S rRNA levels in PTEN $\beta$  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 PTEN $\alpha$  or PTEN $\beta$  was transfected into PTEN $\alpha$  and PTEN $\beta$  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 PTEN $\beta$  knockout cells as indicated in Fig. 5g. (d) rDNA promoter luciferase reporter activity in HeLa cells. PTEN $\alpha$  and PTEN $\beta$  double knockout cells as indicated in Fig. 5g were co-transfected with pHrD-IRES-Luc or pGL3-basic plasmid and exogenous PTEN $\alpha$  or PTEN $\beta$ . 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  $\mu\text{m}$ . (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 PTEN $\alpha$  and PTEN $\beta$  double knockout and wild-type HeLa cells is shown with red asterisks, and the significance of the difference in PTEN $\alpha$  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, PTEN $\alpha$  and PTEN $\beta$  double knockout or PTEN $\alpha$  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  $\pm$  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* PTEN $\beta$  identification experiments, the PTEN $\beta$  coding sequence (CUG<sup>513</sup>-UGA<sup>2240</sup>, PTEN $\alpha$  initiation codon CUG<sup>513</sup> mutated to CUC and canonical PTEN initiation codon AUG<sup>1032</sup> mutated to AUA) was subcloned with a C-terminal His-tag into a pFastBac1 vector for expression in Sf9 insect cells. His affinity chromatography was used for purification of PTEN $\beta$ -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 Human database supplemented with N-terminal extended PTEN sequence (Supplementary Fig. 11a). Search parameters were set as follows: enzyme with semi-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 PTEN $\beta$  identification experiments, a FLAG-purified 70 kDa band (molecular weight lower than PTEN $\alpha$ ) from *Pten*<sup>FLAG</sup> 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 follows: enzyme with 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 PTEN $\beta$  knockout.** Sequences (ACCTCCCGCTCCTGGAGCGG or AGTCGCCTGTCACCATTCC) of the human *PTEN* 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  $\mu$ l 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  $\mu$ g ml<sup>-1</sup> of G418 for 3 days. After G418 selection, cell clones were selected and amplified for mutation sequencing.

**Co-immunoprecipitation.** Immunoprecipitation was performed as previously described<sup>12</sup>. In brief, cells were extracted and lysed in lysis buffer (50 mM Tris-pH 7.5, 150 mM NaCl, 0.5% NP40) freshly supplemented with 1 mM PMSF and protease inhibitor. Cell lysate (500  $\mu$ g) was incubated with antibody 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, PTEN $\alpha$  or PTEN $\beta$ . 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 cells were evaluated with fluorescence microscopy. A Nikon A1 microscope was used for confocal microscopy.

**Cell fractionation.** Nucleoli were isolated in HeLa cells as previously described with some modifications<sup>54</sup>. 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 MgCl<sub>2</sub>, 0.5 mM DTT), 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 purified by two cycles of centrifugation through 0.88 M sucrose, and 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-PTEN $\alpha$ , pSA-PTEN $\beta$  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 Sf9 cells and purified using Ni-NTA agarose (Qiagen). Control FLAG peptides, FLAG-CDK1 and FLAG-PTEN $\beta$  were purified with ANTI-FLAG M2 Affinity Gel (F2426) and 3  $\times$  FLAG Peptide (F4799) from HEK 293T cells after transfection with pCMV vector, pCMV-CDK1 or pCMV-PTEN $\beta$  for 30 h. For kinase assay, purified His-nucleolin on Ni-NTA agarose was incubated with CDK1 in kinase buffer (50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 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-PTEN $\beta$  in dephosphorylation buffer (20 mM HEPES, 1 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT and 0.1 mg ml<sup>-1</sup> 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 two genes per term, and a minimum of 4% of total genes associated with the term for the analysis of the PTEN $\beta$  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 described<sup>56</sup>. Briefly, cells were seeded onto 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 Vectashield (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 FACSVerser (BD Biosciences). Experiments were each performed three times, and at least 10<sup>4</sup> 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 Genebank databases under the accession numbers KX398936 (*Homo sapiens*) and KX421108 (*Mus musculus*). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org/cgi/GetDataset>) via the PRIDE (ref. 57) partner repository with the data set identifier PXD003850. The original immunoblots, polyacrylamide 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

**Supplementary Information** accompanies this paper at <http://www.nature.com/naturecommunications>

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