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# Characterization of nuclear PTEN and its post translational modifications

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ABSTRACT

Somatic loss-of-function mutations of *PTEN* are found in a variety of human malignancies. Our recent work demonstrated that the nuclear function of PTEN is implicated in the maintenance of genome integrity. Proper subcellular localization of PTEN following genotoxic stress is coordinated by a cellular mechanism that involves post-translational modification by SUMOylation and ATM-mediated phosphorylation. Here we summarize biochemical and cell-based methodologies that can be used to characterize the SUMOylation and phosphorylation state of nuclear PTEN in the context of DNA damage. In addition, we describe assays to determine the biological function of SUMO-PTEN in homologous recombination DNA repair. These methods will help elucidate the precise molecular mechanisms of PTEN's role in the maintenance of genomic stability.



## 1. Introduction

Phosphatase and Tensin homolog deleted on chromosome Ten (PTEN) dephosphorylates the D3 position of phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)$P_3$ or PIP3) thereby antagonizing the phosphatidylinositide 3-kinase (PI3K) pathway [1,2]. The PI3K pathway regulates diverse cellular processes, including cell metabolism, survival, proliferation, apoptosis, growth, and migration. These fundamental cellular processes, when deregulated, can contribute to or drive a malignant phenotype. Somatic loss of function mutations of *PTEN* are found in a variety of human cancers including breast, endometrial carcinoma, glioblastoma multiforme, skin and prostate cancers [3].

PTEN localization to the plasma membrane is essential to countering the PI3K pathway. Multiple PTEN regions are implicated in plasma membrane localization. A basic patch at the N-terminus of PTEN facilitates its binding to membranes by interacting with PIP2 [4], whereas the C2 domain promotes membrane recruitment facilitating a productive orientation of the phosphatase domain at the membrane surface [5]. Additional C-terminal interactions with PDZ domain-containing proteins may also contribute to membrane association [6].

Post-translational modifications also impact PTEN's plasma membrane localization. PTEN phosphorylation by CK2 on a cluster of C-terminal sites, including Ser370 and 380, Thr383 and Ser385 causes a conformational change that results in the masking of the PDZ domain-binding site, thereby suppressing PTEN plasma membrane recruitment [7]. Finally, SUMOylation of PTEN at Lys 266 located within the CBR3 loop may further promote binding of PTEN to the plasma membrane via electrostatic interactions [8].

PTEN is also readily found in the nuclei of many cultured cells and tissues, including normal breast epithelium [9], proliferating endometrium [10], normal pancreatic islet cells [9], vascular smooth muscle cells [11], follicular thyroid cells [12], squamous cell carcinoma [13] and primary cutaneous melanoma [14]. While nuclear phosphatidylinositols have been reported, they are part of distinct, partially detergent-resistant proteolipid complexes that are not dynamically regulated and not likely PTEN substrates [15]. Various molecular mechanisms responsible for PTEN nuclear localization have been proposed.

PTEN lacks a typical nuclear localization sequence (NLS), but putative nuclear localization signals within PTEN thought to mediate its interaction with the major vault protein (MVP) have been identified [16,17]. Moreover, N-terminal sequences responsible for Ran-mediated nuclear transport [18] and a potential PI3K signaling-sensitive, cell cycle-regulated PTEN nuclear export mechanism have been proposed [19]. PTEN may also have a cytoplasm-retention/nuclear export sequence within its N-terminus [20]. Interestingly, mutations within this region result in constitutive nuclear localization, precluding PTEN growth-suppressive function at the plasma membrane [20].

In the nucleus, PTEN was found to participate in maintaining genomic integrity. PTEN loss of function is often associated with genetic instability [21,22]. Moreover, genetic deletion of PTEN in

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mouse embryonic fibroblasts (MEFs) causes accumulation of unrepaired DNA double-strand breaks (DSBs) [23]. PTEN loss is thought to contribute to genome integrity via at least two molecular mechanisms [24,25]. Nuclear PTEN associated with the centromeric binding protein CENP-C, which is critical for kinetochore assembly and the Metaphase to Anaphase transition [23]. Further, acting as a co-factor for the transcription factor E2F1, nuclear PTEN appeared to regulate the expression of Rad51, a key component of the DNA repair machinery [23].

Our recent work [26] reports a mechanism whereby SUMOylation of PTEN (on lysine 254) leads to its nuclear retention. SUMOylation is a post-translational modification by which Small Ubiquitin-like Modifier (SUMO) proteins are covalently attached to or detached from a protein to modify its function. We found that SUMOylated PTEN participates in the cellular response to DNA damage. Activated ATM phosphorylates PTEN on threonine 398, and a PTEN mutant that cannot be phosphorylated at this position (PTEN T398A) resists nuclear exclusion following genotoxic stress. Cells lacking nuclear PTEN were found to be hypersensitive to DNA damage and displayed impaired homologous recombination-mediated repair of double-strand DNA breaks [26].

The following review describes methods currently employed in our laboratory for the study of nuclear SUMOylated PTEN and it's role in DNA damage repair pathways.

## 2. Material and methods

### 2.1. Detection of endogenous SUMOylated PTEN

Detection of the SUMOylated form of PTEN by standard non-denaturing purification methods is affected by the sensitivity of SUMO-moieties to deSUMOylases during cell lysis [27]. Inclusion of 20 mM N-ethylmaleamide (NEM), an inhibitor of cysteine-based enzymes, in lysis buffers inhibits deSUMOylases and has been found to improve detection of SUMOylated PTEN. Non-denaturing lysates should be sonicated by two 10-s sonication pulses (lowest setting 1, Misonix microson XL) prior to lysate clarification. Total cell lysates prepared in denaturing conditions such as Laemmli sample buffer without bromophenol blue (60 mM Tris–Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol) should be sonicated or boiled for 5 min prior to clarification by centrifugation. SUMOylated PTEN constitutes only 10% of total PTEN in HEK293 cell lysates [26], thus, we typically load 30–50 μg of total protein in order to detect SUMOylated PTEN by immunoblotting with anti-PTEN antibodies.

Cellular fractionation indicates that SUMOylated PTEN predominantly localizes in the nucleus, specifically within the chromatin fraction [26]. Efficient extraction of endogenous SUMOylated PTEN by immunoprecipitation requires lysis under denaturing conditions [27]. Thus denaturing lysis serves to inhibit the activity of deSUMOylases and facilitate SUMO-PTEN's release from chromatin. For immunoprecipitation of endogenous SUMOylated PTEN, we typically use HEK293 or HeLa cells cultured in 145 mm plates. Following the removal of media and washing in PBS, cells are scraped in 1 mL of PBS at 4 °C, collected by centrifugation at 1000×*g* and washed again with PBS. Lysis directly from a cell pellet minimizes dilution of the lysis buffer from residual PBS. The cell pellet is loosened by gentle agitation then lysed in 0.5 mL of Laemmli sample buffer. The sample is sonicated by two 10-s pulses (lowest setting 1, Misonix microson XL) to facilitate lysis and shear the DNA, then incubated on ice for 10 min to complete lysis. Following another 5-s sonication pulse, the lysate is centrifuged at 15,000×*g* for 10 min at 4 °C. The clarified lysate is then diluted 12 to 15-fold in TBS (50 mM Tris pH 7.5, 150 mM NaCl) containing 1% Triton-x 100 and a protease inhibitor cocktail (P8340, Sigma). We have found SUMOylated PTEN can be efficiently immunoprecipitated from diluted lysate using antibodies reactive towards the PTEN C-terminus, such as the mouse monoclonal 6H2.1 (ABM-2052, Cascade Biosciences) or the rabbit monoclonal 138G6 (#9559, Cell Signaling Technology). One microgram of antibody should be added for each milligram of total lysate protein. Immunoprecipitations should be carried out overnight at 4 °C with gentle end-to-end rotation. Protein A/G-Sepharose is then added for 1 h and the resulting immune complexes washed 4 times with TBS containing 1% Triton x-100. Immunoprecipitates can be visualized by immunoblotting with anti-PTEN antibodies or anti-SUMO-2/3 18H8 (#4971, Cell Signaling Technology).

In HEK293 and HeLa cells, we have often observed endogenous SUMOylated PTEN as a ~75 kDa doublet. This may be a result of slight variations in SDS–PAGE mobility of PTEN species which are mono-SUMOylated at different lysine residues (K254, K266, K289) or other unaccounted for post-translational modifications such as phosphorylation.

### 2.2. In vitro and in vivo characterization of SUMOylated PTEN

#### 2.2.1. In vitro characterization of SUMOylated PTEN

Although the major sites of PTEN SUMOylation have been identified [8,26,28], the relationship with other post-translational modifications is not fully understood. We have found that full-length PTEN is a poor substrate in *in vitro* SUMOylation reactions compared to the shorter PTEN truncation mutants. The C-terminal half of PTEN contains three putative SUMOylation sites and a C-terminal truncation mutant is efficiently SUMOylated *in vitro*, which suggests that the N-terminus may be blocking the accessibility of the SUMOylation machinery.

*In vitro* SUMOylation reactions are performed with recombinant Trx-His-PTEN substrate, the E1 heterodimer SAEI/SAEII, E2 SUMO ligase Ubc9 and SUMO1. Recombinant Trx-His-PTEN and Ubc9 were purified in large quantities from *Escherichia coli* BL21 cells. pET32-PTEN and pGEX4T3-Ubc9 plasmids were generated in house [26]. Starting from a single colony of BL21 transformed with the plasmids pET32-PTEN (N-terminal thioredoxin-6xHis) or pGEX4T3-Ubc9 (N-terminal GST), inoculate 10 mL of LB/Amp and grow in an orbital shaker at 37 °C overnight. In the morning, inoculate a 100 mL culture at an OD600 of 0.05 and grow at 37 °C until the culture reaches an OD600 of 0.6. Induce expression by adding IPTG to a final concentration of 0.5 mM and reduce the temperature to 30 °C for 4 h. The bacterial pellet is collected by centrifugation at 6000×*g* for 10 min and frozen at −80 °C. Bacterial lysis is achieved by 3 freeze–thaw cycles in a dry ice and ethanol bath, followed by a short incubation at 37 °C sufficient to thaw the pellet.

The Trx-His-PTEN containing bacterial pellet is resuspended in 5 mL of His-lysis buffer (20 mM HEPES pH 7.4, 500 mM NaCl, protease inhibitor cocktail). Bacterial lysates should be sonicated in 30 s pulses at power level 4 (misonix microson XL) with 30 s intervals, until the lysate is no longer viscous. Centrifuge the bacterial lysate at 15,000×*g* for 30 min at 4 °C. Imidazole is added to the Trx-His-PTEN containing lysate to a final concentration of 20 mM. Prewash Ni–NTA beads (Qiagen) 3 times in lysis buffer and add 100 μL of Ni–NTA to the clarified lysate and rotate for 4 h at 4 °C. Ni–NTA beads should be washed 4 times in wash buffer (20 mM HEPES pH 7.4, 500 mM NaCl, 20 mM Imidazole). Trx-His-PTEN is then eluted by adding 100 μL of elution buffer (20 mM HEPES pH 7.4, 500 mM NaCl, 300 mM Imidazole) and incubating at 4 °C for 15 min with rotation. Imidazole is removed by dialyzing eluate against TBS with 20% glycerol overnight and purified Trx-His-PTEN is stored at −80 °C.

Resuspend the thawed GST-Ubc9 pellet in lysis buffer (PBS with 1% Triton-x 100, protease inhibitor cocktail and 1 mM DTT) and