CONNECTION BETWEEN POST-TRANSLATIONAL MODULATION OF PTEN AND NON-HOMOLOGOUS END JOINING PATHWAY

Thesis submitted to Jadavpur University

for the award of the degree of

DOCTOR OF PHILOSOPHY IN SCIENCE

by

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2024

DECLARATION

I do hereby, declare that the work embodied in the thesis entitled **"*CONNECTION BETWEEN POST-TRANSLATIONAL MODULATION OF PTEN AND NON-HOMOLOGOUS END JOINING PATHWAY*"** submitted for the award of Doctor of Philosophy (Ph.D.) in science, is the completion of the work carried out under the supervision of Prof. Parimal Karmakar at the Department of Life Science & Biotechnology, Jadavpur University. Neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

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Dedicate this thesis

To

Almighty God, my Parents and my Wife

যাদবপুর বিশ্ববিদ্যালয়
কলকাতা-৭০০০৩২, ভারত

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled ***"CONNECTION BETWEEN POST-TRANSLATIONAL MODULATION OF PTEN AND NON-HOMOLOGOUS END JOINING PATHWAY"*** submitted by **Sougata Ghosh Chowdhury** who got his name registered on **08/12/2016** for the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of Prof. Parimal Karmakar and that neither this thesis nor any part of it has been submitted for either any degree or any other academic award anywhere before.

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PREAMBLE

The thesis consists of four chapters. The first chapter includes a general introduction and literature reviews of PTEN. In the second chapter, we mostly focused on different post-translational modulations of PTEN in the context of the maintenance of nuclear activities and preservation of genomic integrity. In the third chapter, we focused on the role of PTEN in non-homologous end joining pathways. In the last chapter of my thesis, I summarized the results of my research work and discussed the future aspects of post-translational modulation of PTEN and homologous recombination-mediated repair pathways.

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CONTENTS

LIST OF ABBREVIATIONS

AKT	AK strain transforming
ALOX5	Arachidonate 5-lipoxygenase
APC	Anaphase-promoting complex
APH	Aphidicolin
ATM	Ataxia-telangiectasia mutated
BrdU	5-bromo-2´-deoxyuridine
BSA	Bovine serum albumin
CDH1	Cadherin-1
CENP-C	Centromere protein-C
CHK1	Checkpoint kinase 1
CHX	Cycloheximide
CK2	Casein kinase 2
COSMIC	The catalogue of somatic mutations in cancer
COX2	Cyclooxygenase 2
DAPI	4, 6-Diamidino-2-Phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	Double strand break
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N´,N´-tetraacetic acid
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting

FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
FGFR2	Fibroblast growth factor receptor 2
FITC	Fluorescein 5-isothiocyanate
FOXO	Forkhead box protein O
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescence protein
GSK3β	Glycogen synthase kinase 3 beta
H2AX	H2A histone family member X
H3K9me3	Histone 3 lysine 9 trimethylation
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HIF-1	Hypoxia-inducible factor 1
HP1	Heterochromatin protein 1
HR	Homologous recombination
HRP	Horse radish peroxidase
HU	Hydroxyurea
KAP1	Kruppel-associated box domain-associated protein 1
KCl	Potassium chloride
KDM4A	Lysine demethylase 4A
LIG4	DNA ligase 4
LKB1	Liver kinase B1
mAbs	Monoclonal antibodies
MAGI-2	Membrane-associated guanylate kinase inverted 2
MAPK	Mitogen-activated protein kinase
MCM2	Minichromosome maintenance complex component 2
MDM2	Murine double minute 2
MDMX	Murine double minute X

MEF	Mouse embryo fibroblast
MEM	Minimal essential medium
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
MVP	Major vault protein
NaOH	Sodium hydroxide
NEDD4	Neural precursor cell expressed developmentally down-regulated protein 4
NHEJ	Non-homologous end-joining
NLS	Nuclear localization signal
NP-40	Nonidet P-40
OD	Optical density
ORC	Origin recognition complex
OTUB1	OUT deubiquitinase, ubiquitin aldehyde binding 1
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PDB	Protein data bank
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PH	Pleckstrin homology
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PICT-1	Protein interacting with the C terminus 1
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PIP3	Phosphatidylinositol 3,4, 5-trisphosphate
PKC	Protein kinase C
PLK1	Polo-like kinase 1
PMSF	Phenylmethylsulfonyl fluoride
PPI	Proton-pump inhibitor
PTEN	Phosphatase and TENsin homolog deleted on chromosome 10

PTMs	Post-translational modifications
PUMA	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
RH	Relative humidity
RIPA	Radioimmunoprecipitation assay
ROCK	Rho associated coiled-coil containing protein kinase
RPA1	Replication protein A1
RPMI-1640	Roswell park memorial institute-1640
RT	Room temperature
SCC	Squamous cell carcinoma
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SMYD2	SET and MYND domain containing 2
TBST	Tris buffer saline-Tween 20
TCGA	The cancer genome atlas
TEMED	Tetramethyl ethylenediamine
TSC1/2	Tuberous sclerosis complex 1/2
USP	Ubiquitin-specific-processing protease
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein
XLF	XRCC4-like factor
XPC	Xeroderma Pigmentosum complementation group C
XRCC4	X-ray repair cross-complementing protein 4

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LIST OF SYMBOLS

g	Acceleration due to gravity
α	Alpha
&	And
~	Approximate
β	Beta
°C	Degree centigrade
=	Equal
γ	Gamma
gm	Gram
>	Greater than
h	Hour
λ	Lambda
<	Lesser than
µg	Microgram
µl	Microliter
µM	Micromolar
µmol	Micromole
mA	Milliampere
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar

min	Minute
M	Molar
-ve	Negative
±	Plus-minus
+ve	Positive
:	Ratio
rpm	Rotation per minute
x	Times
U	Unit
V	Volt
w/v	Weight/volume

Abstract

ABSTRACT

The fundamental cause of tumor development is the constitutive activation of oncogenic proteins and mutational inactivation of DNA repair proteins. Thus, maintaining genomic stability is the fundamental cause of preventing cancer predisposition as neoplastic transformation shows genomic aberration. PTEN is a multifunctional tumor suppressor protein that was identified in 1997. It is a phosphatase that negatively regulates phosphatidylinositol 3-kinase signaling pathway. PTEN is made up of several domains, such as the N-terminal phosphatase domain, the core C2 domain, and the C-terminal tail which has several phosphorylation sites. However, PTEN has become known as a DNA repair protein and is crucial for preserving genomic stability throughout the last ten years. The replication progression of PTEN-null cells has been demonstrated to be hampered, but no specific studies have been done on the relationship between the catalytic function of PTEN that is controlled by post-translational regulation and the cellular response to replication stress. We found that p-PTEN accumulates in the nucleus after S phase arrest induced by double thymidine block followed by DNA damage induced by etoposide. Accumulation of p-PTEN indicates the PTEN association with chromatin and is necessary for restoration of arrested replication fork which was established in our previous study. Phosphatase-dead PTEN cannot sense replication stress though it can be associated with chromatin. So, it provokes us to investigate the role of post-translation modulation of PTEN in the recovery of stalled replication. Subsequently, we utilized aphidicolin to generate replication stress and explore the necessary post-translational modification of PTEN during replication stress. If the cell does not sense replication stress, aberrant replication may occur which is one of the factors that contributes to genomic instability. Together, our data suggest that the interruption of DNA replication caused by aphidicolin drives the development of heterochromatin by stabilizing and up-regulating H3K9me3 foci, increases CHK1 activation, and enables PTEN

chromatin binding through C-terminal phosphorylation. Thus, PTEN functions as a stress-sensing protein, maintaining genomic stability during replication arrest. Further, p-PTEN promotes heterochromatin formation to arrest replication and allow the repair process initiation.

PTEN can translocate into the nucleus and be associated with chromatin in response to double-strand break. DNA DSB is detrimental as it can cause cancer-driving mutation and chromosomal arrangements unless it is successfully repaired. DSB repair is mediated by either HR or NHEJ which involve several proteins. It has been reported that PTEN is involved in both HR and NHEJ pathways. Mechanistically, it is physically linked to the Rad51 promoter and functions in combination with the transcriptional factor E2F1 to regulate Rad51 synergistically. Though several papers emphasized the role of PTEN in HR but role of PTEN in NHEJ is yet to be studied. There is one report suggesting that PTEN increases NHEJ frequency by up-regulating XLF protein. Further post-translational modulation of PTEN also regulates its activity and chromatin association. Additionally, we found that PTEN phosphorylation is necessary for both DNA repair and chromatin association. Our in-vivo plasmid-based reporter assay also suggests that PTEN-WT increases NHEJ repair frequency. Further, it has been found that loss for PTEN causes chromatin disorganization and compromised expression of HP1α and H3K9me3 protein. H3K9me3 is an epigenetic marker that is critical to the 53BP1 protein-mediated NHEJ repair process. To develop novel therapeutic approaches for targeting the NHEJ pathway in cancer cells, it is necessary to investigate how PTEN post-translational regulation and its catalytic activity influence chromatin modulation and the NHEJ repair pathway in response to DNA damage. In this study, we investigated the epigenetic regulation of PTEN, which is related to NHEJ. Our study reveals that phosphorylation as well as the phosphatase activity of PTEN is needed for the NHEJ-mediated DSB repair. Here, reduced expression of Ku70/80, DNA-PKcs, XRCC4,

and XLF was observed in PTEN-null PC3 cells upon DNA damage. A recent study also indicates that nuclear PTEN may interact with RNA POLII-mediated transcription machinery and thus regulate the expression of many genes including NHEJ factors. PTEN-WT expression can rescue compromised NHEJ unlike phospho and phosphatase-dead PTEN transfected cells. Furthermore, we demonstrated that a DNA-PKcs inhibitor prevents etoposide-induced PTEN C-terminal phosphorylation following DNA damage, suggesting that PTEN may be a target of DNA-PKcs. In addition, we found that PTEN dephosphorylates DNA-PKcs by binding to its C-terminal region. Therefore, after DNA damage, crosstalk between PTEN and DNA-PKcs modulates the NHEJ pathway. PTEN phosphorylation causes its attachment to chromatin, therefore upon DNA damage, PTEN is phosphorylated by DNA-PKcs and attaches to chromatin, resulting in the dephosphorylation of DNA-PKcs and the recruitment of other NHEJ factors on chromatin occurs for efficient execution of the NHEJ pathway. Thus, our research provides a molecular understanding of the epigenetic regulation of PTEN and its significant role in the NHEJ pathway.

Chapter 1

General Introduction & Literature Review

1.1 Introduction to PTEN

Targeted cancer treatments are designed to prevent the growth-promoting process from running rampant. Today, many cancer drugs are designed to block the growth-promoting proteins that are produced by oncogenes [*Lee et al., 2010; Debela et al., 2021*]. The ability to manipulate a particular type of protein involved in the development of cancer has become more challenging. Normally, these proteins prevent abnormal cell growth. However, in cancerous cells, they can lose their function. If these proteins could be turned back on using drugs, this could be a game-changing approach to treating the disease. In studies on mouse models and human cancer cells, researchers were able to identify a way to activate PTEN, which is a tumor suppressor typically silenced in cancer [*Inoue et al., 2013*]. PTEN, which stands for phosphatase and TENsin homolog deleted on chromosome 10, is a well-known tumor suppressor gene that is located in the 10q23 region of chromosome 10 and codes for a 403-amino acid multifunctional protein with phosphatase and lipid activity. [*Abdulkareem et al., 2013*]. Three different research teams independently discovered PTEN in 1977. Two of the teams employed a positional-cloning method, while the third team utilized a biochemical method to hunt down a gene producing a phosphatase with tensin and auxilin homology [*Li DM et al., 1997; Li J et al., 1997; Steck PA et al., 1997*]. The sequence analysis of the gene reveals that it shares a homology with the protein known as tyrosine phosphatase and the cytoskeletal protein known as tensin. These two proteins are known to link integrins to the actin cytoskeleton at sites of adhesion [*Zheng et al., 2013*].

1.2 PTEN and Cancer

PTEN, a crucial tumor suppressor, reduces cell proliferation and raises cellular susceptibility to apoptosis. [*Lu et al., 2016; Wang et al., 2021*]. Since its discovery in 1997, PTEN has been found to play a significant role in inhibiting the growth of cancer cells. Changes in the

expression of this gene and/or protein are now thought to be actionable molecular hallmarks, which means that their presence can influence clinical practice [*Fusco et al., 2020*]. Various forms of cancer have been known to be characterized by the loss or alterations in the PTEN function [*Hollander et al., 2011*]. These are regarded as founder events that trigger the development of the tumors. PTEN mutation rate is highest in cancers such as prostate cancer, endometrial cancer, and glioblastoma [*Mutter 2001; Gbelcová et al., 2022]*. It also affects other types of cancer, such as breast cancer, colon cancer, and lung cancer [*Fusco et al., 2020; Wang et al., 2021*]. In the Western world, sporadic prostate cancer is regarded as the most common type of cancer among men. However, there is still a lot of doubt regarding the exact genetic factors that contribute to its development [*Rawla 2019*]. Studies that involve allelotype and cytogenetic methods have shown that in sporadic prostate cancer, there has been a loss of chromosome 10q heterozygosity [*Carter et al., 1990; Wang et al., 2000*]. Studies that involved deletion mapping revealed that the minimal area of loss was found in chromosome 10q23 [*Macoska et al., 2001*]. Through a microsatellite analysis, the researchers were able to identify 23 cases of deletion of chromosome 10q23 [*Kulke et al., 2001*]. The development of PTEN germline mutations can be characterized by various disorders and deficits. These include neurological disorders, multiple hamartomas, developmental disorders, and an increased risk of endometrial, breast, and thyroid cancers (Table 1.1) [*Misra et al., 2021*]. Some of the other disorders that are known to be associated with this condition include Cowden syndrome and Lhermitte-Duclos disease [*Al-Noman et al., 2023*]. Individuals suffering from Cowden syndrome have a greater likelihood of developing uterine, thyroid, kidney, colon, or other cancers. It is also associated with non-cancerous lumps in the uterus, thyroid, and gastrointestinal tract. Currently, more than 2700 PTEN mutations have been identified in 28 different tumor types in the COSMIC cancer database, while 1120

mutations have been identified in 27 different tumor types in the cBio portal of The Cancer Genome Atlas (TCGA) [*Hopkins et al., 2014*].

Table 1.1 *Incidence and therapeutic significance of PTEN alterations in different human cancers.*

Cancer type	***Therapeutic implications of PTEN loss***
Prostate cancer	Development of metastasis, early recurrence after surgery, strongly associated with adverse oncological outcomes
Liver cancer	Increased recurrence, associated with high tumor, high α-fetoprotein expression
Breast cancer	Resistance to HER2-targeted therapy, resistance to anti-estrogens tamoxifen and fulvestrant
Colorectal cancer	Lack of response to EGFR-targeted mAbs
Endometrial cancer	Associated with obesity, uninterrupted production of tumorigenic PIP3
Pancreatic cancer	Increased recurrence and metastasis, associated with increased liver metastasis, poor survival rate
Thyroid cancer	Other genetic alterations and more aggressiveness
Leukemia	Shorter survival
Lung cancer	Reduced susceptibility to apoptosis in EGFR-mutant cells through EGFR and AKT activation

EGFR: epidermal growth factor receptor; HER2: human epidermal growth factor receptor 2; mAbs: monoclonal antibodies; PIP3: phosphatidyl inositol (3,4,5)-triphosphate.

1.3 Structure of PTEN

PTEN crystal structure showed that the protein is divided into two main parts, one at the N-terminus, which contains the phosphatase domain, and the other at the C-terminus, which is primarily made up of a C2 domain. There are three more PTEN functional domains. A C-terminal tail with two PEST repeats and a C-terminal PDZ binding domain follows the C2 domain, while an N-terminal PIP2-binding (PBD) domain precedes the phosphatase domain (Figure 1.1). The N-terminus of PTEN is known to play a significant role in the activity of the lipid phosphatase in the membrane [*Mondal et al., 2020*]. PTEN residues 6 to 15 are a

potential PIP2 binding motif that directs PTEN to the membrane to dephosphorylate PIP3 as a substrate. PTEN has been demonstrated to be allosterically activated by PIP2, which increases PTEN phosphatase activity against PIP3 and appears to depend on residues Lys13, Arg14, and Arg15 for this interaction [*Jang et al., 2021*]. Additionally, the PTEN N-terminus has several localization sequences that change the subcellular localization of PTEN. It is hypothesized that residues 20-25 (GFDLDL) are involved in the cytoplasmic localization of PTEN, as 10 clinically significant mutations in these residues cause nuclear import [*Chen et al., 2018*]. Alternatively, PTEN cannot accumulate in the nucleus when residues 13-16 (LRRY) are deleted or Tyr26, Tyr27, and Tyr29 are mutated. This indicates that these N-terminal portions are most likely nuclear localization sequences. [*Gil et al., 2006*].

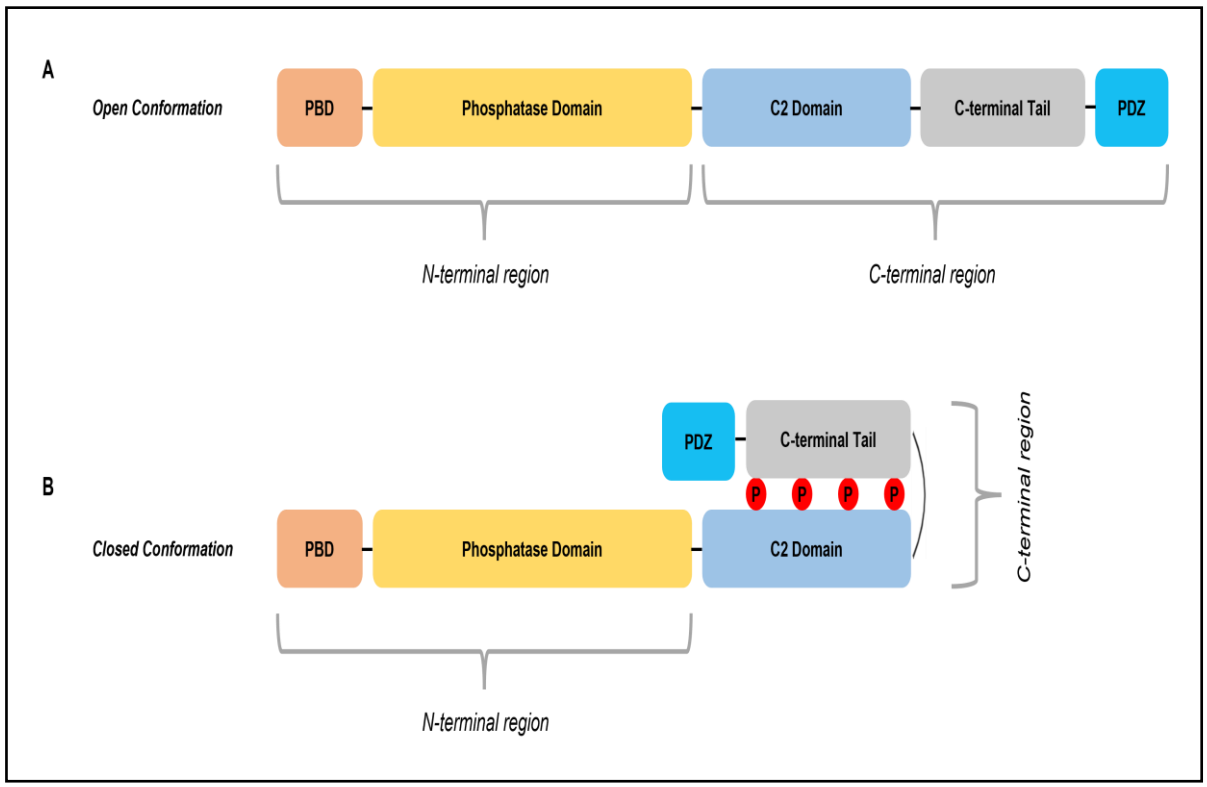


Figure 1.1 *PTEN protein structure contains the PIP2 Binding Domain (PBD), Phosphatase Domain, C2 Domain, C-terminal Tail Domain, and PDZ Binding Domain. (A) PTEN exists in open conformation when dephosphorylated favoring its accumulation in the nucleus and (B) In closed conformation when phosphorylated favoring accumulation in the cytoplasm.*

It is believed that PTEN's C-terminal C2 domain plays a role in the interaction between the plasma membrane and the lipid-metabolizing enzymes such as phospholipase A2 [*Tu et al., 2020*]. Although it is independent of Ca^{2+}, the PTEN C2 domain is similar to that of other phospholipid metabolism enzymes. Additionally, the C2 domain controls PTEN nuclear import by using certain residues. A protein known as MVP can function as a nuclear transporter [*Ho et al., 2020*]. PTEN's interaction with this protein was disrupted by mutations in the amino acids 160–164 (PD) and 265-269 (C2D), which can result in abnormal localization. PTEN's 50 amino acids unstructured C terminus serves as a focal point for regulatory interactions and changes. The regulation of protein stability is significantly influenced by several phosphorylation residues in the carboxyl terminus. Numerous protein-protein interactions have been demonstrated to be mediated by the PDZ binding site, which is made up of the three C-terminal amino acids.

1.4 Proteoforms of PTEN

Proteoforms are particular molecular variations of protein products produced by a single gene that has various structural and functional characteristics. Numerous mechanisms, including allelic differences, pre-translational modifications, functional transformations, and conformational dynamics, can be delivered by a single gene to produce a wide variety of proteoform variants, which can aid in the emergence of illness [*Guzman et al., 2021*]. Single nucleotide variations in PTEN can lead to the development of proteoforms that are known to be tumor drivers. PTEN-L, -M, -N, and -O protein products are translated as a result of several non-canonical translation initiation codons that are present in 5' upstream of the traditional AUG start site [*Tzani et al., 2016; Malaney et al., 2017*]. Additionally, PTEN mRNA alternately splices to produce 10 different isoforms, including PTEN-delta, PTEN-B, DelE5, DelE6, PTEN-3a, -3b, -3c, and PTEN-5a, -5b, -5c (Figure 1.2) [*Agrawal et al.,*

2006]. This section addresses these naturally occurring alternative translational and alternative splice variants, emphasizing their functions and structures.

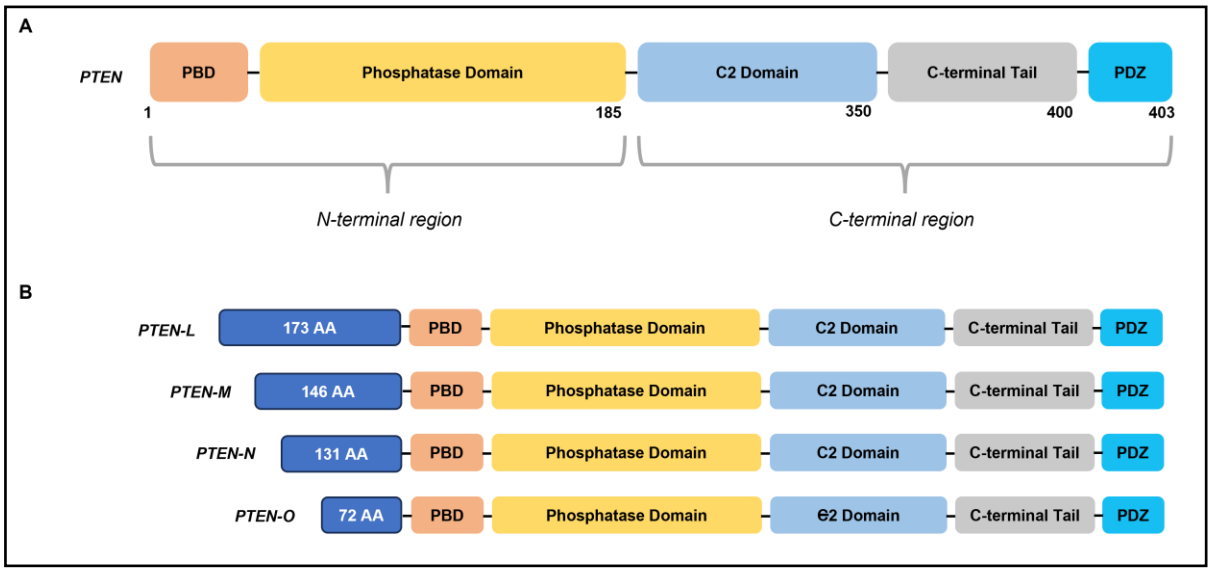


Figure 1.2 *(A) The 403 amino acids that comprise the structure of the canonical PTEN isoform are arranged into five functional domains. (B). The four translational variants of PTEN, designated PTEN-L, -M, -N, and -O, each have an extra N-terminus and have different subcellular localization and activities. For every variant, translation results in N-terminal extensions that start at a translation site upstream of the canonical initiation sequence.*

1.4.1 Alternative Translational Proteoforms

The N-terminus of every alternative translational isoform has been lengthened, and they all have PTEN lipid phosphatase activity. PTEN-O lacks endogenous expression, but PTEN-L, -M, and -N are all endogenously expressed in a variety of cell lines, with PTEN-M being the most prevalent [*Tzani et al., 2016*]. Even though PTEN-M is the alternative translational variation with the highest level of expression, PTEN-L (also called PTEN-Long) has special structural and functional characteristics and therefore it has been the subject of

most of the research [*Milella et al., 2015*]. In addition to maintaining its activity as a lipid phosphatase, the PTEN-L protein also has other localization abilities. These include its N-terminal extension sequence motif, which can help in penetrating cells. Studies have shown that PTEN-L can localize to the mitochondria's inner membrane. It can also maintain the function and structure of the mitochondria and increase the activity of cytochrome c oxidase [*Bononi et al., 2015*]. The N-terminal segment of PTEN-L acts as a cell-penetrating agent, making it an effective tumor suppressor. This can be utilized in the treatment of cancers that have low or compromised levels of PTEN expression [*Dillon et al., 2014*].

1.4.2 Alternative Splice Proteoforms

Numerous naturally occurring splice variants of PTEN result in distinct proteoforms with varied functionalities. PTEN protein truncations are a common outcome of alternative splicing. The intronic region is inserted after exons 8 and 5, respectively, resulting in the generation of two splice variants, PTEN-delta and PTEN B (Table 1.2). In patients with renal cell carcinoma, the PTEN-delta protein's expression can be associated with an improved prognosis [*Shin Lee et al., 2003*]. In human kidney carcinoma cells, the stable overexpression of PTEN-delta caused a decrease in the MAPK and AKT signaling, as well as a reduction in the cell's migration capacity [*Breuksch et al., 2018*]. Eight additional PTEN splice variants were later discovered by Agrawal et al. These variants kept portions of intron 3 (variants 3a, 3b, and 3c), and parts of intron 5 (variants 5a, 5b, and 5c). While intron 5 is present immediately 3' of exon 5 in variants -5a, -5b, and -5c, intron 3 is present directly 3' of exon 3 in variants -3a, -3b, and -3c. Patients with Cowden Syndrome and breast cancer displayed distinct expression patterns for these splice variants. Splice variants 5b and 5c exhibited cyclin D1 promoter activity, which was the opposite of the behavior of full-length PTEN [*Dillon et al., 2014*].

Table 1.2 *Details of PTEN N-terminally extended Proteoforms.*

Proteoforms	***N-terminal extended AA***	***Total AA***	***MW (kDa)***
PTEN-L	173	576	64.9
PTEN-M	146	549	62.5
PTEN-N	131	534	61.0
PTEN-O	72	475	55.0

1.5 Subcellular localization and function of PTEN

Previous research suggests that PTEN can only be located in the cytoplasm and can transiently associate with the plasma membrane depending on the concentrations of PIP2 and PIP3 [*Bononi et al., 2015*]. In addition, studies have shown that PTEN can localize to certain subcellular compartments. These include the nucleus, mitochondria, nucleolus, and ER. In vitro, the C2 domain and PBD can mediate the PTEN binding to acidic lipid vesicles. But, in vivo, PTEN is believed to be either nuclear or cytoplasmic. In particular cell lineages and under certain circumstances, PTEN can only be found to migrate to the plasma membrane [*Vazquez et al., 2006*]. It is widely believed that the protein's subcellular localization is dependent on its function. This is especially true about PTEN. It is therefore important to determine the exact location of PTEN and its function in a specific region.

1.5.1 In plasma membrane

The essential regulatory step of binding to the plasma membrane is necessary for PTEN activity in suppressing the PI3K signaling pathway [*Georgescu et al., 2010*]. Most mammalian cells don't seem to have a clear link between the plasma membrane and PTEN. Instead, PTEN is primarily located in the cytoplasm and the nucleus. PTEN's membrane targeting is facilitated by the presence of an N-terminal PIP2-binding motif, a C2 domain,

and a C-terminal tail [*Bononi et al., 2015*]. The PTEN crystal structure revealed that the C2 domain is a region that contains basic residues that are required for membrane binding. Additionally, a group of serine and threonine phosphorylation sites (S370, S380, T382, T383, and S385) located in the flexible C-terminal tail of PTEN control the protein's stability, activity, and adhesion to the cell membrane [*Liu et al., 2019*]. The C-terminal tail of PTEN can be phosphorylated by a variety of kinases. According to a theory put up by Vazquez et al., phosphorylated PTEN adopts a tight shape that prevents membrane attachment and renders PTEN inactive in the cytoplasm [*Vazquez et al., 2000*].

1.5.2 In cytoplasm

In the early stages of studies on PTEN localization, it was found in the cytoplasm, but these studies mainly focused on tumorigenic tissues and cell lines [*Bononi et al., 2015*]. The functions of PTEN that are dependent on phosphoinositide 3 kinase have been well-researched in several kinds of literature. The PI3K is a component of the growth and survival pathways that are frequently deregulated or hyperactivated in human cancer. This suggests that it plays an important role in the development of tumors. In response to extracellular signals, the catalytic subunits of class I PI3Ks, p110, are recruited to active membrane receptors through their regulatory subunits, p85. Phosphoinositide 3,4,5-triphosphate is immediately dephosphorylated at the D3 position by the lipid phosphatase PTEN, which functions as the primary negative regulator of PI3K [*Denley et al., 2009*]. Activated PI3Ks catalyze the production of PIP3 from PIP2. The serine-threonine kinase AKT is an essential PIP3 downstream effector that is drawn to the membrane by PIP3 binding to its PH domain. Phosphorylation of Thr308 and Ser473 by PDK1, the rapamycin-insensitive mTOR complex (mTORC2), or potentially by another kinase, results in its complete activation [*Liu et al., 2009*]. In addition to being involved in the regulation of cell motility, PTEN also plays a role

in the development and maintenance of cell adhesion. It can dephosphorylate SHC, which can prevent downstream pathways, such as the MAPK pathway [*Milella et al., 2015*]. In addition, it can reduce the activity of FAK by interacting with it and reducing its potential impact on cell migration and cell adhesion [*Tamura et al., 1999*]. The PTEN-FAK interaction significantly lowers the tyrosine phosphorylation of Tyr397 and its downstream effectors, which can negatively affect the integrin-catalyzed cell adhesion and migration. The loss of PTEN's function in HepG2 cells increased in the expression of various proteins that are known to induce angiogenesis, such as MMP9, VEGF, and HIF-1 [*Tian et al., 2010*]. The nude mice model further confirmed that PTEN suppressed the HepG2-induced angiogenesis and decreased the expression of VEGF and HIF-1.

1.5.3 In nucleus

In the nucleus, PTEN has a vital role in tumor suppression. Nuclear PTEN is a useful prognostic marker in some cancers since its absence can result in more aggressive tumors [*Luongo et al., 2019*]. The precise process of PTEN localization is still unknown since there is no traditional signal for nuclear localization. Various molecular mechanisms can be used to explain the mechanism of PTEN's interaction with the major vault protein (MVP) [*Ho et al., 2020*]. Many different biochemical processes, such as simple diffusion and active shuttling controlled by a putative nuclear localization signal (NLS), have been proposed to explain how PTEN interacts with the major vault protein [*Minaguchi et al., 2006*]. The PTEN protein also has a cytoplasmic signal, which is required for localization, in the amino acid sequence 19-25. This signal can be used to identify the site of nuclear export. A potential mechanism to control the accumulation of PTEN nuclear and cytoplasmic components by interacting with the GTPase Ran is based on the presence of numerous nuclear exclusion motifs in different PTEN regions and its N-terminus [*Gil et al., 2006*].

The nuclear PTEN can maintain chromosomal stability by stimulating the development of centromere protein CENP-C [*Shen et al., 2007*]. Additionally, it contributes to responses that damage DNA by increasing the transcription of the Rad51 gene [*Hou et al., 2019; Milella et al., 2015*]. In addition, it can regulate the cell cycle by inducing G0 -G1 arrest, which is most likely triggered by cyclin D1 regulation [*Brandmaier et al., 2017*]. Finally, it can regulate the development of cellular senescence through an anaphase-promoting complex [*Bononi et al., 2015*]. PTEN was subsequently found in the nuclei of healthy primary neurons, endothelial cells, myoepithelial cells lining healthy breast ducts, and healthy follicular thyroid cells [*Gimm et al., 2000*]. Numerous sporadic cancers have been identified to specifically lose nuclear PTEN. It can promote the activity of a tumor suppressor protein known as p53 by directly interacting with it [*Mayo et al., 2002*]. In the presence of PTEN, MAP kinase can be triggered to inhibit cyclin D1's expression. Additionally, it was discovered that PTEN works with E2F1 to promote Rad51 expression and hence improve DNA repair [*Chen et al., 2018*]. When nuclear PTEN function is disrupted, the double-stranded DNA breakage rate is shown to rise, which may be explained by the association between PTEN and Rad51.

1.5.4 In mitochondria and ER

The ability of PTEN to interact with organelles that possess intracellular membranes has recently come to light through several evidence. Studies using FRAP revealed that PTEN exhibited a unique characteristic whereby its nuclear form diffused rapidly and did not seem to be tethered, but its cytoplasmic form diffused more slowly and dispersed with time [*Bononi et al., 2015*]. It was crucial to determine if PTEN's attachment to cytoplasmic components stops it from exerting its action at the plasma membrane or if it localizes PTEN to particular subcellular domains where it suppresses tumors through different mechanisms. The presence of PTEN on the surface of mitochondria has been demonstrated in various

experiments [*Bononi et al., 2015; Thomas et al., 2009*]. These include studies on the effects of apoptotic stimulation using staurosporine (STS) in primary rat hippocampal and myocytes during ischemia-reperfusion [*Zhu et al., 2006*]. The presence of mitochondria-localized PTEN contributes to the development of ROS and causes apoptosis [*Zu et al., 2011*]. In vitro studies revealed that PTEN interacts with inositol-1,4,5-trisphosphate receptors (IP3Rs) to phosphorylate AKT, which counteracts lower Ca^{2+} release [*Bononi et al., 2013*]. It has been suggested that PTEN localization in ER is involved in the apoptotic process by the Ca^{2+}-catalyzed induction of apoptotic cells. Experiments with live imaging revealed that there is further accumulation of PTEN at the ER during the process.

1.6 Nucleo-cytoplasmic shuttling of PTEN

Although PTEN does not contain a typical nuclear import signal, it can still enter and exit the nucleus through its nucleocytoplasmic transport. Various mechanisms can facilitate this process, such as simple diffusion, the Ran GTPase's active protein movement, phosphorylation-dependent protein transfer, and monoubiquitylation [*Bauer et al., 2015*]. Researchers noted that simple diffusion can allow PTEN to enter the nucleus. This process can only be performed on proteins with a molecular weight of less than 60,000 Da [*Liu et al., 2005*]. Its size could allow it to enter the structure through passive diffusion. The presence of Ran-mediated PTEN import in human glioblastoma cells has been observed. The researchers noted that the protein was not able to enter the nucleus of the cells that were deficient in the Ran GTPase and the various mutations that affect the protein's phosphorylation can alter its nucleocytoplasmic localization [*Fata et al., 2012*]. The researchers also noted that the S370A phosphorylation site mutation caused PTEN to localize to cytoplasm, just like its wild-type form [*Stumpf et al., 2016*]. On the other hand, the T382A variant had a similar distribution of nucleo-cytoplasmic distribution. This finding is different from the findings of the work done

on human breast cancer cells, which showed that the presence of similar mutations did not affect PTEN's nucleocytoplasmic localization [*Weng et al., 1999*]. MVP plays a role in yet another possible method of PTEN import. In yeast two-hybrid tests, PTEN attaches to a protein called MVP, which is believed to be a general nuclear-cytoplasmic transport carrier molecule [*Fata et al., 2012*]. NLS1 to NLS4 are four unconventional NLS-like sequences that have been discovered through studies in MCF-7 cells [*Planchon et al., 2008*]. PTEN localization is unaffected by mutations in a single sequence. However, PTEN is not found in the nucleus when NLS2 and NLS3 or NLS3 and NLS4 are targeted as combinations of two sequences. The stability/degradation of the PTEN protein and its subcellular distribution seem to be in delicate balance with one another. The development of new therapeutic targets based on the unique localization of PTEN may be possible through future studies on the various regulatory mechanisms.

1.7 Canonical signaling pathways regulated by PTEN

The main signal for the activation of growth factors is PIP3 accumulation. PIP3 recruits downstream proteins, such as AKT, to the plasma membrane by binding to their pleckstrin homology (PH) domain, providing a lipid moiety. The affinity of PIP3 to the PH domain can alter the protein's confirmation, which can then be triggered by phosphorylation. These proteins' confirmation is altered by PIP3 binding to the PH domain so that they can later be activated by phosphorylation. By reducing the amounts of PIP3 within the cell, PTEN prevents the activation of the downstream proteins of the PI3K pathway (Figure 1.3), including the serine/threonine kinase AKT and the protein kinase C (PKC) [*Liu et al., 2009*]. The AKT protein is a well-known component of the PTEN signal's downstream effector. Numerous biological processes, including cell proliferation, differentiation, migration, and regulation of organ size, depend on it. Through direct interaction with its PH domain, AKT

can enter the plasma membrane upon PI3K activation due to the buildup of PIP3. AKT's binding to PIP3 exposes its sites and permits it to pass through the membrane. It has been demonstrated that PDK1, a distinct PH domain-containing kinase, phosphorylates the protein at Thr308 [*Chu et al., 2020*]. Thr308 phosphorylation is required for AKT's initial activation. On the other hand, the activation of AKT by mTORC2 requires the phosphorylation of Ser473. These molecules' activities are controlled by phosphorylation by AKT and are crucial for numerous cellular processes.

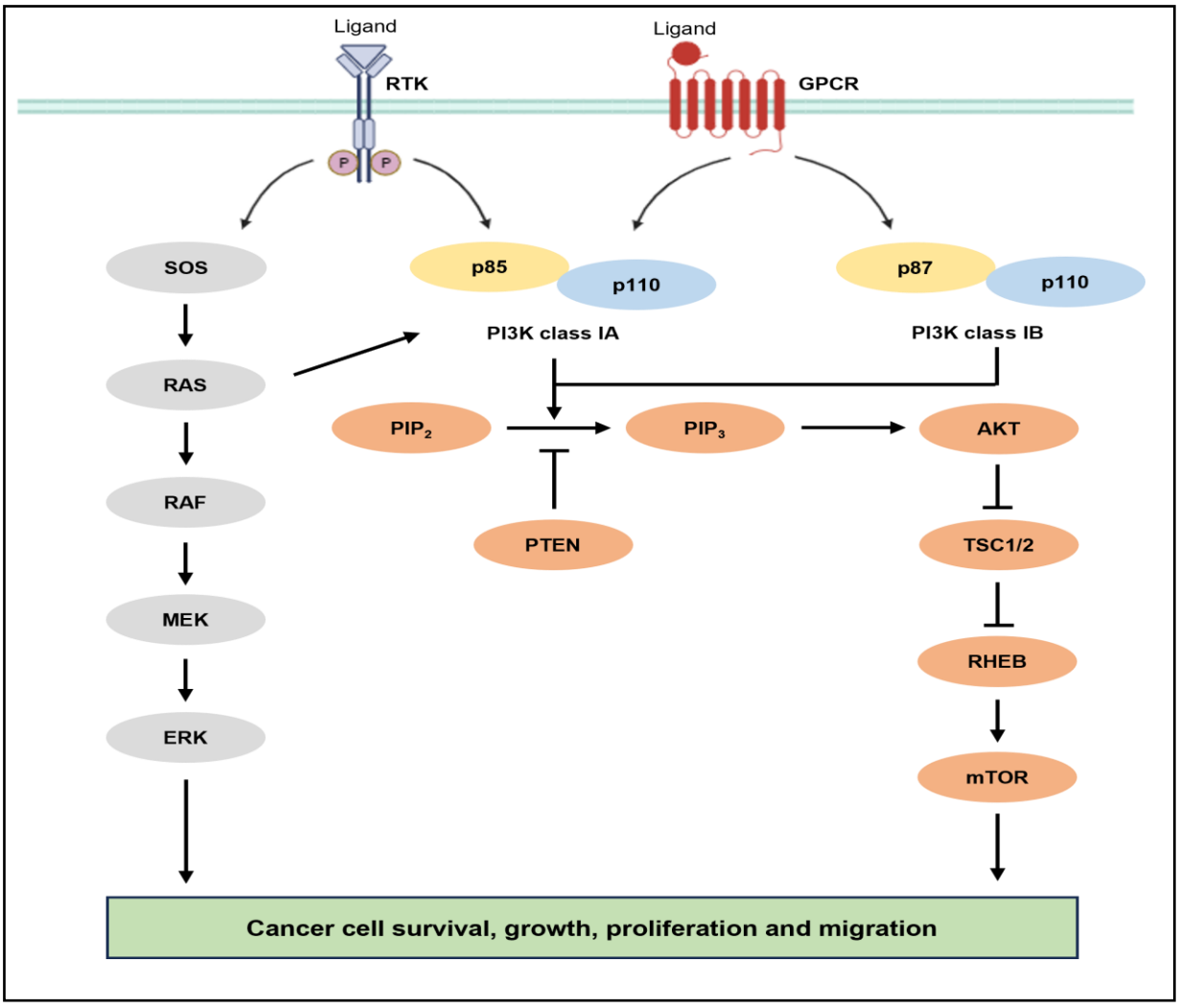


Figure 1.3 *Classical tumor-suppressing activity of PTEN antagonizing the PI3K pathway.*

For instance, the pro-apoptotic proteins BAD, caspases 3 and 9, and p38 MAPK are phosphorylated by AKT, rendering them inactive and promoting cell survival. AKT similarly phosphorylates MDM2 and MDMX directly [*Chen et al., 2018*]. The 14-3-3 proteins that the phosphorylated MDM2 and MDMX bind to stabilize the MDM2-MDMX complexes, which then mediate the degradation of p53 to maintain the low level of p53 in the cells [*Falcicchio et al., 2020*]. Additionally, forkhead transcriptional factors (FOXO) are phosphorylated by AKT, which promotes their association with 14-3-3 proteins [*Tzivion et al., 2011*]. AKT targets multiple forkhead transcriptional factor subunits, including FOXO1 and FOXO3, which regulate cell proliferation, metabolic changes, and survival [*Zhang et al., 2011*]. These forkhead transcriptional factor binding elements are highly abundant in the target cells. For instance, FOXO3a binds to the promoters of the genes Bim and PUMA, resulting in the transcription of these genes, which can initiate apoptotic cascades [*You et al., 2006*]. Through the transcriptional activation of p21 and p27, FOXO1 prevents cell growth. Additionally, through the PI3K/AKT signaling pathway, insulin signaling also causes several metabolic consequences that are likely caused by these forkhead transcriptional factors.

To mediate crosstalk between the PI3K/AKT signaling pathway and other signaling pathways, two AKT substrates, GSK3β and the tuberous sclerosis complex TSC1/2, are crucial [*Chen et al., 2018*]. In Wnt signaling, GSK3β is a crucial regulator. By phosphorylating β-catenin, it triggers ubiquitin-mediated destruction of the protein. Some of the effects of PTEN on the control of stem cell maintenance and G0-G1 cell cycle regulation may be explained by the interaction between PTEN and Wnt signaling. TSC1/2 is essential for coordinating the regulation of cell growth and proliferation with the regulation of metabolism and cell size. The mTOR (mammalian target of rapamycin) function is suppressed by the heterodimer of TSC1 and TSC2. When phosphorylated by AKT, TSC2 activity is suppressed [*Zhou et al., 2010*]. As a result, AKT stimulates mTOR activity by

acting on TSC2, which in turn triggers mTOR activation's downstream processes, such as metabolic changes, protein translation, and cell proliferation [*Saxton et al., 2017*]. This crosstalk between PTEN and another tumor suppressor, LKB1, is made possible by the control of mTOR by the AKT-TSC-mediated signal.

1.8 Function of PTEN in cell cycle and genome stability

The constitutive activation of oncogenic proteins and mutational inactivation of DNA repair proteins are the primary causes of tumor formation. As neoplastic transformation exhibits genomic aberration, preserving genomic stability is the key cause to prevent cancer predisposition. A wide variety of PTEN-deficient human malignancies exhibit frequent PTEN mutation and genomic instability, albeit the underlying mechanism is yet unknown. The nuclear PTEN protein is known to exert tumor suppression in a lipid phosphatase-independent manner [*Song et al., 2011*]. It is noteworthy that PTEN's C-terminus allows for a range of phosphatase-independent activities in the nucleus. PTEN binds to the E3 ligase APC/C and strengthens its interaction with CDH1 to target mitotic cyclins and kinases, glutaminase, and PFKFB3 for degradation and promote growth inhibition and a tumor-suppressive metabolic state. To decatenate DNA during the G2/M cell cycle phases, PTEN interacts with topoisomerase II to stabilize the protein. PTEN/CENP-C binding also controls centromere stability (Table 1.4) [*Hou et al., 2017*]. Furthermore, genotoxic stress stimulates PTEN to bind with H2AX and Rad52, two DNA damage repair proteins, or to initiate autophagy mediated by AMPK. As a result, nuclear PTEN uses PPIs via its C-terminus to carry out its tumor-suppressive functions, which include preserving genome stability and regulating the cell cycle [*Bassi et al., 2013*]. The correct kinetochore assembly and the metaphase to anaphase transition during mitosis depend on the nuclear PTEN and CENP-C co-localizing in MEFs. PTEN-deficient cells express less Rad51. It interacts with the Rad51

promoter physically and works in concert with the E2F1 transcriptional factor to regulate Rad51 synergistically [*Orhan et al., 2021*].

Table 1.3 *PTEN interactors involved in the regulation of cell cycle progression and its exit.*

Cell Cycle	***Target***	***Mode of Action***
G1-S transition	RB	Activation/dephosphorylation by PTEN
	Cyclin A	Downregulation by PTEN
	Cyclin D	Downregulation by PTEN
	Cyclin E	Downregulation by PTEN
	$p21^{Cip1}$	Downregulation by PTEN
	p53	Acetylation by PTEN-associated p300
S phase	Chk1	Association with PTEN on replication fork
	Rad51	Association with PTEN on replication fork to promote fork restart
	MCM2	PTEN dephosphorylation to limit replication fork progression
G2-M transition	Chk1	Inhibition of PI3K/AKT-mediated phosphorylation
	CDK1	Association with PTEN
	Cyclin B1	Association with PTEN
M phase	Plk1	Downregulation/dephosphorylation by PTEN
	γ-tubulin	Downregulation by PTEN
	EG5	Association with PTEN to preserve spindle architecture

Nevertheless, additional research revealed that no contact between centromeric DNA and PTEN was detected in wild-type cells, suggesting that the genomic instability in PTEN-deficient cells could not be caused by a loss of PTEN at centromeres. The intrinsic genomic instability of PTEN-deficient cancers makes them vulnerable to the impacts of drugs that exacerbate this deficiency. PTEN expression is diminished in skin papillom and squamous

cell carcinoma (SCC) compared to skin devoid of these diseases [*Ming et al., 2009*]. Xeroderma Pigmentosum C (XPC) synthesis is inhibited by PTEN loss through the AKT/p38 signaling pathway. Mice with PTEN C-terminal deletion develop several spontaneous malignancies, such as cancers and B-cell lymphomas. PTEN has been shown to serve as a component of the cell cycle checkpoint for the G1/S and G2/M transitions in numerous studies. PTEN inhibits the G1-S transition by up-regulating p27Kip1 and down-regulating cyclin D1, which prevents the growth of tumors [*Brandmaier et al., 2017; Radu et al., 2003*]. Apart from blocking cyclin D1, nuclear PTEN can interact with p300 to promote p53 acetylation and stability, leading to G1 arrest. Additionally, metastatic human prostate cancer generally leads to loss of PTEN which results in the overexpression of cdc6 and cyclin E2. PTEN expression led to cdc6 and cyclin E2 level inhibition, which was connected to G1 arrest [*Wu et al., 2009*]. PTEN null cell displays early G2/M exit in response to genotoxic stress. The PI3K/AKT pathway hyperphosphorylates Chk1 (Ser 280) in a manner that prevents G2 cell cycle arrest when PTEN is knocked out [*Puc et al., 2005*].

1.9 PTEN's function in transcriptional regulation and chromatin integrity

Through its PPIs, nuclear PTEN can affect gene transcription. It interacts with E2F1 to stop the activity of cyclin D1, cyclin E1, and other genes that are involved in promoting cell proliferation [*Malaney et al., 2018*]. Through its binding to p53, PTEN can enhance the protein's transcription and binding to p53-dependent genes. It is also associated with BMI1 to deregulate the activity of p16INK4 and p14ARF, which are involved in cell cycle modulation [*Li et al., 2011*]. In addition to acting as a transcription factor, PTEN can also interact with chromatin and its associated histone proteins. These interactions can regulate the stability of chromatin and its condensation. PTEN's actions at the chromatin can hinder the transcription of important genes that are required for cancer cell proliferation. According to the researcher,

PTEN got connected with chromatin after DNA damage and this association requires PTEN to be phosphorylated at Ser366 and Thr370 sites [*Choi et al., 2013*]. Furthermore, they discovered that PTEN's nuclear translocation is similarly caused by the C2 domain. Considering that PTEN interacts with both Plk1 and centromere, as reported in previous studies, it could be pertinent to propose that Plk1 phosphorylates PTEN upstream, given that C-terminally truncated PTEN is unable to interact with centromere. [*Li et al., 2014*]. Additionally, Plk1 phosphorylates PTEN at Ser380, which is an important site for the development of p-PTEN and its interaction with chromatin [*Kotelevets et al., 2020*]. The presence of p-PTEN at Ser380 is essential for normal mitotic progression, as replacing Ser380 with Ala led to a significant reduction in the number of mitotic cells. In addition to repressing polyploid cell populations, PTEN can also dephosphorylate Plk1. This leads to mitotic arrest and prevents genome segregation [*Zhang et al., 2016*]. However, phosphorylated PTEN is unable to dephosphorylate Plk1 as it decreases phosphatase activity. It is tempting to think that there are mechanisms that can prevent PTEN from being dissociated from chromatin. In addition, a study suggested that Plk1 can phosphorylate PTEN and prevent its nuclear entry, which could promote tumor development [*Li et al., 2014*].

1.10 Post-translational modulations (PTMs) of PTEN

PTEN expression and activity are influenced by almost all levels of transcriptional, translational, and post-translational regulation. The most important post-translational modifications that affect PTEN regulation are phosphorylation, oxidation, acetylation, methylation, ubiquitination, sumoylation, and interactions with other proteins (Figure 1.4) [González-García et al., 2022]. These covalently linked alterations affect its cellular transport, protein-protein interactions, and enzymatic function directly.

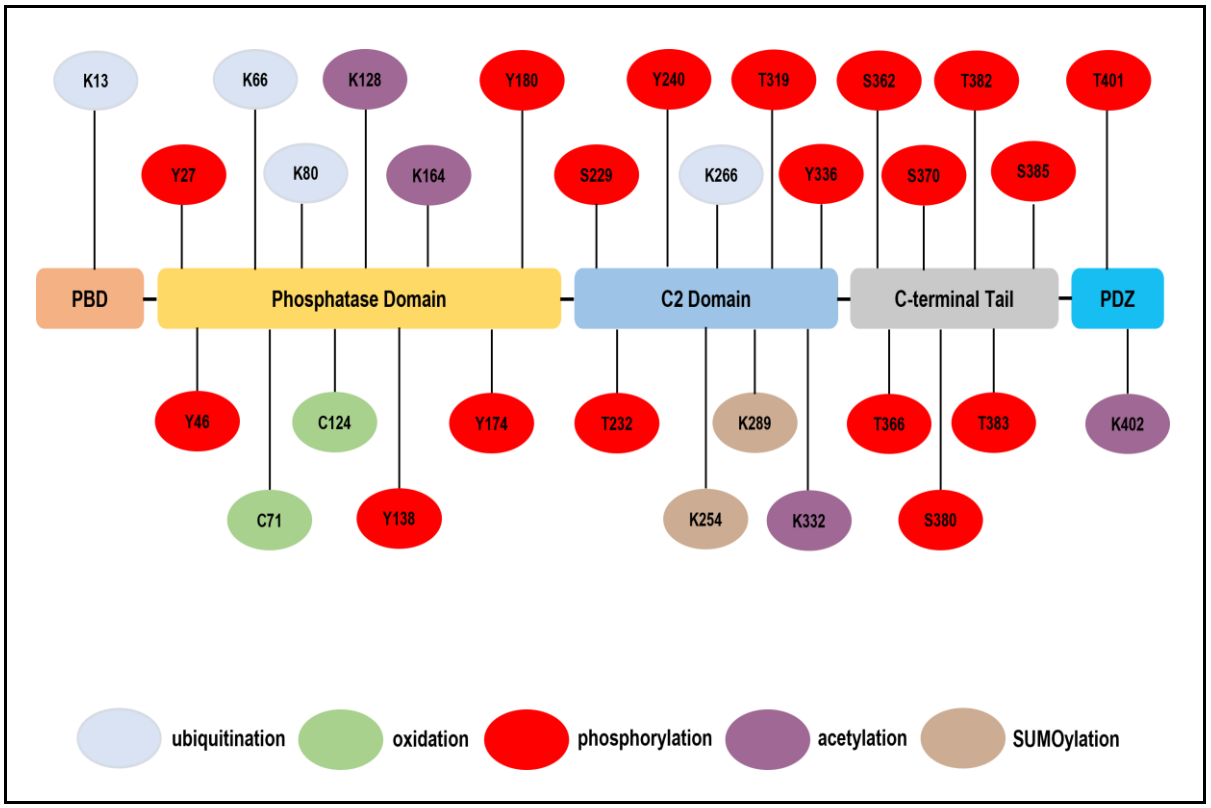
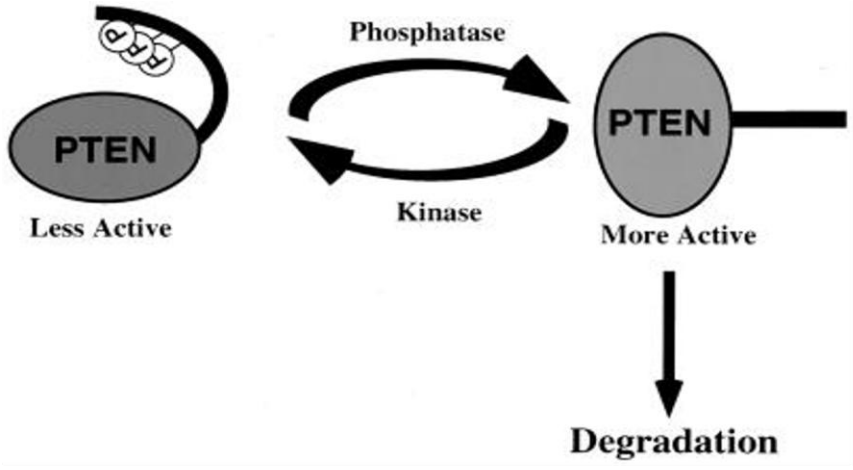


Figure 1.4 *Mutation status of human cancers at significant PTEN post-translational modification (PTM) sites. Diagrammatic representation of mutation numbers at each site for the five distinct types of PTMs are identified by indicated color codes.*

1.10.1 Phosphorylation

PTEN function, conformation, stability, and subcellular localization are all directly regulated by phosphorylation, the most extensive PTM of PTEN. PTEN has several phosphorylation sites in both its C2 and C-terminal domains [*Dempsey et al., 2021; Masson et al., 2020*]. When CK2 (casein kinase 2) phosphorylates Ser380, Thr382, Thr383, and/or Ser385 there is a conformational shift that leads the C-tail to fold back on the core PD and C2D. This conformation increases PTEN stability by blocking the ubiquitination sites that would otherwise cause PTEN to be degraded by proteasomes and digested by caspase-3 [*Chen et al., 2016; Odriozola et al., 2007; Rahdar et al., 2009*]. Additionally, the intramolecular C-tail/C2D interaction precludes the PTEN PDZ binding motif from interacting with MAGI-2

proteins and other PTEN-associated complex members [*Wu et al., 2000*]. This supramolecular complex must remain intact to prevent PTEN membrane recruitment and consequent p-AKT inhibition. PTEN is thereby rendered inactive and is retained in the cytoplasm when phosphorylation occurs at the C-tail cluster, despite improving stability (Figure 1.5).



Adopted from Vazquez F, Ramaswamy S, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail regulates protein stability and function. Mol Cell Biol. 2000;20(14):5010-8

Figure 1.5 *Phosphorylation of the PTEN tail and its regulation.*

Under oxidative stress, p70S6K phosphorylates PTEN at Ser380, leading to PTEN deubiquitylation and nuclear export and influencing PTEN nuclear activities [*Wu et al., 2014*]. It's interesting to note that the PTEN C-terminal interacting protein PICT-1 controls Ser380 phosphorylation and that Ser380 phosphorylation levels were drastically decreased in PICT-1 knockout mice [*Okahara et al., 2004*]. A second Plk1 phosphorylation site on PTEN, Ser385, increases PTEN stability by preventing polyubiquitination but prevents PTEN monoubiquitylation from excluding it from the nucleus [*Li et al., 2014*]. Ser385 phosphorylation by Plk1 also activates the PI3K pathway, which is linked to a metabolic condition that promotes cancer [*Li et al., 2014*]. PTEN can also undergo auto-

dephosphorylation, which controls its stability and activity by concentrating on the phosphorylated amino acid cluster at the end of the protein. Notably, when compared to PTEN wildtype protein, a phosphorylation deficient mutant with alanine substitutions at Ser380, Thr382, Thr383, and Ser385 exhibits increased lipid phosphatase activity and preferentially localizes to the nucleus [*Vazquez et al., 2000; Vazquez et al., 2001*]. Ser362 by GSK3, Ser370 by CK2 or Plk3, and Ser366 by GSK3 or Plk3 are additional C-tail phosphorylation sites that control its stability [*Maccario et al., 2007; Miller et al., 2002*]. PTEN function, localization, and stability are controlled collectively through the regulation of phosphorylation at all C-tail phosphorylation sites. Tyr240 phosphorylation, which may have been caused by FGFR2 following ionizing radiation, encouraged PTEN to interact with chromatin and Ki-67, attracting molecules that repair DNA such as Rad51 [*Ferrarelli et al., 2019*]. On the other hand, PTEN is destabilized and the PI3K pathway is initiated when Src phosphorylates Tyr240, Tyr336, and Tyr315 [*Lu et al., 2003*]. In GBM patients, Tyr240 phosphorylation is linked to a reduced overall survival rate, resistance to EGFR inhibitor therapy, and an active involvement in promoting resistance to EGFR inhibition in vitro [*Fenton et al., 2012*].

1.10.2 Acetylation

PTEN is acetylated at Lys125 and Lys128 by K acetyltransferase 2B (KAT2B, also known as PCAF), and this activity is dependent on growth factor stimulation [*Shi et al., 2012*]. On the other hand, PTEN function is improved by acetylation at Lys163 through membrane translocation [*González-García et al., 2022*]. CREB binding protein (CBP) acetylates PTEN at lysine 402 to enhance interactions with PDZ domain-containing proteins like MAGI-2 and hDLG, which are well-known regulators of cell shape and polarity [*Kotelevets et al., 2020*]. PTEN aids in the preservation of cell polarity and shape via binding to the MAGI/Par-3

complex [*von Stein et al., 2005*]. Consequently, it is plausible that PTEN acetylation directly regulates cell polarity, behavior, and shape. Histone deacetylases such as SIRT1, HDAC6, HDAC I, and HDAC II inhibit PTEN acetylation [*Chae et al., 2011; Meng et al., 2016*]. PTEN gets more acetylated when Sirtuin 1 is active. In mouse embryonic stem (mES) cells lacking SIRT1, PTEN becomes hyperacetylated and is removed from the nucleus [*Chae et al., 2011*].

1.10.3 Ubiquitination

In vivo, protein degradation frequently occurs by ubiquitination. PTEN stability and localization are regulated by PTEN ubiquitination at lysine residues 13, 66, and 289 in the protein [*Xia et al., 2020*]. Lys13 and Lys289 of PTEN can be mono- and polyubiquitinated by the E3 ligase NEDD4. By inhibiting its polyubiquitination and transferring it to the nucleus, the rise in monoubiquitylation levels stabilizes PTEN. PTEN is targeted for proteasomal destruction by the E3 ligases XIAP, WWP2, and NEDD4-1 when it is polyubiquitinated [*Xia et al., 2020*]. Since PTEN expression in NEDD4-1 positive or negative MEFs showed no discernible difference and PTEN was not demonstrated to interact with NEDD4-1, the function of NEDD4-1 remains unclear [*Fouladkou et al., 2008*]. PTEN expression levels in cancer are inversely correlated with elevated E3 ligase expression, indicating that these ligases adversely regulate PTEN expression to accelerate the onset of the disease [*Amodio et al., 2010*]. It's interesting to note that polyubiquitination by E3 ligase RFP lowers PTEN's phosphatase activity while not affecting PTEN stability or localization [*Lee et al., 2013*]. Another PTEN polyubiquitination site, Lys66 residue, controls protein stability [*Gupta et al., 2016*]. Moreover, molecules other than E3 ligases can control PTEN ubiquitination. PTEN ubiquitination is enhanced by tankyrase-mediated PTEN ribosylation and the NEDD4-1 activators Ndfip1 and Ndfip2 [*Mund et al., 2010*]. PTEN is de-monoubiquitinated by

HAUSP/USP7, which lowers the nuclear PTEN pool; nevertheless, PTEN is directly bound by USP13 and OTUD3, which deubiquitinate it to make it more stable [*Yuan et al., 2015*].

1.10.4 SUMOylation

A small ubiquitin-related modifier, or SUMO, is known to modify three specific locations on PTEN. PTEN is anchored to the plasma membrane by modifications at Lys266 and Lys289, where it can dephosphorylate the PIP3 substrate and inhibit the PI3K pathway [*Huang et al., 2012*]. PTEN is directed to the nucleus by SUMO modification at Lys254 [*Bassi et al., 2013*]. PTEN's subcellular location is thus determined by SUMOylation.

1.10.5 Oxidation

PTEN oxidation impairs phosphatase activity by forming a disulfide bond with the nearby Cys71 residue at the catalytic Cys124 residue [*Connor et al., 2005*]. AKT and other downstream signaling effectors are directly phosphorylated and activated as a result of oxidative inactivation. A layer of complexity has been added to control PTEN by thioredoxin by the discovery that thioredoxin-1, which decreases and reactivates PTEN, forms a disulfide bond with C2D of PTEN [*Schwertassek et al., 2014*]. This disulfide bond suppresses lipid phosphatase activity and stops PTEN from connecting with the plasma membrane. PTEN's binding to peroxiredoxin I prevents it from oxidizing [*Cao et al., 2009*]. It is unknown which oxidative species target the cysteine residues. However, in colorectal cancer, COX-2 or ALOX5 deletion and its inhibitors increased PTEN activity and inhibited the PI3K/AKT pathway, which in turn stopped the growth of cells and adenomas [*Covey et al., 2007*]. All together, these studies have demonstrated that reactive oxygen species generation can enhance AKT pathway signaling through PTEN inactivation via active site residue oxidation.

1.10.6 Methylation

The methylation of PTEN at lysine and arginine residues has recently been demonstrated, with unique functional effects [*Feng et al., 2019*]. PTEN's Ser380 phosphorylation is increased when SMYD2 methylates Lys313 of PTEN, which results in a closed, inactive conformation [*Kotelevets et al., 2020*]. The PTEN-mediated inhibition of the PI3K pathway, on the other hand, was strengthened by PRMT6's methylation of Arg159 [*Feng et al., 2019*]. Since PTEN has multiple conserved arginine residues that frequently undergo mutation in cancer, methylation at these arginine sites may reveal novel methods for influencing PTEN activities.

1.11 PTEN and DNA repair

All organisms must maintain their genomes properly through ongoing cell division cycles to ensure appropriate development, reproduction, and the prevention of various disorders, including cancer. DNA strand breaks brought on by ineffective topoisomerase I and topoisomerase II activity [*Kelley et al., 2014; McClendon et al., 2007*]. DNA mismatches occasionally introduced during DNA replication [*Nowosielska et al., 2008*], and ROS [*Srinivas et al., 2019*] created from common metabolic by-products that can attack DNA are just a few examples of endogenous mechanisms that can cause DNA damage. It is important to note that unrepaired DNA does not always prevent DNA replication from continuing because some polymerases that do not require exact base pairing can get around the obstruction. To defend themselves against these dangers, cells have created sophisticated surveillance systems that are capable of spotting DNA alterations and alerting pathways to their presence, delaying the advancement of the cell cycle, repairing the alterations, or eliminating the genetically unstable cells by inducing cell death.

The DNA of our cells can become damaged by a variety of external and internal factors, which can result in bases being absent or altered, bubbles being created by the insertion or deletion of a nucleotide, or a mixture of cross-linked strands and linked pyrimidines [*Martin et al., 2005*].

The primary cytotoxic lesion brought on by ionizing radiation and radio-mimetic substances is a DNA DSB, while it can also result from DNA polymerase encountering a DNA single-strand break and stalling replication forks. DSBs are known to influence cell viability and genomic instability and to be more hazardous and cytotoxic. Two distinct processes are primarily used by eukaryotic organisms to repair these damages. One of these is homologous recombination (HR), while the other is non-homologous end joining (NHEJ). DSBs are regarded as the biggest threat to DNA integrity because they disrupt both double helix strands, leaving no intact complementary strand to serve as a template for repair. Because homologous sequences, typically from sister chromatids, are employed to prime repair synthesis, HR is regarded as an "error-free" process for DSB repair. NHEJ, on the other hand, is typically regarded as an error-prone process because it does not need a DNA template. It should be emphasized that NHEJ is active at any phase of the cell cycle, making it the most frequent method for repairing DSBs. HR is restricted to only functioning during the S and G2 phases of the cell cycle since it requires a sister chromatid [*Brandsma et al., 2012*].

1.11.1 Homologous recombination (HR)

HR and NHEJ are the two main repair processes by which mammalian cells fix DSBs. While NHEJ is prone to errors and occurs throughout the cell cycle [*Bétermier et al., 2014*], HR is a more precise and error-free mechanism that mostly happens in the S phase [*Elbakry et al., 2021*]. When one or both of these processes are compromised, mutations that cause cancer accumulate and genomic instability occurs. A variety of human malignancies have been

linked to PTEN mutations. Through a variety of downstream effectors, constitutively activated levels of AKT result from PTEN inactivation, which supports cell growth, proliferation, survival, and migration [*Fusco et al., 2020*]. However, more data pointed to PTEN's involvement in DNA damage repair and other processes unrelated to PI3K/AKT signaling [*Ming et al., 2012*]. PTEN loss results in genomic instability and spontaneous DSBs in mouse embryonic fibroblasts [*Ming et al., 2012*]. Numerous research groups' use of an *in vivo* plasmid-based assay indicates that PTEN depletion reduced the frequency of HR repair [*Mansour et al., 2018*]. HR frequency increased considerably when they ectopically transfected PTEN in PTEN null cells. Through encircling the Rad51 promoter or modifying chromatin, PTEN mechanistically upregulates Rad51 transcription by allowing E2F1 to regulate Rad51 transcription [*Grundy et al., 2020*]. However, a different study contends that the PTEN C-terminal phospho mutant represses transcription at the promoter region of several genes by physically interacting with E2F1 [*Malaney et al., 2018*]. According to a different study, Rad51 protein expression was markedly up-regulated in wild-type PTEN and phosphatase dead PTEN following ionizing radiation-induced DNA damage, but not in sumo defective PTEN transfected cells [*Zhao et al., 2017*]. Through direct dephosphorylation of 53BP1, researchers discover that SUMOylated PTEN downregulates NHEJ repair while promoting HR repair [*He et al., 2023*]. PTEN was effectively interacting with phosphorylated p14ARF during DNA damage responses, which encouraged PTEN to SUMOylate as an atypical SUMO E3 ligase [*He et al., 2023*]. Remarkably, in DNA-break locations, SUMOylated PTEN was subsequently recruited to the chromatin. This occurred as a result of the SUMO-interacting motif of BRCA1, which is situated at the center of 53BP1 foci on the chromatin during the S/G2 stage, recognizing and binding SUMO1 conjugated to PTEN [*He et al., 2023*].

1.11.2 Non-homologous end joining (NHEJ)

In the mammalian system, the NHEJ pathway plays a significant role in DNA double-strand break repair. Compared to other repairing strategies, it is more flexible [*Lieber et al., 2010*]. It is thought to be the primary reason for DSB error-prone repair because it commonly occurs in conjunction with minor deletions at the repair break location.

According to recent research, PTEN increases the NHEJ repair pathway by directly inducing the expression of NHEJ1/XLF, a factor involved in DNA end ligation and bridging [*Sulkowski et al., 2018*]. Again, acetylated PTEN at Lys128 interacts with the promoter of XLF to recruit PCAF and CBP, histone acetyltransferase, and cause activation of the gene [*Okumura et al., 2006*]. However, a different study claimed that nuclear PTEN inhibits the error-prone NHEJ pathway by poly(ADP-ribosylating) Ku70 and preventing Ku70 from attaching to DNA DSB [*Guan et al., 2016*]. Consequently, DNA-PKcs cannot bind DNA breaks to carry out the repair process. They also demonstrate the importance of PTEN sumoylation for this function, rather than the requirement for phosphatase activity. PTEN may indirectly positively control NHEJ via the 53BP1-mediated pathway, since PTEN loss results in chromatin disorder, impaired H3K9me3 protein production, and foci formation [*Svobodová Kovaříková et al., 2018*]. The epigenetic marker H3K9me3 is significant for the 53BP1 protein-mediated NHEJ's potential to function effectively. To better understand the treatment strategy for targeting the NHEJ pathway in cancer cells, more research into the molecular involvement of PTEN and its post-translational regulation in controlling the NHEJ repair pathway is required.

1.12 PTEN and DNA replication

If cells are unable to fix their DNA through a checkpoint inaccuracy, then mutations will appear in the genome. This process can lead to the emergence of daughter cells that carry the mutations from their parents. To prevent cancer development, the regulation of DNA

replication must be tightly controlled. When DNA replication is de-regulated, cells may start to initiate unsystematically and generate genomic instability and DNA damage. When the genome is unstable, PTEN can potentially function as a part of the machinery that replicates DNA to maintain its integrity [*Hou et al., 2017*]. Various reports are suggesting that PTEN can be involved in DNA replication [*Hou et al., 2017*]. In comparison to wild-type cells, PTEN null cells showed more anaphase bridges. Additionally, the impaired replication process in PTEN-deficient cells implies endogenous replication stress [*He et al., 2015*]. Compared to wild-type cells, the treatment of PTEN nulls with either Hydroxyurea or Aphidicolin significantly hinders the replication fork's ability to restart [*Fan et al., 2020; He et al., 2015*]. After experiencing stress, the protein RPA1 binds to the DNA strands near the newly synthesized ones. In addition to recruiting OTUB1, the PTEN promotes accumulation by interacting with the RPA1's C-terminal domain. This prevents the organism from ubiquitinating, which stabilizes the replication fork [*Wang et al., 2015*]. A report also noted that the PTEN's activity can limit the movement of the replication fork due to external replication stress by affecting the phosphatase activity [*Misra et al., 2022*]. For the activation of the intra-S checkpoint, ssDNA coated with RPA acts as a common intermediate structure. As a result, PTEN preserves the integrity of the intra-S phase checkpoint [*Hou et al., 2017*].

The Rationale behind the study

The Rationale behind the study

Mutational inactivation of DNA repair proteins and the constitutive activation of oncogenic proteins are the primary causes of tumor formation. Therefore, keeping genomic stability is the primary factor in preventing cancer propensity as neoplastic transition exhibits genomic aberration. The role that PTEN has been given up to this point shows that it possesses tumor-suppressive properties that go beyond its capacity to obstruct AKT and also include DNA damage repair. To develop a strategy to combat cancer and other diseases associated with PTEN mutations, it is imperative to have a thorough understanding of the role of the tumor suppressor protein PTEN, particularly the role of nuclear PTEN in DNA damage repair. This is because the majority of chemotherapeutic drugs currently in use target proteins involved in the DNA repair pathway. PTEN's catalytic function is further maintained by a variety of post-translational modifications, which are also responsible for its trafficking within cells and the nucleus. Additionally, it has been revealed that nuclear PTEN is resilient to destruction caused by ubiquitination. It appears that PTEN function in the nucleus is finely regulated by post-translational regulation. Therefore, it is crucial to investigate how PTEN's post-translational alterations appear to relate to its nuclear activity. As a result, efforts were made to investigate PTEN's additional role in post-translational modulation of its DNA repair dynamic. Therefore, if we are aware of how PTEN modulation affects its localization and DNA repair, we can devise a strategy for treating cancers in which PTEN has been altered or deleted.

Chapter 2

Phosphorylation and phosphatase activity of PTEN are responsible for the maintenance of nuclear activities and preservation of genomic integrity

Phosphorylation and phosphatase activity of PTEN are responsible for the maintenance of nuclear activities and preservation of genomic integrity

2.1 Introduction

A combination of structural and functional damage at several levels, such as the genome, epigenome, and metabolome can lead to cancer. The combined effects of genetic and epigenetic modifications drive transformation among these numerous aberrant events, while metabolic responses provide selective pressures that contribute to the clonal development of cancer [*Sun et al., 2022*]. The dysregulation of replication origin activation in cancer cells leads to an accumulation of fragile, unreplicated, or highly replicated DNA, which ultimately causes genomic instability and aggressive proliferation capacity [*Boyer et al., 2016*]. Even more intriguingly, research employing PTEN models has revealed its critical functions in controlling metabolism, the epigenome, and the genome.

Understanding PTEN's involvement in its several tumor suppressor capabilities has drawn more attention in recent decades. PTEN is either mutated or inactivated in a variety of tumor lineages, including glioblastoma, breast, prostate, hepatocellular carcinoma, melanoma, and endometrial cancer [*Álvarez-Garcia et al., 2019; Milella et al., 2015*]. PTEN functions to boost its tumor suppressor activity in the nucleus by directing and maintaining genomic stability, as well as in the cytoplasm by downregulating the PI3K-AKT pathway [*Chen et al., 2018*]. It becomes the protector of the genome by regulating many essential cellular processes, such as the G1 and G2 checkpoints of the cell cycle, DNA damage repair, appropriate chromosome segregation, and the advancement of DNA replication [*Brandmaier et al., 2017; Fan et al., 2020*]. Compared to wild-type cells, PTEN null cells have been shown to exhibit an early S-phase departure and to be well-adapted to overcome intra-S-phase checkpoints. Aberrant DNA replication and early S-phase exit induce genomic

instability in daughter cells and are considered probable causes of cancer promotion. Replication stress is brought on by any type of DNA damage during the S-phase, and if it goes undetected and compromises checkpoint machinery, it has become a significant cause of genomic instability [*Técher et al., 2017*]. Genomic stability is seriously threatened by the "Achilles' heel" of controlling and monitoring DNA damage during the S-phase, which needs to be strictly controlled. Replication stress is essential for remodeling the chromatin and establishing an effective framework that allows DNA repair machinery to function. Run-on replication can result in chromosome fragility, gene loss, or mutations during replication stress [*Briu et al., 2021*]. Checkpoint malfunctions under replication stress lead to failed transmission of DNA damage response signals, which in turn causes more aneuploidy [*Branzei et al., 2009*]. The influence of epigenetic modifications made to chromosomes during carcinogenesis on cancer propensity and early prognosis has recently increased.

Furthermore, it has been discovered that PTEN localizes at centromeres and that centromeric instability results from the disruption of PTEN's interaction with centromeres [*Fan et al., 2020; Shen et al., 2007*]. Nevertheless, it is still unclear how precisely nuclear PTEN regulates the centromere and other nuclear components. A distinct compact chromatin structure called heterochromatin makes up the mammalian centromere, which is necessary for proper centromere stability and function [*Allshire et al., 2018*]. Some of the epigenetic changes that produce the heterochromatin include histone hypoacetylation, restricted histone H3K9me3, and the accumulation of the non-histone protein HP1α, which attracts in other essential downstream heterochromatin modifying proteins [*Allshire et al., 2018*]. It is noteworthy that several malignancies, including those of the breast, brain, colon, and thyroid, have decreased expression of the HP1α, which is a sign of defective chromatin condensation or organization [*Aggarwal et al., 2019*]. Prostate cancer is also reported to have significantly lower levels of H3K9me3, another histone epigenetic marker, as compared to prostate tissue

that is not malignant [*Ellinger et al., 2010*]. However, H3K9me3 enrichment is seen in breast cancer, and this may be related to invasion and metastasis [*Zhou et al., 2022*]. A flaw in any one of the heterochromatin-associated proteins could cause widespread epigenetic instability, which could affect the expression patterns of genes and ultimately result in aberrant characteristics of growth. Chromatin accessibility is increased when the epigenetic signature linked to late replicating heterochromatin, such as H3K9me3, is disrupted [*Ellinger et al., 2010*]. As a result, heterochromatin preservation is essential for cancer cell genetic stability, which is closely related to the dynamics of replication, including initiation, space, time, and progression. Therefore, PTEN plays a role in both maintaining heterochromatin and controlling DNA replication. Moreover, it has been found that post-translational modifications such as SUMOylation, phosphorylation, acetylation, and methylation at different locations are necessary for PTEN to protect or repair DNA [*Ramazi et al., 2021*]. The C-terminal region of PTEN exhibits a favorable correlation with genetic stability via lysine residue 254 and 349 methylation and SUMOylation, respectively [*Liu et al., 2019*]. This led us to look into the roles that PTEN's various post-translational modifications play in DNA replication. Using the PTEN-null PC3 cells as a model system, we demonstrated how PTEN binds to chromatin when its C-terminal is phosphorylated, leading to the formation of heterochromatin in response to exogenous replication stress. Thus, to prevent cell division under stressful circumstances, PTEN triggers CHK1 through heterochromatin formation. It has also been observed that the phosphatase activity of PTEN is necessary for the detection of replication stress, but not SUMOylation. Therefore, PTEN serves as a stress-sensing protein that efficiently reduces genomic instability by blocking replication in stressful circumstances.

2.2 Aims and Objectives of Work

- To create various PTEN mutant clones by site-directed mutagenesis e.g., phospho-dead mutant, phospho-mimetic mutant, phosphatase-dead mutant, and SUMOylation dead mutant PTEN from PTEN-WT clone.
- How exogenous DNA damage changes the phosphorylation status of PTEN.
- To study the effect of PTEN and its different mutants in DNA replication dynamics after replication stress.

2.3 Results

2.3.1 Generation of different PTEN clones by site-directed mutagenesis and their expression

Using an Agilent site-directed mutagenesis kit, we create several PTEN mutant clones from PTEN-WT by the manufacturer's instructions. Following transformation, a single colony was isolated, sequenced, and verified with the aid of a sequencing primer (see the methods and materials section for more information).

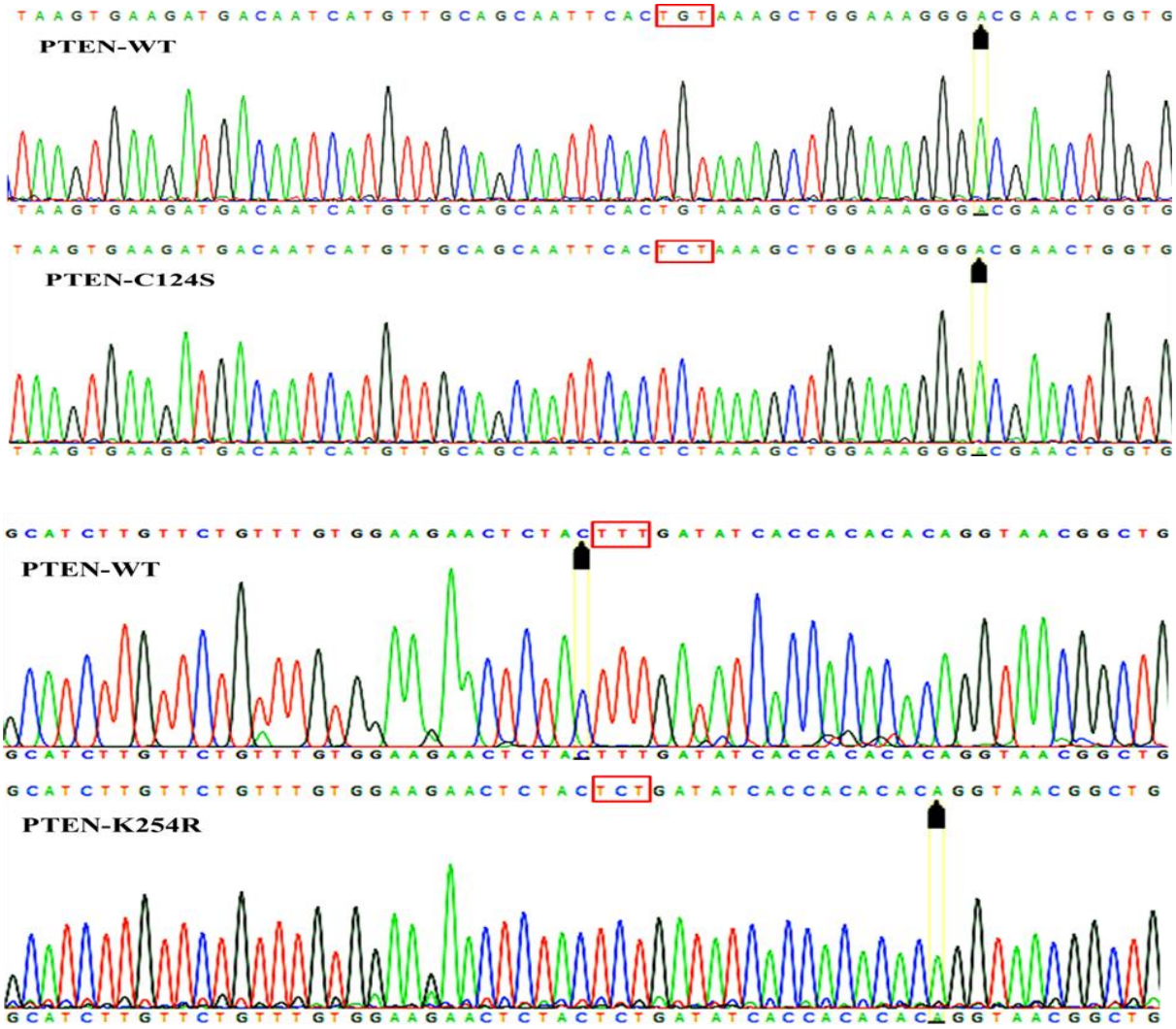


Figure 2.1 *DNA sequencing result of Phosphatase-dead PTEN (C124S) and sumo-dead PTEN (K254R).*

2.3.2 3D structure prediction of PTEN and its different mutants

The previously obtained nucleotide sequences were translated to protein sequences (FASTA format) using the Expasy Translate Tool. The protein sequence thus obtained was then introduced to the iTASSER webserver for the structure prediction (Figure 2.2).

The protein sequences obtained for PTEN and its mutants in FASTA format are given below:

PTEN-WT:

MTAIIKEIVSRNKRRYQEDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFLD
SKHKNHYKIYNLCAERHYDTAKFNCRVAQYPFEDHNPPQLELIKPFCEDLDQWLSE
DDNHVAAIHCKAGKGRTGVMICAYLLHRGKFLKAQEALDFYGEVRTRDKKGVTIPS
QRRYVYYYSYLLKNHLDYRPVALLFHKMMFETIPMFSGGTCNPQFVVCQLKVKIYS
SNSGPTRREDKFMYFEFPQPLPVCGDIKVEFFHKQNKMLKKDKMFHFWVNTFFIPGP
EETSEKVENGSLCDQEIDSICSIERADNDKEYLVLTLTKNDLDKANKDKANRYFSPNF
KVKLYFTKTVEEPSNPEASSSTSVTPDVSDNEPDHYRYSDTTDSDPENEPFDEDQHTQ
ITKV

PTEN-A4:

MTAIIKEIVSRNKRRYQEDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFLD
SKHKNHYKIYNLCAERHYDTAKFNCRVAQYPFEDHNPPQLELIKPFCEDLDQWLSE
DDNHVAAIHCKAGKGRTGVMICAYLLHRGKFLKAQEALDFYGEVRTRDKKGVTIPS
QRRYVYYYSYLLKNHLDYRPVALLFHKMMFETIPMFSGGTCNPQFVVCQLKVKIYS
SNSGPTRREDKFMYFEFPQPLPVCGDIKVEFFHKQNKMLKKDKMFHFWVNTFFIPGP
EETSEKVENGSLCDQEIDSICSIERADNDKEYLVLTLTKNDLDKANKDKANRYFSPNF
KVKLYFTKTVEEPSNPEASSSTSVTPDVSDNEPDHYRYADAADADPENEPFDEDQHT
QITKV

PTEN-C124S:

MTAIIKEIVSRNKRRYQEDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFLD
SKHKNHYKIYNLCAERHYDTAKFNCRVAQYPFEDHNPPQLELIKPFCEDLDQWLSE
DDNHVAAIHSKAGKGRTGVMICAYLLHRGKFLKAQEALDFYGEVRTRDKKGVTIPS
QRRYVYYYSYLLKNHLDYRPVALLFHKMMFETIPMFSGGTCNPQFVVCQLKVKIYS
SNSGPTRREDKFMYFEFPQPLPVCGDIKVEFFHKQNKMLKKDKMFHFWVNTFFIPGP
EETSEKVENGSLCDQEIDSICSIERADNDKEYLVLTLTKNDLDKANKDKANRYFSPNF
KVKLYFTKTVEEPSNPEASSSTSVTPDVSDNEPDHYRYSDTTDSDPENEPFDEDQHTQ
ITKV

PTEN-C124S-A4:

MTAIIKEIVSRNKRRYQEDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFLDS
KHKNHYKIYNLCAERHYDTAKFNCRVAQYPFEDHNPPQLELIKPFCEDLDQWLSEDD
NHVAAIHSKAGKGRTGVMICAYLLHRGKFLKAQEALDFYGEVRTRDKKGVTIPSQRR
YVYYYSYLLKNHLDYRPVALLFHKMMFETIPMFSGGTCNPQFVVCQLKVKIYSSNSG
PTRREDKFMYFEFPQPLPVCGDIKVEFFHKQNKMLKKDKMFHFWVNTFFIPGPEETS
EKVENGSLCDQEIDSICSIERADNDKEYLVLTLTKNDLDKANKDKANRYFSPNFKVKL
YFTKTVEEPSNPEASSSTSVTPDVSDNEPDHYRYADAADADPENEPFDEDQHTQITKV

PTEN-K254R:

MTAIIKEIVSRNKRRYQEDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFLDS
KHKNHYKIYNLCAERHYDTAKFNCRVAQYPFEDHNPPQLELIKPFCEDLDQWLSEDD
NHVAAIHCKAGKGRTGVMICAYLLHRGKFLKAQEALDFYGEVRTRDKKGVTIPSQR
RYVYYYSYLLKNHLDYRPVALLFHKMMFETIPMFSGGTCNPQFVVCQLKVKIYSSNS
GPTRREDKFMYFEFPQPLPVCGDIRVEFFHKQNKMLKKDKMFHFWVNTFFIPGPEET

SEKVENGSLCDQEIDSICSIERADNDKEYLVLTLTKNDLDKANKDKANRYFSPNFKVK

LYFTKTVEEPSNPEASSSTSVTPDVSDNEPDHYRYSDTTDSDPENEPFDEDQHTQITKV

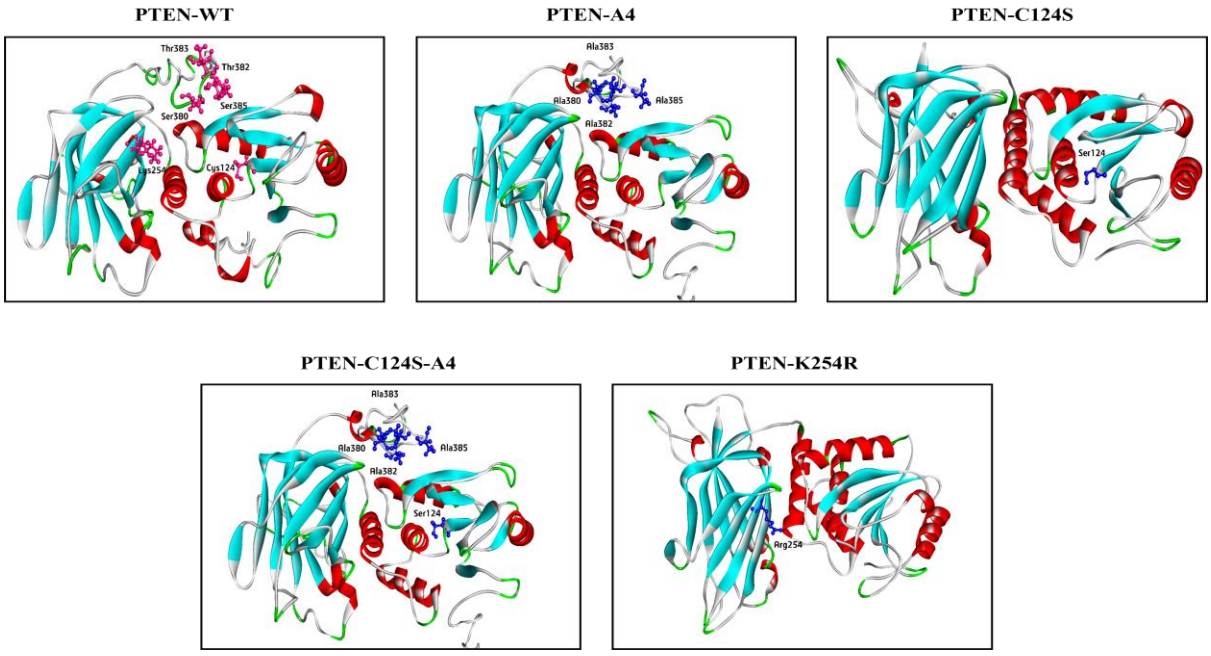


Figure 2.2 *3D structures of PTEN and its mutants.*

2.3.3 Different mutations of PTEN do not effect the stability of mutant proteins

Protein stability involves the balance of forces determining whether a protein will either remain in its native form or be denatured. Although it is difficult to rationalize protein stabilization, a detailed analysis of some carefully chosen mutants could provide us with valuable information on the underlying mechanisms. The stability of a protein is usually regarded as the Gibbs free energy (ΔG) that plays a role in the biophysical process of folding. Its magnitude is determined by its interactions with the surrounding environment and the amino acid residues. Therefore, the alteration in the thermodynamic stability of proteins (i.e., ΔΔG) brought forth by mutations is crucial to daily life. Lower ΔG indicates more stable protein structures and positive entropy change contributes negatively, or stabilizes, to the free

energy of protein folding. It is also believed that the presence of hydrophobic interactions contributes to the stabilization of protein structures in an aqueous environment. These interactions have been regarded as important for stabilizing protein structures and driving aggregation. In this study, we present a thermodynamic examination of PTEN mutants that have undergone one or more amino acid substitutions that have dramatically stabilized the protein (Table 2.1 & 2.2). We examine how hydrophobicity affects the functional effects of various PTEN mutations and observed that wild-type PTEN and different PTEN mutants show nearly no difference in hydrophobicity percentage confirming the fact that mutation has no impact on PTEN protein stability (Table 2.1) (Figure 2.3). The stability of the mutations of each predicted protein was studied using DynaMut and mCSM servers.

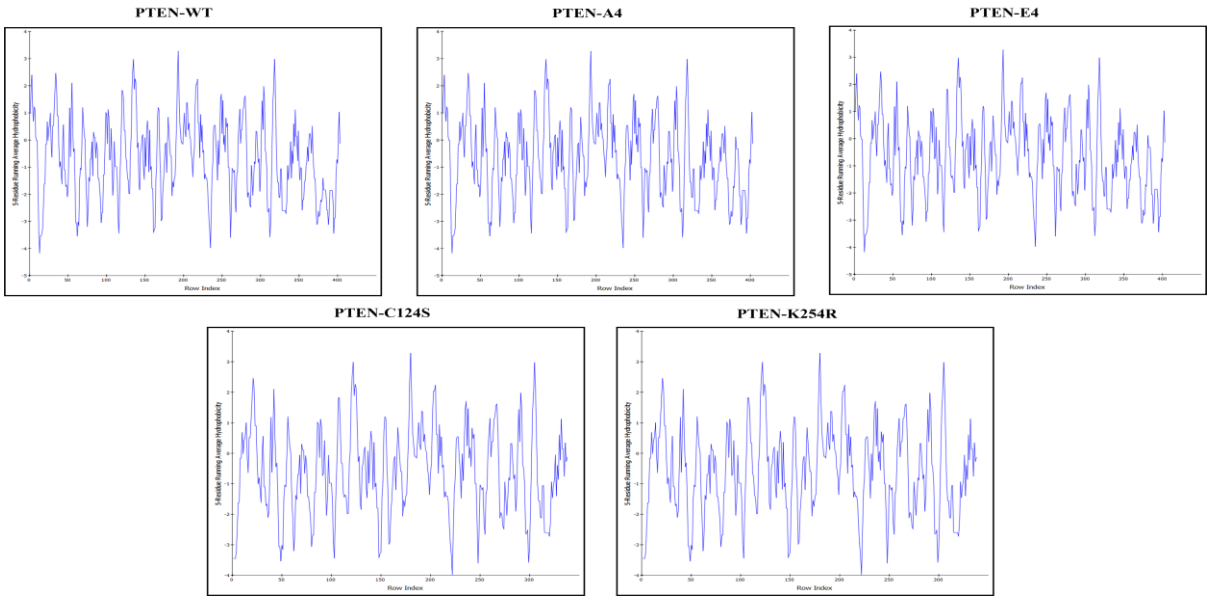


Figure 2.3 *Hydrophobicity plot of wild-type and different PTEN mutants.*

Table 2.1 *Change in Stability due to Mutations and % Hydrophobicity.*

Clones	***Mutations***	***ΔΔG (Mutation stability) kcal/mol***		***ΔΔS (Vibrational Entropy) kcal/mol***	***% Hydrophobicity***
		Dynamut	***mCSM***	***Dynamut***	
PTEN-WT					Hydrophobic: 36.48% Acidic: 15.38% Basic: 16.63% Neutral: 31.51%
PTEN-A4	S380A	1.024 (Stabilizing)	-0.987 (Destabilizing)	-0.209 (Decrease of molecule flexibility)	Hydrophobic: 37.47% Acidic: 15.38% Basic: 16.63% Neutral: 30.52%
	T382A	0.101 (Stabilizing)	0.346 (Stabilizing)	0.090 (Increase of molecule flexibility)	
	T383A	0.528 (Stabilizing)	0.904 (Stabilizing)	0.180 (Increase of molecule flexibility)	
	S385A	-0.145 (Destabilizing)	-0.497 (Destabilizing)	-0.129 (Decrease of molecule flexibility)	
PTEN-E4	S380E	1.078 (Stabilizing)	-0.989 (Destabilizing)	-0.209 (Decrease of molecule flexibility)	Hydrophobic: 37.47% Acidic: 15.38% Basic: 16.63% Neutral: 30.52%
	T382E	0.101 (Stabilizing)	0.346 (Stabilizing)	0.090 (Increase of molecule flexibility)	
	T383E	0.467 (Stabilizing)	0.854 (Stabilizing)	0.180 (Increase of molecule flexibility)	
	S385E	-0.245 (Destabilizing)	-0.612 (Destabilizing)	-0.129 (Decrease of molecule flexibility)	
PTEN-C124S	C124S	-0.466 (Destabilizing)	-0.997 (Destabilizing)	0.278 (Increase of molecule flexibility)	Hydrophobic: 36.48% Acidic: 15.38% Basic: 16.63% Neutral: 31.51%
PTEN-K254R	K254R	1.437 (Stabilizing)	-1.213 (Destabilizing)	-0.420 (Decrease of molecule flexibility)	Hydrophobic: 36.48% Acidic: 15.38% Basic: 16.63% Neutral: 31.51%

Table 2.2 *Energetics of wild-type PTEN and its mutants.*

Protein	Potential Energy (kcal/mol)	Van der Waals Energy (kcal/mol)	Electrostatic Energy (kcal/mol)
PTEN-WT	-12306.81294	-3267.46839	-12024.63796
PTEN-A4	-12063.66456	-3185.17424	-11870.25236
PTEN-C124S-A4	-12070.83151	-3173.38795	-11902.91691
PTEN-C124S	-12296.28863	-3355.88021	-12684.54896
PTEN-K254R	-12545.31310	-3306.57274	-12010.14278

2.3.4 Double thymidine block arrests S-phase leading to accumulation of p-PTEN in the nucleus

In a previous study, our lab and another one found that, following DNA damage induced by etoposide and IR, respectively, phosphorylated PTEN accumulates in the nucleus [*Misra et al., 2019; Wei et al., 2018*]. We wish to investigate the state of phosphorylated PTEN in replication when it builds up in the nucleus as a result of DNA damage. In this study, the cells were double thymidine blocked to arrest them in the S phase, and both the etoposide-treated cells and the S-phase blocked cells were immune stained with the p-PTEN and total PTEN antibodies. The phosphorylated PTEN, however, behaved very similarly to what we had observed in etoposide-treated cells when we synchronized WI-38 cells at S-phase by double thymidine block, but total PTEN was distributed throughout the cells (Figure 2.4). These findings suggest a possible connection between phosphorylated PTEN and DNA replication.

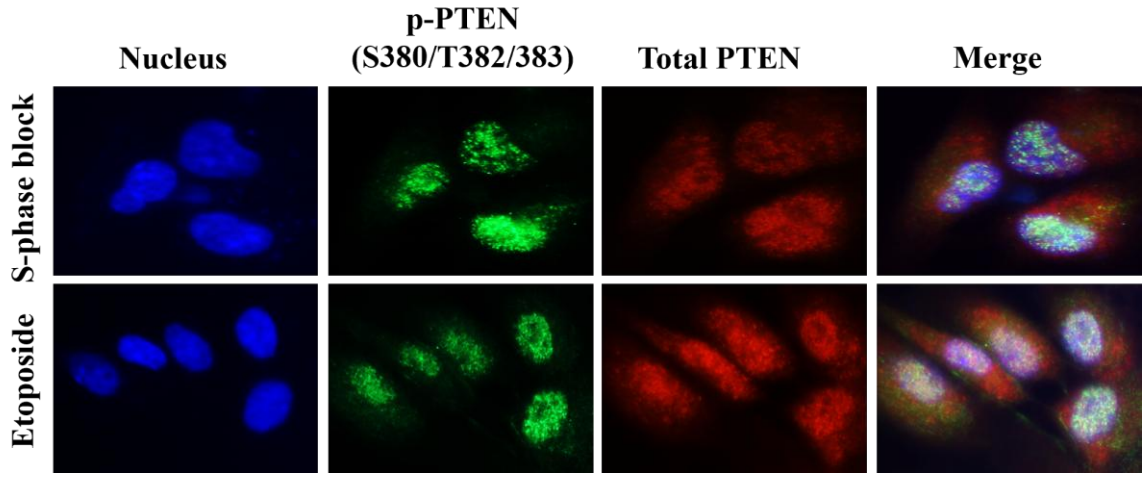


Figure 2.4 *Representative photomicrographs of S-phase arrested control and etoposide-treated WI-38 cells immune labelled with total PTEN (red) or p-PTEN (S380/T382/383) (green).*

2.3.5 Aphidicolin-mediated replication arrest is associated with phosphatase activity and carboxy-terminal phosphorylation of PTEN

We examine whether the post-translational regulation of the PTEN is related to DNA replication in light of our discovery that the accumulation of phosphorylated PTEN in the nucleus is related to S-phase. The PTEN isogenic cell line models were initially created by overexpressing plasmids bearing HA fusion constructs with PTEN wild-type (PTEN-WT) and PTEN C-terminal phosphorylation-dead mutant (PTEN-A4), PTEN C-terminal phosphorylation-mimetic mutant (PTEN-E4), PTEN phosphatase-dead mutant (PTEN-C124S), and PTEN SUMOylation-dead mutant (PTEN-K254R) in PTEN-negative PC3 cells. After 1 h of treatment; the DNA replication inhibitor aphidicolin (APH) was released, BrdU labelling was performed on them, and the BrdU-positive cells were then analyzed. Compared to PTEN-null cells (42.25%), the percentage of BrdU-positive cells was much lower (17.9%) when wild-type PTEN was expressed (Figure 2.5.A). Similarly, there were fewer BrdU-positive cells in the cells harboring sumoylation-mutant plasmids. On the other hand, the cells

expressing the phosphorylation-dead, phosphorylation-mimetic, and phosphatase-dead PTEN mutants had a significantly higher percentage of cells with BrdU signal (30.66%, 31.44%, and 29.81%, respectively) in comparison to the cells expressing the wild type PTEN (Figure 2.5.A). In light of these findings, it may be concluded that replication stress might be alleviated in PTEN null cells by the expression of wild-type PTEN and that this process was unaffected by PTEN's SUMOylation. However, this mechanism can be impacted by the PTEN C-terminal phosphorylation cluster and its phosphatase activity.

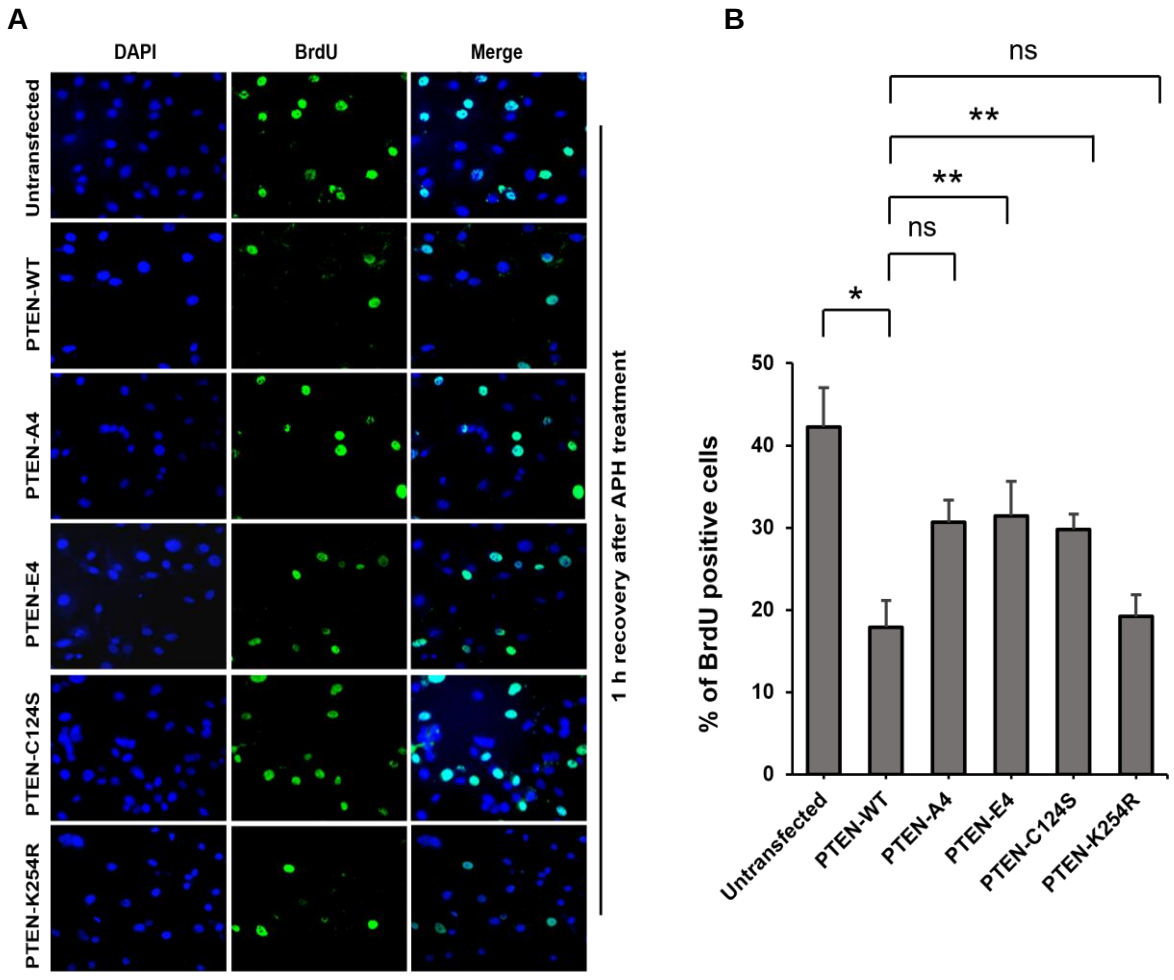


Figure 2.5 *(A) BrdU (green) immunostaining image of untransfected and various PTEN clone transfected cells after 1 h release from APH treatment (3 h). (B) Quantitative data of BrdU staining. Values are the mean ± SD of three independent experiments. *P < 0.05.**P < 0.005.*

2.3.6 Elevated DNA damage co-exists with CHK1 inactivation in phospho-dead and phosphatase-dead PTEN transfected cells

We transfected different PTEN clones into PC3 cells to see if replication restraint with APH is linked to the persistence of DNA damage and assessed the amount of γH2AX foci after 3 h of APH treatment. We found that compared to untransfected cells, PTEN transfected cells have significantly fewer, less intense γH2AX foci (Figure 2.6.A). Again, phospho and phosphatase dead PTEN transfected cells exhibit higher γH2AX foci intensity than PTEN-WT transfected cells. These findings show that cells with mutant PTEN or those that are not transfected continue to multiply even with their damaged DNA.

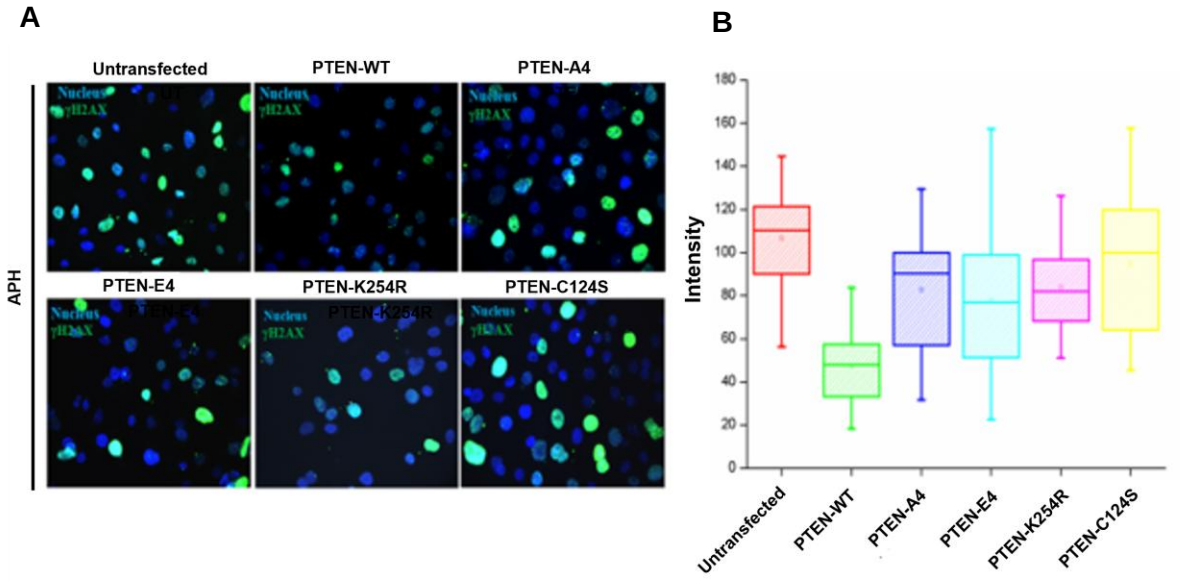


Figure 2.6 *(A) A representative photomicrograph shows the green γH2AX foci on both untransfected and mutant PTEN clones transfected cells. (B) Quantification of γH2AX intensity per nucleus in transfected cells expressing PTEN and PTEN mutants following APH treatment.*

We examined the CHK1 phosphorylation status (Ser345) in several PTEN clone transfected cells to see whether the accumulation of damaged DNA is connected to restricted CHK1 activation, as active CHK1 can provide replication fork stability. We found that CHK1 activation is compromised in A4, E4, and phosphatase-dead transfected cells (Figure 2.7.A). This finding suggests that PTEN null and mutant PTEN transfected cells are responsible for unchecked replication in stressful situations with DNA damage due to reduced CHK1 activation.

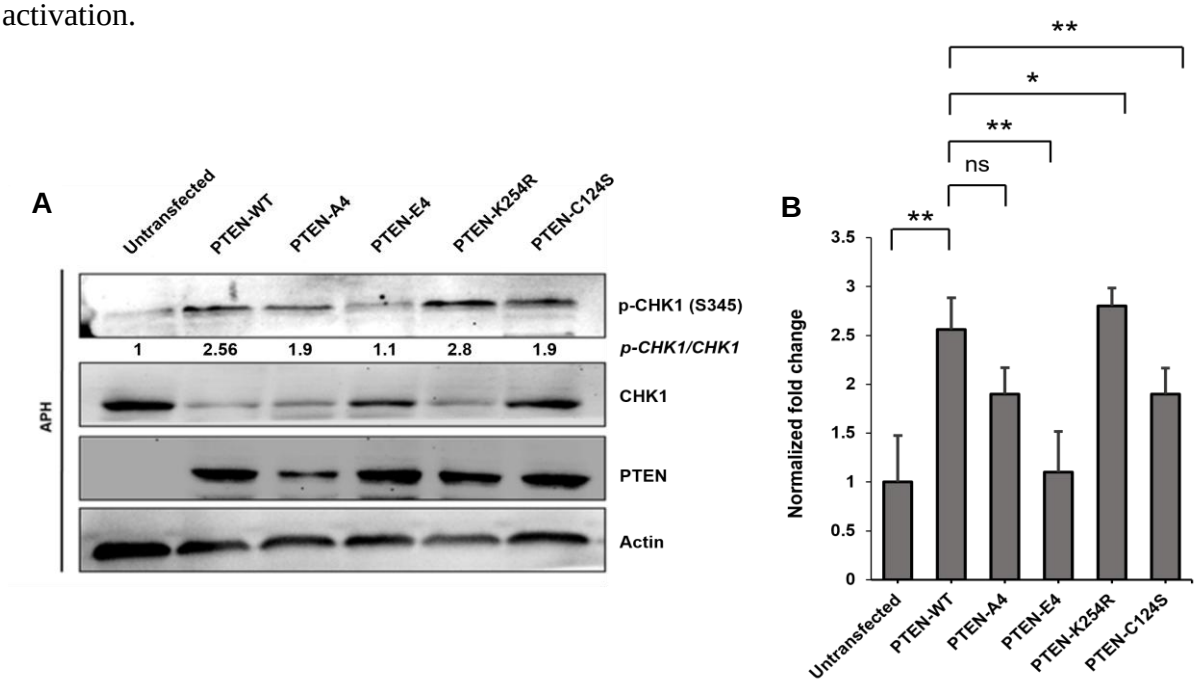


Figure 2.7 *(A) The aforementioned PTEN clones were transfected into PTEN-null PC3 cells. APH was applied to the cells for 3 h after a 24 h incubation period. Following the preparation of the whole-cell lysate, anti-CHK1, anti-p-CHK1(Ser 345), and anti-PTEN antibodies were used in western blotting. β-actin was used as a loading control. ImageJ was used to measure band intensities. The ratio of p-CHK1 (Ser 345) to total CHK1 intensity is shown here. An untransfected control sample was normalized to 1. (B) The fold change is shown on the graph, normalized to CHK1. The untransfected sample has been normalized to 1. Values are the mean ± SD. *p < 0.05, and **p < 0.005.*

2.3.7 C-terminal phosphorylation is necessary for chromatin association of PTEN

Since aphidicolin-mediated replication arrest is caused by the phosphorylation of PTEN at its C-terminus, we looked into whether this would impact PTEN's ability to associate with chromatin. Western blotting was carried out following the preparation of the cytoplasmic and nuclear high salt fractions from the cells expressing both the wild-type and mutant PTEN in the presence of aphidicolin. Following treatment with aphidicolin, western blot analysis revealed a decrease in the amount of H3K9me3 protein expression in phospho-dead PTEN in the nuclear high salt chromatin fractions relative to wild-type PTEN transfected cells (Figure 2.8). Simultaneously, a modest rise in the level of sumoylation-dead and phosphatase-dead PTEN on cells treated with aphidicolin showed a potential involvement for these in the recruitment of PTEN into chromatin during replication stress.

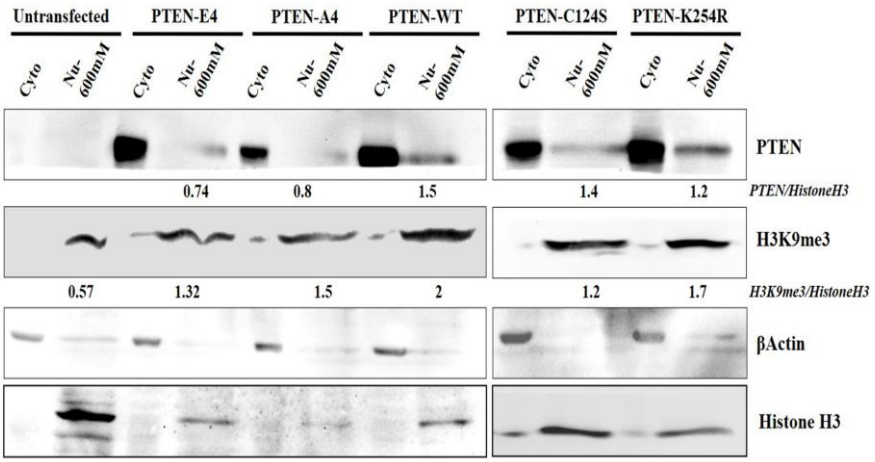


Figure 2.8 *The aforementioned PTEN clones were transfected into PTEN-null PC3 cells. The nuclear high salt fraction was prepared following an incubation period of 24 h. After that, anti-HA and anti-H3K9me3 antibodies were used to immunoblot the cytoplasmic and nuclear high salt fractions. As a loading control, histone H3 was used.*

2.3.8 Post-translational modulation of PTEN regulates heterochromatin

Constitutive heterochromatin, marked by H3K9me3, is a region of high-density repeating DNA elements that control genomic integrity by preventing chromosome missegregation, recombination, and DNA replication [*Rivera et al., 2015*]. Since previous research indicated that heterochromatin is destabilized in the absence of PTEN, we aimed to investigate how post-translational modulation of PTEN affects heterochromatin stability. In this case, PTEN null PC3 cells showed lower expression of transcriptional silent heterochromatin marker HP1α and H3K9me3 (Figure 2.9) compared to wild-type PTEN expressing cells, which is consistent with the previous study [*Gong et al., 2015*].

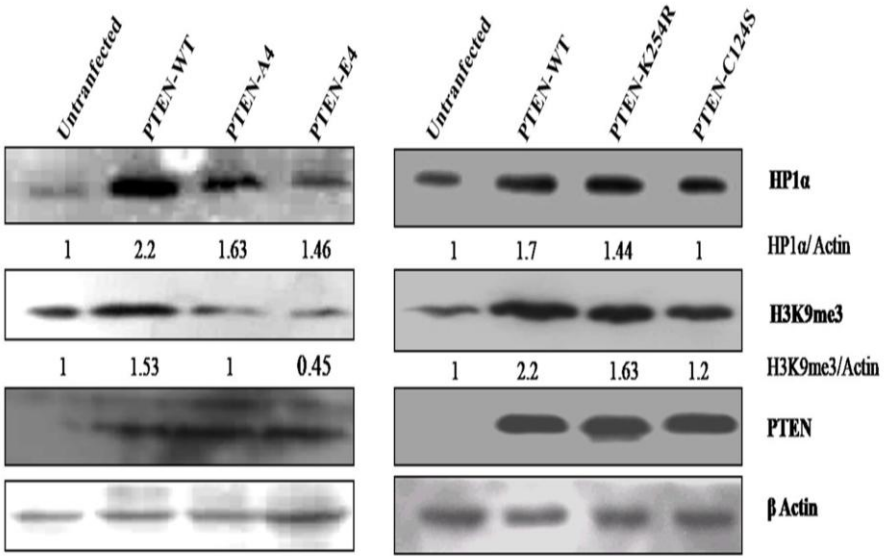


Figure 2.9 *The aforementioned PTEN clones were transfected into PTEN-null PC3 cells. Whole-cell lysate was made after 24 h of incubation, and anti-HP1α, anti-H3K9me3, and anti-PTEN antibodies were then used to perform western blotting. β-actin was used as a loading control. ImageJ was used to measure band intensities. Untransfected control was normalized to 1.*

We also assess the development of H3K9me3 heterochromatin foci after transfecting different PTEN clones. We found that 16.2% of untransfected cells show > 5 foci/cell, whereas 38.4% of PTEN-WT transfected cells show > 5 foci. PTEN transfected cells with both phosphatase-dead and phospho-dead PTEN exhibited a significantly reduced number of foci compared to PTEN-WT transfected cells (Figure 2.10.A). In the case of SUMOylation-deficient PTEN, the H3K9me3 foci count was nearly identical to wild-type PTEN (Table 2.3). As a result, this open chromatin maintenance may be linked to unaffected replication during replication arrest, which could result in faulty replication by getting past the checkpoint barrier, a trait that is associated with a higher risk of developing cancer. As a result, PTEN helps to detect replication arrest via maintaining heterochromatin, or it may do so by detecting replication arrest through phosphatase-dependent and C-terminal phosphorylation.

Table 2.3 *Quantitative data representing percentage of cells with H3K9me3 foci.*

Clone	% of cells with H3K9me3 foci
Untransfected	16.2 ± 5.86
PTEN-WT	38.4 ± 6.12
PTEN-A4	18.4 ± 6.56
PTEN-E4	21.6 ± 9.58
PTEN-C124S	20.5 ± 4.37
PTEN-K254R	32.6 ± 8.24

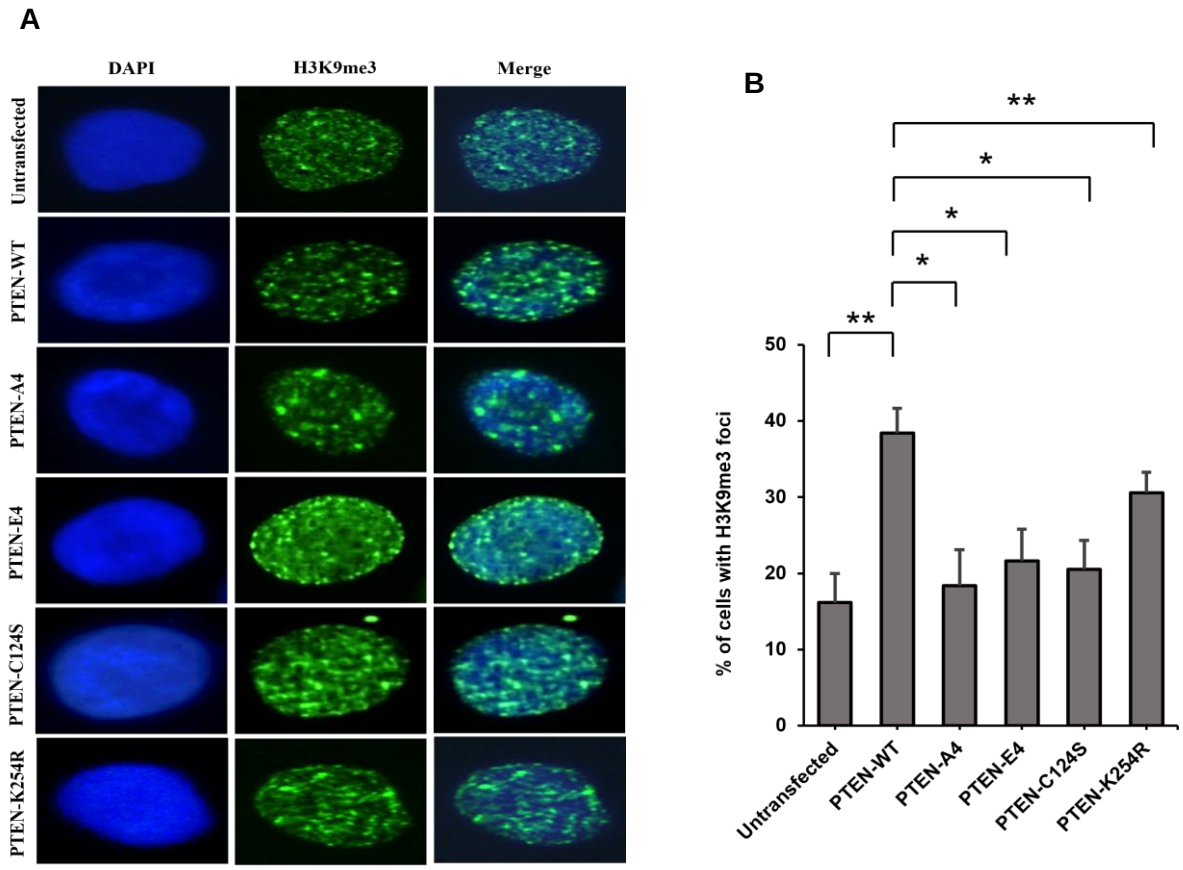


Figure 2.10 *(A) H3K9me3 foci (green) in untransfected and mutant PTEN-transfected cells are shown in a representative photomicrograph. The nucleus is shown by DAPI (blue). (B) Quantification data reflects the percentage of cells with more than five H3K9me3 foci.*

2.4 Discussion

For the suppression of neoplastic development, the capacity to retain genomic integrity during external DNA damage or replication is entirely necessary. Replicated DNA in daughter cells must be accurate and faithful for life to exist. In addition to its well-known role as a tumor suppressor, PTEN is also being identified as a DNA repair protein due to its ability to block the PI3K/AKT pathway, which promotes the formation of cancer. Even while past research looked at the relationship between PTEN's structure and function, only a few studies were done to investigate the function of phosphorylated PTEN in DNA metabolism [*Sellars*

et al., 2020]. The primary site of PTEN's phosphorylation is at its 50 amino acid residues at the C-terminus, which is important for maintaining PTEN's stability, nuclear localization, and phosphatase activity [*Masson et al., 2020*]. According to reports, PTEN C-terminal mutations are linked to several human malignancies [*Georgescu et al., 1999*] and are also said to be necessary for tumor suppressor activity [*Vazquez et al., 2000*].

The PTEN C-terminus has recently been shown to be necessary for genomic stability, although tumor suppression and phosphatase activity are not significant [*Sun et al., 2014*]. The phosphorylation of Ser380 has also been demonstrated not to be necessary for the translocation of PTEN into the nucleus [*Choi et al., 2013*] rather phosphorylation of Ser366/Thr370 is necessary. On the other hand, it has also been shown that Ser380 phosphorylation is directly related to PTEN accumulation on chromatin [*Choi et al. 2014*]. However, our results are by the discovery that PTEN translocation to the nucleus is triggered by oxidative stress and is reliant on phosphorylation of the Ser380 residue [*Chang et al., 2008*]. Ser366/Thr370 phosphorylation is conceivable in the A4-PTEN construct, but we have frequently seen that the A4-PTEN mutant is not restricted to the nucleus after etoposide treatment. One possibility is that the Ser366/Thr370 residues must first be phosphorylated, either entirely or in part, on the STT motif in the C terminal.

The time of DNA replication appears to have a direct impact on mutagenesis. Genomic instability results from a buildup of DNA damage brought on by increased replication stress in cancer cells [*Salmaninejad et al., 2021*]. According to various human illnesses and mouse models, malfunctions in the transmission of the cellular response to replication stress, such as those caused by an improper DNA damage checkpoint, or DNA repair, can result in tumors [*Rydell-Törmänen et al., 2019*]. Our research indicates that PTEN null/mutant cells proliferate quickly even when subjected to replication stress brought on by aphidicolin. An accumulating phenotype of PTEN-deficient cells depicts genomic instability (aneuploidy,

micronucleus, and nuclear bridge) brought on by replication under stressful conditions [*Fan et al., 2020; Hou et al., 2017*]. Mutagenesis appears to be directly impacted by the timing of DNA replication. Remarkably, we found that p-PTEN accumulates in the nucleus following S phase arrest by a double thymidine block, similar to DNA damage caused by etoposide. Thus, phosphorylated PTEN may have an impact on S-phase dynamics. It is widely known from several publications that PTEN plays a role in DNA repair [*Hou et al., 2017; Ming et al., 2012; Ogino et al., 2016*]. Additionally, it has been noted that PTEN-null cells exhibit a down-regulation of the heterochromatin markers H3K9me3 and HP1α [*Dillon et al., 2014*]. Therefore, elevated HP1α and H3K9me3 expression in PTEN-positive cells may prevent unfettered ORC admittance. When growth conditions or DNA damage cause replication stress, the use of flexible origins may be increased, or a nearby latent origin may be triggered to complete DNA replication; nevertheless, these approaches may be ineffective [*Zeman et al., 2014*]. DNA damage has already been shown to trigger the HP1/KAP1/Suv39h1axis to H3K9me3 spanning tens of kilobases around the DSBs, leading to chromatin compaction [*Legartová et al., 2019*]. Here, we discovered that PTEN-WT transfected cells exhibit enhanced expression and chromatin association of HP1α and H3K9me3 dependent on C-terminal phosphorylation, whereas both phospho-dead (PTEN-A4) and phospho-mimetic (PTEN-E4) PTEN expressing cells have defective heterochromatin. Thus, by maintaining heterochromatin foci, PTEN may function as a silencing factor during replication stress. Additionally, the deletion of the global H3K9me3 mark impairs the progeroid cells' ability to respond to DNA damage by limiting ATM activation and reducing the development of γH2AX foci [*Zhang et al., 2016*], which may also serve as a cue for the abnormal DNA damage signal in PTEN-null cells. We have previously shown that genotoxic stress elevated PTEN's C-terminal phosphorylation and generate nuclear foci. Consequently, we discovered in this study that phosphorylated PTEN is also required for the preservation of heterochromatin.

Important kinase CHK1 controls origin firing in response to replication blocks and maintains replication stability [*Petermann et al., 2010*]. According to a prior publication, PTEN depletion inhibits CHK1 activation, which results in the build-up of DSBs [*Puc et al., 2005*]. Our research further shows that PTEN null cells exhibit significantly reduced levels of p-CHK1. In PTEN null cells, PTEN-WT transfection prevents DNA damage and restores CHK1 activation following replication stress, whereas in A4 and E4 transfected cells, CHK1 activation is reduced. Interestingly, although CHK1 activation is increased in phosphatase-dead transfected cells, it is not as robust as in PTEN-WT transfected cells. Furthermore, it has been shown that PTEN dephosphorylates MCM2 in response to replication stress, hence promoting genomic stability [*Liu et al., 2022*]. We also discovered that PTEN's phosphatase activity may be crucial for detecting replication stress. PTEN phosphorylation preserved PTEN on the chromatin by preventing it from ubiquitin-mediated degradation, and phosphatase activity is necessary for dephosphorylating MCM2, as evidenced by the fact that phosphatase-dead PTEN shows a strong association with chromatin [*Chen et al., 2018*]. As phosphorylation appears to be tightly regulated during replication stress, phospho-PTEN exhibits reduced phosphatase activity [*Moiseeva et al., 2018*]. Despite having constitutive phosphatase activity, phospho-mutant PTEN may not be able to recognize aphidicolin-mediated replication arrest due to decreased chromatin association and instability. PTEN-expressing cells with SUMOylation mutations can behave like wild-type PTEN at least during replication arrest since they have both phosphorylation sites and phosphatase activity. This finding may be consistent with a previous discovery that PTEN's chromatin association in DNA interstrand crosslinks caused by mitomycin C is independent of SUMOylation [*Vuono et al., 2016*]. Prerequisite stability is required for PTEN to function in a phosphatase-dependent way, and this could be the reason that phosphorylation of PTEN's C-terminal Ser/Thr residues limits its phosphatase action [*Masson et al., 2020*]. PTEN must continue to function as a phosphatase to dephosphorylate itself at its C-terminal tail [*Leslie et al., 2004;*

Liu et al., 2022], which may momentarily activate phosphate activity. Overall, our current understanding suggests that for PTEN to properly function in the conventional axis and the non-canonical nuclear activities associated with preserving genomic integrity, there must be a precise control between its phosphatase activity and phosphorylation.

2.5 Materials & Methods

2.5.1 Chemicals and antibodies

The following chemicals were purchased from HiMedia: RPMI 1640, DMEM, PBS, Paraformaldehyde, Tris base, Glycine, SDS, Penicillin/Streptomycin, Amphotericin-B. FBS was from Gibco. MTT, Tween-20, Low Melting Point Agarose, Triton X-100, EDTA, NaCl, EGTA, Sodium Metavanadate, PMSF, NP-40, Acrylamide/bis-acrylamide, 5% non-fat dry milk, and Tween-20 were from SRL. DAPI from Vector Laboratories. The protease and Phosphatase inhibitor cocktail were from Abcam. BSA, Bradford's reagent, Etoposide, DMSO, Etoposide, and Aphidicolin were from Sigma Aldrich. PTEN, p-PTEN (S380/T382/383), p21, HP1α, histone H3, CHK1, and p-CHK1(Ser345) were purchased from Cell Signaling Technology. β-actin, HA antibody, γH2AX, β-actin, Lamin A/C were purchased from Santa Cruze Biotechnology. H3K9me3 from Abcam. Anti-mouse or anti-rabbit IgG conjugated with HRP and anti-rabbit or anti-mouse IgG conjugated with FITC or anti-mouse IgG conjugated with Texas Red (Invitrogen) were used as secondary antibodies.

2.5.2 Plasmids and clones

Gene-constructs pSG5L-HA-PTEN-WT (HA-PTEN) encodes hemagglutinin tagged wild type PTEN protein; phospho-deficient pSG5L-HA-PTEN-A4 (Ser380Ala, Thr382Ala, Thr383Ala, Ser385Ala) and phospho-mimicking pSG5L-HA-PTEN-E4 (Ser380Glu, Thr382Glu, Thr383Glu, Ser 385Glu) encode hemagglutinin tagged mutant PTEN proteins

was a kind gift of Prof. Dr. W.R. Sellers, Harvard Medical School. The site-directed mutagenesis kit was used to construct pSG5L-HA-PTEN-C124S (phosphatase-dead), and pSG5L-HA-PTEN-K254R (SUMOylation-dead), as per the manufacturer's instructions. Sequencing was used to confirm the results. The Primer X software was used to develop the mutagenic primers listed below.

For phosphatase-dead mutant, PTEN-C124S the following primer was used:

```
      Mutation site at center:       Yes
-------------------------------------------------------------------------
3 primer pair(s) generated.
Primer pair 1
                                    *
     Forward: 5' GTTGCAGCAATTCACTCTAAAGCTGGAAAGGG 3'
     Reverse: 5' CCCTTTCCAGCTTTAGAGTGAATTGCTGCAAC 3'
                                   *
      GC content: 46.88%              Location: 355-386
      Melting temp: 76.7°C            Mismatched bases: 1
      Length: 32 bp                   Mutation: Substitution
      5' flanking region: 16 bp       Forward primer MW: 9897.55 Da
      3' flanking region: 15 bp       Reverse primer MW: 9750.46 Da
Primer pair 2
                                    *
     Forward: 5' GTTGCAGCAATTCACTCTAAAGCTGGAAAGGGAC 3'
     Reverse: 5' GTCCCTTTCCAGCTTTAGAGTGAATTGCTGCAAC 3'
                                    *
      GC content: 47.06%              Location: 355-388
      Melting temp: 77.9°C            Mismatched bases: 1
      Length: 34 bp                   Mutation: Substitution
      5' flanking region: 16 bp       Forward primer MW: 10499.95 Da
```

For Sumo dead mutant, PTEN K254R the following primer was used:

```
2 primer pair(s) generated.
Primer pair 1
                                    *
  Forward: 5' GTGTGTGGTGATATCAGAGTAGAGTTCTTCCAC 3'
  Reverse: 5' GTGGAAGAACTCTACTCTGATATCACCACACAC 3'
                                   *
  GC content: 45.45%              Location: 745-777
  Melting temp: 76.5°C            Mismatched bases: 1
  Length: 33 bp                   Mutation: Substitution
  5' flanking region: 16 bp       Forward primer MW: 10214.74 Da
  3' flanking region: 16 bp       Reverse primer MW: 10050.68 Da
```

2.5.3 Cell lines and Culture conditions

PTEN null PC3 cells were obtained from the National Centre for Cell Science in Pune, India, and maintained in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 1X non-essential

amino acid, penicillin (100 U/ml), and streptomycin (100 U/ml) at 37°C, 5% CO_2, and 95% relative humidity (RH).

2.5.4 Cellular transfection

Before transfection, PC3 cells were seeded for an overnight period in antibiotic-free media. According to the manufacturer's instructions, Lipofectamine 3000 transfection reagent (Invitrogen) was utilized for transient transfection. 5 µg of DNA, 10 µl of p3000, and 10 µl of Lipofectamine 3000 reagent were mixed and incubated for 30 min for transfection in a 60 mm plate. The 60 mm plate was then filled with this mixture, and it was left to incubate overnight.

2.5.5 BrdU incorporation assay

On sterile coverslips measuring 18 mm, cells were seeded. Following APH (6 M) treatment, cells were permeabilized with 0.2% Triton X-100 on ice for 10 min, fixed in freshly made 4% paraformaldehyde solution for 15 min, and then incubated with BrdU for 1 h. Cellular DNA was then denatured with 2 M HCl, rinsed with 1X PBS, and pre-incubated in blocking solution (1% BSA in 1X PBS) before being incubated with anti-BrdU antibody (1:100) overnight at 4 °C in wash buffer (0.1% BSA and 0.05% Tween 20 in 1X PBS). Following six rounds of washing with wash buffer, cells were probed for 1 h at RT with FITC-conjugated anti-Mouse antibody (1:200). Cells were washed properly, put in a mounting solution containing DAPI (Vector Laboratories), and examined with a fluorescence microscope (Leica).

2.5.6 Western blotting

Cell pellets were collected and cell lysates were prepared using cell lysis buffer (50 mM Tris HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and phosphatase inhibitor) following transfection with several PTEN clones. Cell

lysates were boiled with 1X protein loading dye, resolved by 10% SDS-PAGE, and then transferred to PVDF membranes that had been activated by methanol. After blocking the membrane with TBS containing 0.1% (w/v) Tween-20% and 5% (w/v) non-fat milk for 1 h at RT, the membrane was incubated with primary antibodies for overnight at 4 °C.

2.5.7 Indirect immunofluorescence

On 18 mm sterile coverslips, cells were plated. Following treatment, cells were permeabilized with 0.2% Triton X-100 on ice for 10 min before being fixed in a fresh 4% paraformaldehyde solution for 15 min. Before overnight incubation with anti-H3K9me3 antibody in wash buffer (0.1% BSA and 0.05% Tween 20 in 1X PBS), fixed cells were pre-incubated in blocking solution (1% BSA in 1X PBS). Following six rounds of washing with wash buffer, cells were probed for 1 h at RT with an Alexa Fluor 488-conjugated anti-mouse antibody (1:200). Cells were properly washed before being put in DAPI-containing mounting solution (Vector Laboratories) and observed with a fluorescence microscope (Leica).

2.5.8 Double thymidine Block

In a 10 cm culture dish, 30–40% confluent WI-38 cells were seeded, and after an overnight incubation at 37°C, 2 mM thymidine-containing media was added. The thymidine-containing medium was removed after 18 h of incubation, rinsed with PBS, and then incubated for 9 h with fresh medium. After that, a 2 mM thymidine-containing mixture was added, and it was cultured in a CO2 incubator for an additional 18 h. The immune-fluorescence assay was then performed on the cells.

2.5.9 Cellular fractionation

After rinsing the cells in 1X PBS, they were again suspended in 200 µl of solution A, which comprises 1 mM DTT, 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, and protease and phosphatase inhibitors. After being incubated on ice

for 10 min, the nuclear and cytoplasmic fractions were separated by centrifugation at 1500 g for 5 min. After that, the nuclear fraction was twice cleaned with solution A and then incubated in 150 µl of solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease and phosphatase inhibitors) on ice for 15 min. The soluble nuclear fractions were then separated using centrifugation, which was run for 4 min at 1700 g. After that, the pellet is rotated at 4 °C for 30 min while being incubated in 300 µl of high salt buffer (10 mM Tris pH 7.4, 2 mM $MgCl_2$, 2 mM EGTA, 0.1% Triton X-100, 600 Mm NaCl). The lysate was then centrifuged for 15 min at 13,200 rpm. After removing the pellet, the separated chromatin fraction was boiled in 1X Laemmli sample buffer. The high-salt fractions of the nucleus and cytoplasm were examined using western blotting.

2.5.10 Statistical analysis

The student's t-test was used to calculate the statistical differences between the groups. The criteria for statistical significance were $P < 0.05$ and 0.001. The data was analyzed using Origin Pro v.8 (Origin Lab) software.

Chapter 3

PTEN is required for promoting the NHEJ pathway by regulating DNA-PKcs

PTEN is required for promoting the NHEJ pathway by regulating DNA-PKcs

3.1 Introduction

In the previous chapter, we found that PTEN plays an important role during replication stress and the catalytic function of PTEN is regulated by its post-translational modulation. This function of PTEN is essential to maintain genomic integrity. Thus, being a tumor suppressor protein PTEN is linked to several physiological processes, including DNA replication [*Ho et al., 2020*], DNA repair [*Ming et al., 2012*], cell migration [*Fedorova et al., 2022*], cellular senescence [*Bermúdez et al., 2015; Leslie et al., 2004*], and preservation of chromatin structure [*Masson et al., 2020*]. The lipid phosphatase-encoding gene PTEN regulates the PI3K pathway, which in turn modulates pro-tumorigenic behaviors [*Chalhoub et al., 2009*]. A wide range of cell types express PTEN, and at the cellular level, it is present almost everywhere, with the cytoplasm and nucleus being the two most frequent compartments. PTEN affects the functions of nuclear proteins or modifies the activities of these proteins to mediate their nuclear function in preserving the integrity of the genome [*Ho et al., 2020*]. PTEN's non-canonical activity is thus caused by post-translational modifications including phosphorylation, SUMOylation, methylation, and acetylation [*González-García et al., 2022*]. Numerous investigations suggest that PTEN may have novel nuclear roles, such as regulating the transcription of the Rad51 gene, whose product is essential for HR to repair DNA breaks [*Pei et al., 2022*]. It is clear from another study that, in response to DNA damage brought on by ionizing radiation, Rad51 protein production was considerably increased in PTEN-WT and phosphatase dead PTEN but not in cells transfected with sumo defective PTEN [*Guan et al., 2016*]. Although the link between PTEN and HR has been widely studied, it is still unclear how various PTEN modulations control NHEJ. The PI3K/AKT cascade is activated by PTEN loss, and tumor cells that have constitutive AKT activation are believed to be

resistant to chemotherapy and radiation therapy [*Teng et al., 2021*]. This is because AKT enhances DNA repair by phosphorylating and activating DNA-PK [*Liu et al., 2022*]. DNA-PK is necessary for the DNA DSB repair process by NHEJ, which normally occurs in the G1 phase of the cell cycle [*Davis et al., 2014*]. To prevent genomic instability through the repair of DSBs, NHEJ is the predominant template-independent repair process in human cells. Genomic instability results from the loss of the essential NHEJ components Ku70/80, DNA-PKcs, DNA Ligase 4 (LIG4), XRCC4, and XLF [*Davis et al., 2013*]. Evidence has shown that improper repair of defective NHEJ increases genomic instability, which promotes carcinogenesis [*Sishc et al., 2017*]. Ku70/80 hetero dimer typically binds to the DNA broken end during the first stage after DNA DSB. DNA-PKcs are recruited once the Ku heterodimer is attached to the DSB [*Zahid et al., 2021*]. This complex is called DNA-PK. The mechanism by which binding to the Ku-DNA complex enhances DNA-PKcs' catalytic activity is yet unclear. DNA-PKcs is autophosphorylated at several places as a result of binding to DNA ends, which separates DNA PK from DNA ends. The Ku heterodimer is released from the forked dsDNA as a result of DNA-PKcs phosphorylating Ku, and the subsequent liberation of the dsDNA ends enables Exo1-mediated DNA end resection [*Fell et al., 2015*]. Therefore, there must be a mechanism in place to stop the immediate phosphorylation of these proteins; otherwise, the NHEJ pathway will be impaired, which leads to genomic instability. By the time the repair is complete, the phosphorylated protein must either be destroyed, or the phosphate group liberated. Although much has been learned about how the phosphorylation status of DNA-PKcs regulates the activity of the protein, the precise role of these phosphorylations in NHEJ and other DSB responses remains unknown and will remain a subject of considerable interest for research. It is yet unknown what function DNA-PKcs' kinase activity performs in NHEJ, what substrates are relevant to NHEJ, and what components and particular mechanisms are needed for the complex's dissolution after repair

is finished. Further, it will be interesting to see if chromatin modification in PTEN-deficient cells affects the NHEJ repair pathway. Given that PTEN regulates chromatin condensation, which affects both gene expression and DNA repair, changes in the architecture of DNA packaged as chromatin are required for NHEJ for repair to occur.

We were able to show that PTEN positively regulates the NHEJ pathway through phosphatase activity and C-terminal phosphorylation in PTEN-null PC3 cells using a fluorescent reporter expressing plasmid. The NHEJ proteins Ku80, XLF, and DNA-PKcs have more chromatin-based stability as a result of PTEN's C-terminal phosphorylation. Our research also shows that PTEN dephosphorylates DNA-PKcs and aids in its successful recruitment to DNA damage sites, effectively carrying out the ensuing repair process. Thus, PTEN positively regulates NHEJ by recruiting NHEJ proteins to chromatin in response to C-terminal phosphorylation, and then dephosphorylating DNA-PKcs to keep it attached to DNA damage sites.

3.2 Aims and Objectives of Work

- How various post-translational modulation of PTEN regulates the NHEJ pathway.
- To identify the functional and physical interaction between PTEN and NHEJ proteins.
- How PTEN regulates chromatin association of various NHEJ proteins after DNA damage.
- How PTEN modulation influences NHEJ pathway through chromatin modulation upon DNA DSB.

3.3 Results

3.3.1 Generation of different mutant PTEN clones by site-directed mutagenesis and their expression

Following the manufacturer's instructions, we generate several PTEN mutant clones from PTEN-WT using an Agilent site-directed mutagenesis kit. With the aid of a sequencing primer, a single colony was isolated following transformation before being sequenced and verified (see the materials & methods section).

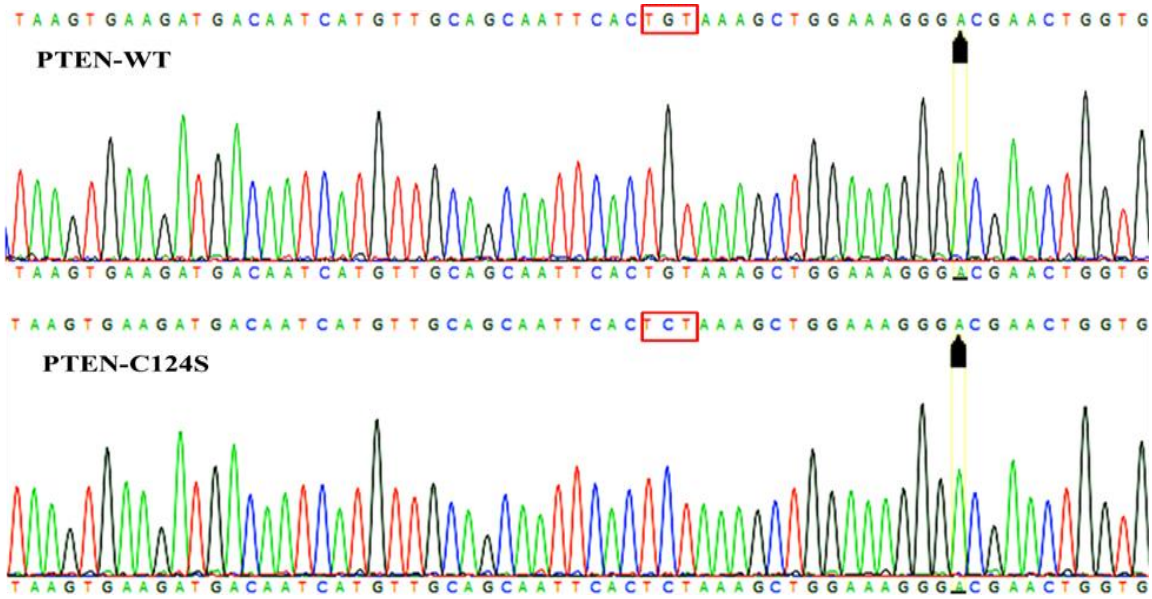


Figure 3.1 *DNA sequencing result of wild-type PTEN (PTEN-WT), and Phosphatase-dead PTEN (C124S).*

3.3.2 Generation of NHEJ repair assay system

Using the Mlu1 restriction enzyme, the cDNA of GFP from the eGFPC1 plasmid containing the promoter (pCMV) was subcloned into the DsRed vector. Agarose gel electrophoresis and the production of both fluorescent proteins after transfection served as indicators of a successful ligation. DsGFP is the name of this hybrid plasmid. The restriction enzyme Sbf1, which is present in the DsRed gene, was used to linearize this plasmid and the agarose gel elution procedure was used to collect this linear fragment (Figure 3.2.C).

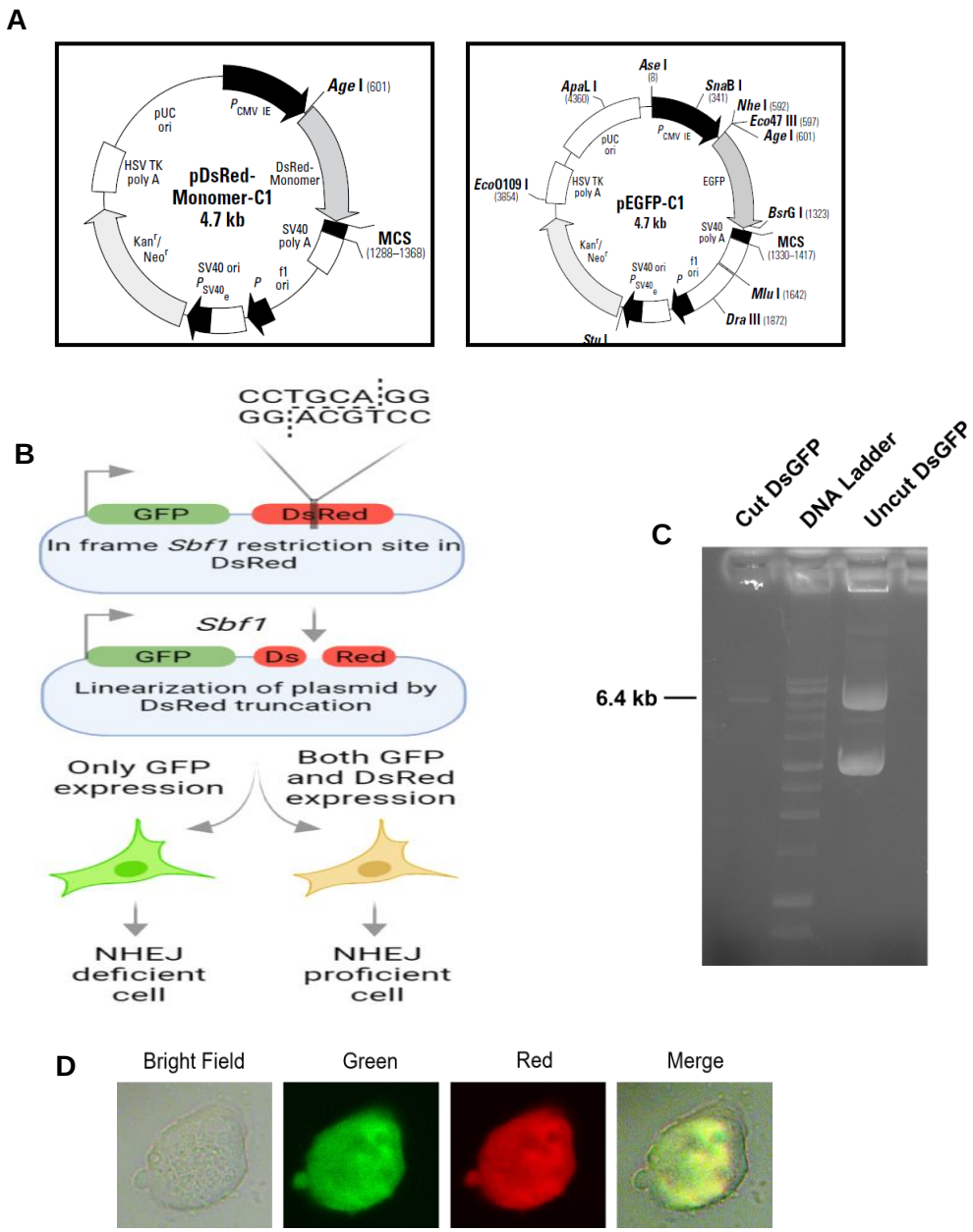


Figure 3.2 *(A) Pictorial representation of plasmid maps. (B) A schematic representation of the NHEJ reporter system. The DsRed vector was used to sub-clone the GFP plasmid's cDNA. With the use of a restriction enzyme (Sbf1) found in the DsRed gene, this hybrid plasmid was made linear. (C) Analysis of restriction enzyme digestion reactions by agarose gel electrophoresis. Samples were either undigested (right) or digested (left) with Sbf1. (D) Transfection of the hybrid DsGFP plasmid in PC3 cells with different reagents. Bright-field and fluorescence images of PC3 cells taken at the 24th h after transfection.*

3.3.3 Validation of NHEJ repair assay system

A reporter-based *in vitro* NHEJ repair assay system was designed to investigate the impact of PTEN on NHEJ. To validate our assay system, we have employed two methods: pharmacologic inhibition of DNA-PKcs and knockdown of the core NHEJ repair protein XRCC4. By using siRNA XRCC4 transfection, PC3 cells' XRCC4 expression was knocked down to examine the impact of XRCC4 on these cells. The amounts of XRCC4 protein were quantified via western blot analysis. According to the findings, XRCC4 protein levels in cells transfected with XRCC4-siRNA were significantly lower than those in cells transfected with control-siRNA (Figure 3.3.A). We employed flow cytometry to assess the NHEJ efficiency after co-transfecting linearized recombinant DsGFP plasmid with empty vector in XRCC4+ PC3 cells and XRCC4 knocked down PC3 cells, respectively. The percentage of DsRed cells in the GFP+ population was estimated, and it was discovered that in comparison to empty-vector transfected XRCC4+ PC3 cells, the percentage of DsRed cells in empty-vector transfected XRCC4 knockdown PC3 cells was over 50% lower (Figure 3.3.B). Briefly, we employed flow cytometry to quantify the NHEJ efficiency after co-transfecting linearized recombinant DsGFP plasmid with empty-vector and PTEN wild type (PTEN-WT) plasmid in PC3 cells with or without DNA-PKcs inhibitor (NU7026). The percentage of DsRed cells in the GFP+ population was determined, and it was discovered that NHEJ inhibitors dramatically reduced the DsRed cell percentage. (Figure 3.3.D).

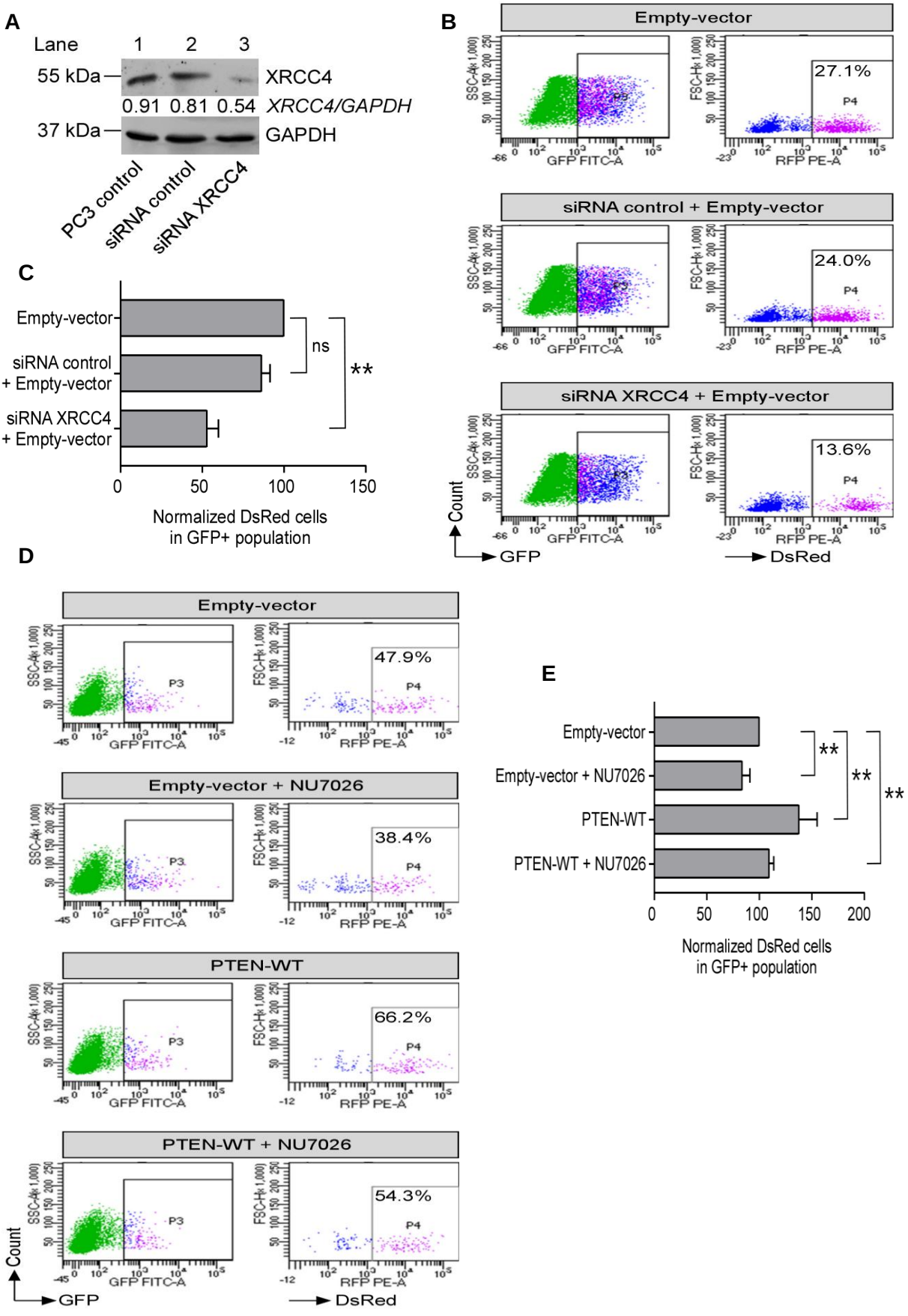

A
Lane 1 2 3
55 kDa
XRCC4
0.91 0.81 0.54 XRCC4/GAPDH
37 kDa
GAPDH
PC3 control
siRNA control
siRNA XRCC4
B
Empty-vector
27.1%
siRNA control + Empty-vector
24.0%
siRNA XRCC4 + Empty-vector
13.6%
Count
GFP
DsRed
C
Empty-vector
siRNA control + Empty-vector
siRNA XRCC4 + Empty-vector
ns
**
0 50 100 150
Normalized DsRed cells in GFP+ population
D
Empty-vector
47.9%
Empty-vector + NU7026
38.4%
PTEN-WT
66.2%
PTEN-WT + NU7026
54.3%
E
Empty-vector
Empty-vector + NU7026
PTEN-WT
PTEN-WT + NU7026
0 50 100 150 200
Normalized DsRed cells in GFP+ population

Figure 3.3 *(A) After transfecting PC3 cells with either non-targeted control siRNA or siRNA targeting XRCC4, extracts were subjected to western blot analysis using an XRCC4 antibody 48 h post-transfection. We used GAPDH as the loading control. As determined by ImageJ software, the value shows the intensity ratio of XRCC4 with GAPDH. (B) A linearized 2.5 µg DsGFP plasmid and a 2.5 µg empty vector were co-transfected into PC3 cells. Following a 24 h incubation period, the cells were ready for flow cytometry analysis to determine the proportion of DsRed cells in the GFP+ population. Before analysis, cells were treated with the specified siRNA. The gated GFP+ population is shown by P3 in this image, while the DsRed population within the gated GFP+ population is represented by P4. (C) The quantification of normalized DsRed cells in the GFP+ population is shown in the graph. The GFP+ population of the empty-vector transfected sample's DsRed cell percentage was standardized to 100. Values are the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01. (D) Linearized 2.5 µg DsGFP plasmid and 2.5 µg empty-vector or PTEN-WT plasmid were co-transfected into PC3 cells with or without DNA-PKcs inhibitor (NU7026). Following a 24 h incubation period, the cells were ready for flow cytometry analysis to determine the percentage of DsRed cells in the GFP+ population. The gated GFP+ population is shown by P3 in this image, while the DsRed population within the gated GFP+ population is represented by P4. (E) The normalized DsRed cell quantification in the GFP+ population is shown in the graph. The GFP+ population of the empty-vector transfected sample's DsRed cell percentage was standardized to 100. Values are the mean ± SD of three independent experiments. *p < 0.05, **p < 0.01.*

3.3.4 PTEN phosphorylation and its phosphatase activity are essential for NHEJ-mediated DNA-DSB repair

By co-expressing the linearized recombinant DsGFP plasmid with either PTEN wild type (PTEN-WT), phosphorylation-dead mutant (PTEN-A4), PTEN phosphatase-dead mutant (PTEN-C124S), or PTEN double mutant (PTEN-C124S-A4) in PTEN negative PC3 cells, we were able to assess the impact of PTEN modulation on NHEJ using this reporter-based assay system. The percentage of DsRed cells in the GFP+ population demonstrated that the expression of wild-type PTEN significantly increased the frequency of NHEJ by almost 50% in comparison to empty-vector transfected cells. In contrast, cells expressing PTEN-A4 and phosphatase-dead PTEN (PTEN-C124S) showed 20% and 15% lower NHEJ frequency, respectively, in comparison to PTEN-WT (Figure 3.4.A). However, compared to cells expressing PTEN in its wild-type form, cells expressing the double mutant PTEN (PTEN-C124S-A4) showed a 40% decrease in NHEJ frequency (Figure 3.4.B). These results imply that PTEN phosphatase activity and phosphorylation may both increase NHEJ efficiency. These plasmids were individually transfected into PTEN null PC3 cells, and equal expression of all PTEN clones was confirmed by western blotting with PTEN antibody (Figure 3.4.C).

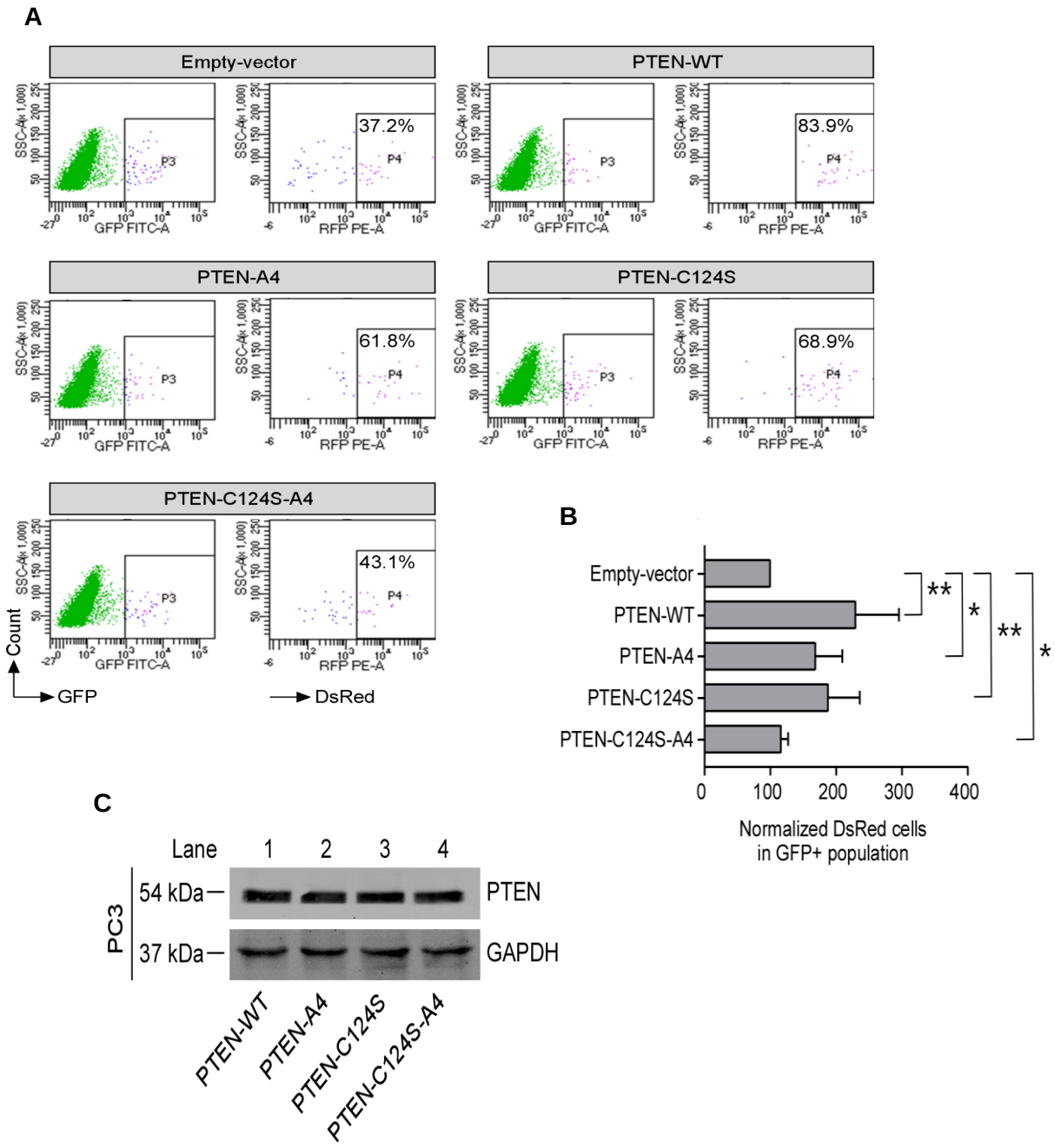


Figure 3.4 *(A) PC3 cells were co-transfected with linearized 2.5 µg DsGFP plasmid with either 2.5 µg PTEN-WT or other different PTEN mutant clones. After 24 h of incubation cells were prepared for analysis of the percentage of DsRed cells in the GFP+ population by flow cytometer. In this figure, P3 denotes the gated GFP+ population and P4 denotes the DsRed population amongst gated GFP+ population. (B) The graph represents the quantification of normalized DsRed cells in the GFP+ population. The percentage of DsRed cells in the GFP+ population of the empty-vector transfected sample was normalized to 100. Values are the*

*mean ± SD of three independent experiments. *p < 0.05, **p < 0.01. (C) Equal expression of PTEN and its mutants were confirmed by western blotting with PTEN antibody. GAPDH was taken as loading control. The value represents the intensity ratio of PTEN with GAPDH measured by ImageJ software.*

3.3.5 Post-translational modulation of PTEN leads to the change in the expression of NHEJ repair proteins

We examined the degree of NHEJ repair protein expression in cells that were transfected with the previously reported PTEN clones. In PTEN-deficient PC3 cells, we transfected the empty vector, wild type, and different mutants of PTEN and then treated the cells with or without etoposide for 3 h before analyzing the expression levels of various NHEJ repair proteins using western blotting (Figure 3.5.A). Ku70, DNA-PKcs, and XLF protein expression levels increased over two-fold in PTEN-WT transfected cells compared to empty-vector-transfected cells, while Ku80 and XRCC4 protein expression levels increased almost 1.5-fold (Figure 3.5.B). Following etoposide-induced DNA damage, transfected cells expressing phospho-dead (PTEN-A4), phosphatase-dead (PTEN-C124S), and double mutant PTEN (PTEN-C124S-A4) expressed fewer of the aforementioned proteins in comparison to PTEN-WT cells. The observed outcome, nevertheless, might have something to do with how cells adjusted to PTEN's lack. Therefore, we wanted to make sure that the PTEN knockdown using siRNA produced the same outcomes. PTEN expression was reduced in HeLa cells by siRNA PTEN transfection to study the impact of PTEN in HeLa cells. Following a 48 h transfection period, the PTEN knockdown cells and control HeLa cells underwent mock treatment or etoposide treatment for 3 h. Western blotting was then used to determine the expression levels of several NHEJ repair proteins. Western blot was used to quantify the amounts of PTEN protein. According to the findings, PTEN protein levels were considerably

lower in cells transfected with PTEN-siRNA than in cells transfected with control-siRNA (Figure 3.5.C). The expression levels of the proteins Ku70, Ku80, DNA-PKcs, XRCC4, and XLF in control HeLa cells were about 1.5 times higher than in PTEN knockdown HeLa cells (Figure 3.5.D).

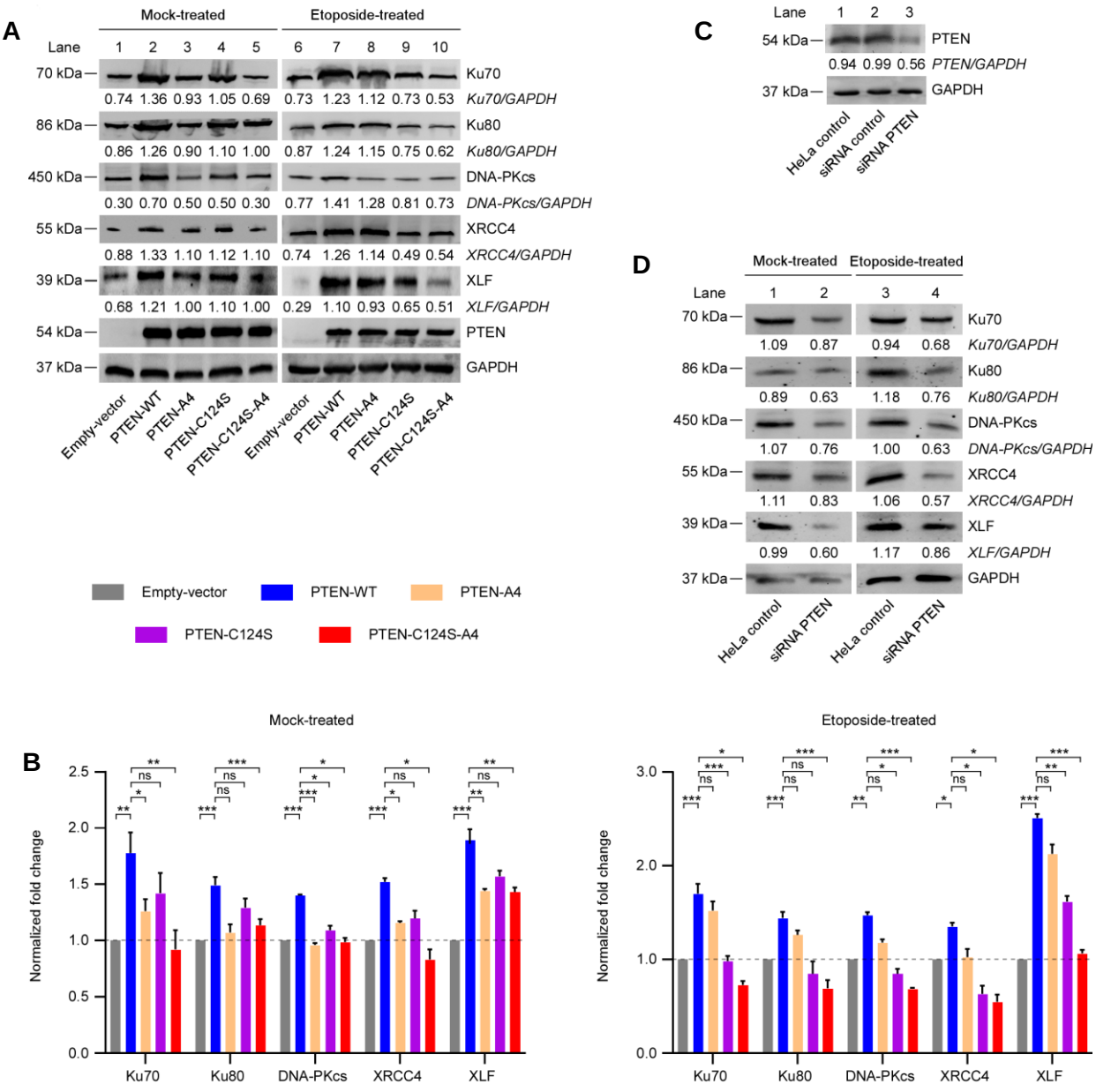


Figure 3.5 *(A) PTEN clones and empty-vector transfections were performed on PTEN-null PC3 cells. The cells were mock-treated for 24 h and then given etoposide for 3 h. Following the preparation of the entire cell lysates with cell lysis buffer, western blotting with the*

*appropriate anti-Ku70, anti-Ku80, anti-DNA-PKcs, anti-XRCC4, anti-XLF, and anti-PTEN antibodies was carried out. We used GAPDH as a loading control. ImageJ was used to measure band intensities. As determined by Image J software, the value represents the intensity ratio of Ku70, Ku80, DNA-PKcs, XRCC4, and XLF with GAPDH. (B) The proteins' fold change about GAPDH is shown on the graph. The relative intensity of the transfected empty-vector sample was standardized to 1 in every instance. Values are the mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001. (C) 48 h after transfecting HeLa cells with siRNA targeting PTEN or non-targeted control siRNA, cell lysates were subjected to western blot analysis using a PTEN antibody. We employed GAPDH as a loading control. As determined by ImageJ software, the value shows the intensity ratio of PTEN with GAPDH. (D) siRNA-PTEN was used to transfect HeLa cells. The cells were mock-treated and treated to etoposide for 3 h following a 48 h incubation period. Western blotting was used to identify the protein expression of Ku70, Ku80, DNA-PKcs, XRCC4, and XLF. We employed GAPDH as a loading control. As determined by ImageJ software, the value indicates the intensity ratio of Ku70, Ku80, DNA-PKcs, XRCC4, and XLF with GAPDH.*

3.3.6 Stability of NHEJ Protein is decreased in PTEN null cells

The stability of those proteins will next be evaluated in PC3 cells that have been transfected with either an empty vector or PTEN-WT. The protein half-life assay with CHX treatment reveals that NHEJ proteins in cells transfected with empty vector are significantly more unstable than in cells transfected with PTEN-WT (Figure 3.6.A). We discovered that these proteins' stability substantially decreases after 8 h of CHX exposure and that their expression patterns are almost nonexistent after 24 h. As a result, these NHEJ proteins were stabilized by PTEN expression.

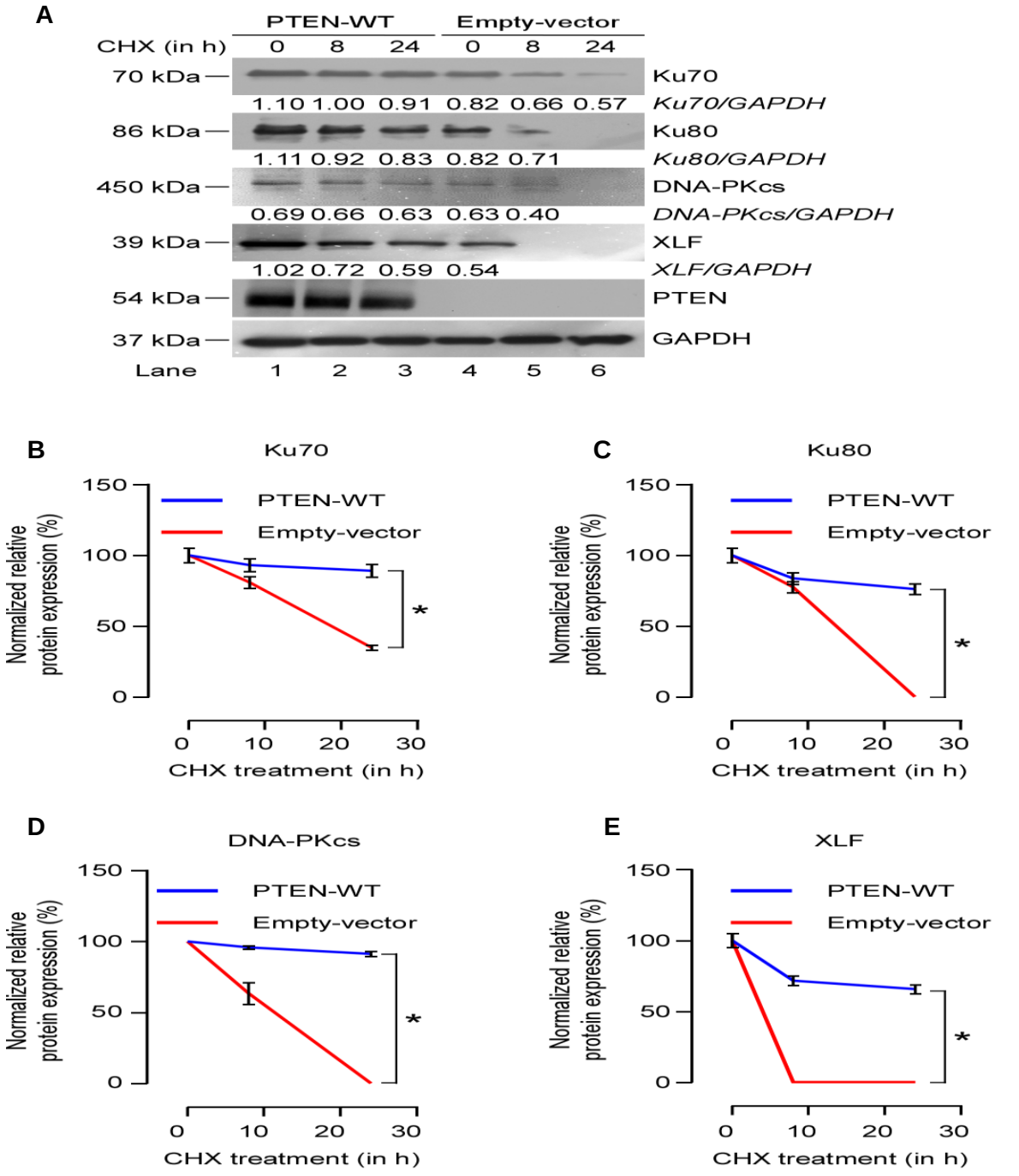


Figure 3.6 *(A) Western blot analysis was performed on PC3 cells transfected with the wild-type PTEN and empty vector after they were treated for 8 and 24 h with 100 µg/ml of cycloheximide. We used GAPDH as a loading control. Using ImageJ software, band intensities were expressed as a ratio of target protein to GAPDH. The Ku70, Ku80, DNA-PKcs, and XLF proteins were rapidly degraded in PC3 cells transfected with an empty vector*

*that lacks PTEN. (B, C, D, and E) The relative protein expression % is shown on the graph, normalized to GAPDH. In every instance, the relative intensity of the transfected empty-vector sample (0 h) was standardized to 100. Values are the mean ± SD. *p < 0.05.*

3.3.7 Stability of NHEJ protein expression is restored in the presence of proteasome inhibitor MG132

Subsequently, we aimed to examine whether proteasomal pathway inhibition restores NHEJ protein expression in PTEN null cells. We found that the expression of these repair proteins was markedly increased following 16 and 24 h of MG132 treatment in the presence of CHX, in contrast to cells that were simply treated with CHX (Figure 3.7.A). Thus, it may be stated that NHEJ protein expression can be increased again by inhibiting the proteasomal pathway.

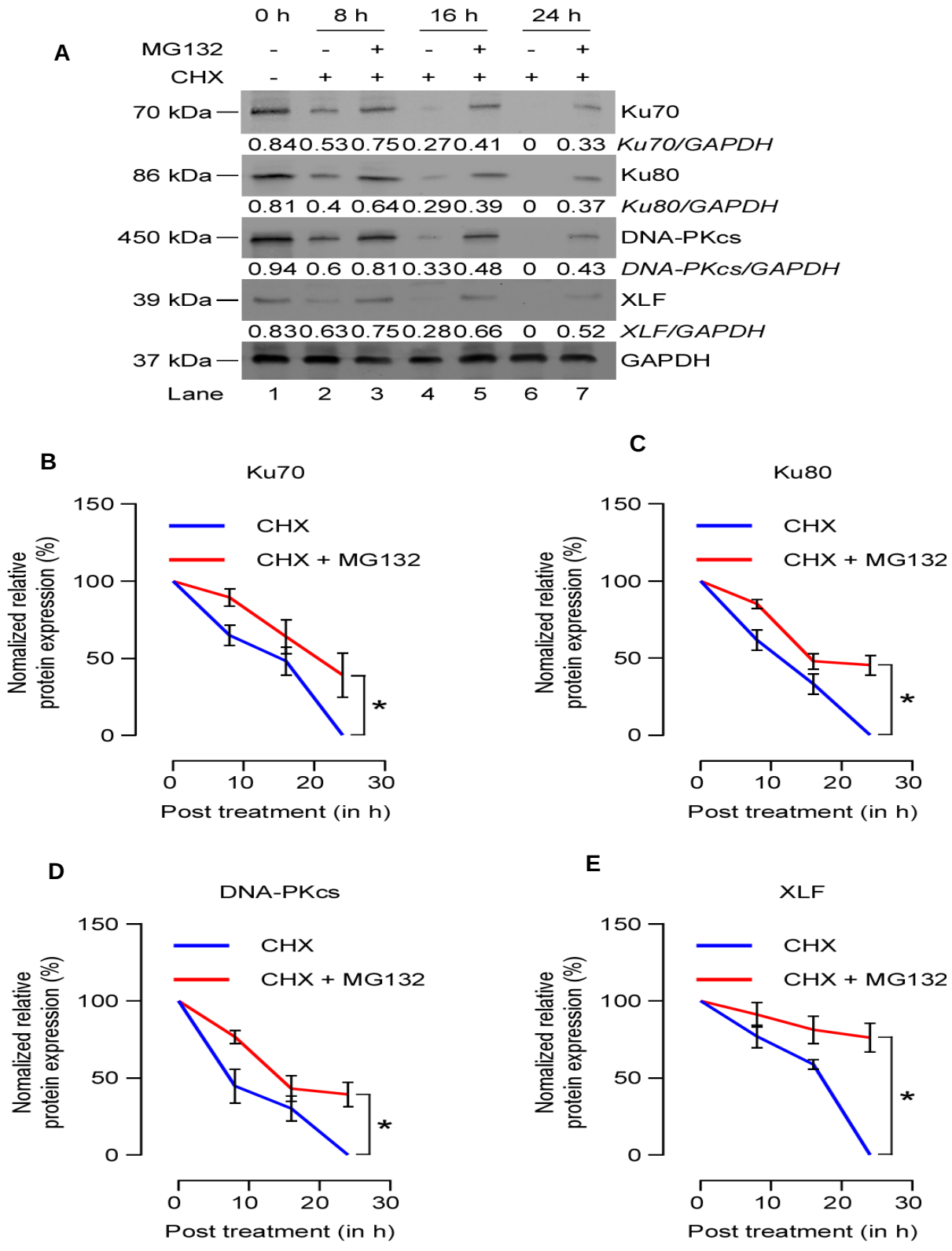


Figure 3.7 *(A) Proteasomal inhibitor MG132 was present or absent during the 0, 8, 16, and 24 h treatments of cells with cycloheximide at a dosage of 100 µg/ml. For loading control, GAPDH is used. Using ImageJ software, band intensities were expressed as a ratio of desired*

*protein/GAPDH. (B), (C), (D), and (E) The relative protein expression % is shown as a graph, normalized to GAPDH. In every instance, the relative intensity of the transfected empty-vector sample (0 h) was standardized to 100. Values are the mean ± SD. *p < 0.05.*

3.3.8 DNA damage-induced PTEN phosphorylation is attenuated by NU7026

Next, we looked into the regulation of DNA-PKcs and PTEN-WT. By reversibly binding to the kinase domain, the inhibitor NU7026 prevents DNA-PK from functioning [*Davidson et al., 2017*]. Using a western blot, it was possible to see whether NU7026's suppression of DNA-PKcs activity alters how PTEN is phosphorylated in response to DNA damage. NU7026 was pretreated for 2 h on WI-38 (normal lung fibroblast) cells, then etoposide (35 µM) was added either with or without NU7026 (1µM) for 3 h. Following the removal of etoposide, an inhibitor was added and incubated for 19 h. Following incubation, cell lysates were prepared and used for western blot analysis. We found that in both control and etoposide-treated cells, PTEN phosphorylation is significantly reduced when an inhibitor is present as opposed to when it is not (Figure 3.8.A). Exogenous demonstration of this was achieved by transfecting PTEN-null PC3 cells with PTEN-WT, followed by the same experimental conditions for the western blot (Figure 3.8.B), immunofluorescence (Figure 3.9.C), and flow cytometry (Figure 3.9.E) analysis.

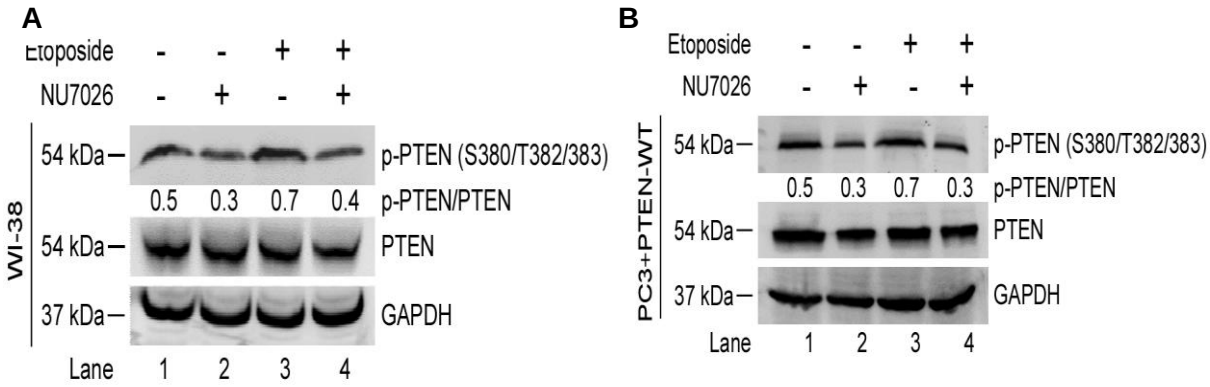


Figure 3.8 *(A) Mock and etoposide treatments of WI-38 cells were performed for 3 h in both the presence and absence of DNA-PKcs inhibitor (NU7026). After that, a cell lysate was prepared and subjected to western blotting using antibodies against p-PTEN, total PTEN, and GAPDH. (B) PTEN-deficient PC3 cells were transfected with PTEN-WT and then subjected to etoposide treatment both with and without NU7026. After that, a cell lysate was made and used in a western blotting experiment with the p-PTEN, total PTEN, and GAPDH antibodies. In all instances, band intensities were calculated as a ratio of p-PTEN/Total PTEN using ImageJ software.*

Additionally, we noticed that the NU7026 treatment lowers PTEN phosphorylation. The results of an immunofluorescence analysis demonstrate that p-PTEN foci colocalized with γ-H2AX following etoposide treatment (Figure 3.9.A). This suggests that PTEN is localized on DNA-damaged sites and that p-PTEN foci dramatically increase following etoposide-induced DNA damage in comparison to undamaged cells. According to an immunofluorescence investigation, PTEN-WT transfected cells are more likely to develop p-PTEN foci when they are damaged compared to undamaged cells, but not when they are damaged in the presence of an inhibitor (Figure 3.9.C). The phosphorylation of PTEN is also increased by etoposide treatment, but it is decreased by the presence of an inhibitor (Figure 3.9.E). As a result, a DNA-PKcs inhibitor (NU7026) blocks the phosphorylation of PTEN brought on by etoposide, suggesting that PTEN might be a DNA-PKcs target. Thus, DNA-PKcs may

phosphorylate the PTEN C terminus in response to DNA damage, either directly or through an unidentified indirect mechanism.

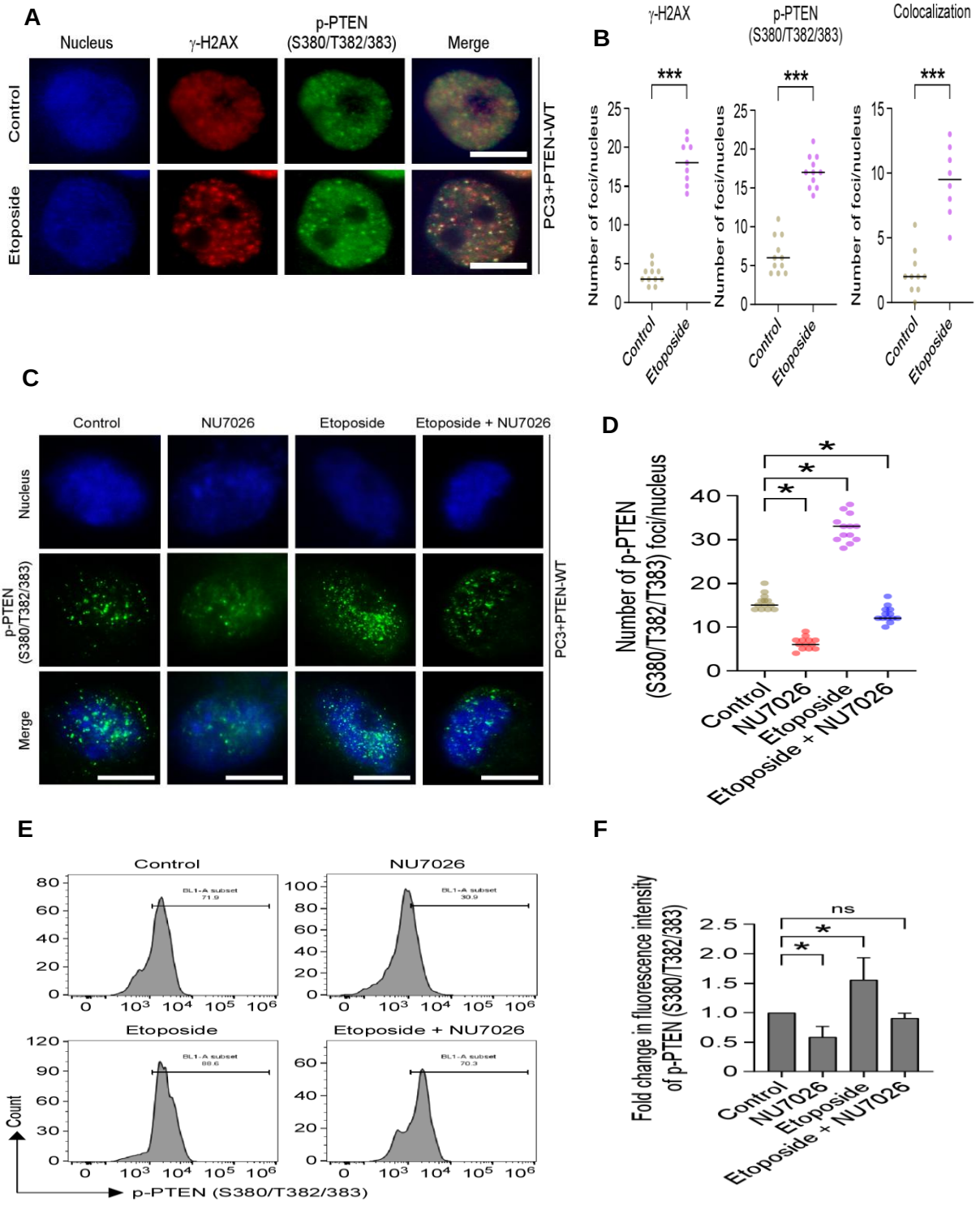


Figure 3.9 *(A) The PTEN-WT plasmid was transfected into PC3 cells, which were then fixed and stained with the appropriate antibodies after either a mock treatment or an etoposide*

*treatment for 3 h. Colocalization between γ-H2AX (red) and p-PTEN (S380/T382/383) (green) is demonstrated by the yellow foci in the merge. Scale bar = 20 µm. (B) The quantification of γ-H2AX, p-PTEN, and colocalization foci per nucleus in PTEN-WT transfected PC3 cells following mock or etoposide treatment is shown in the graph. Approximately 50 nuclei were measured in each case. Values are the mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001. (C) In PTEN-WT transfected PC3 cells treated with etoposide in the presence or absence of NU7026, a representative photomicrograph of p-PTEN (S380/T382/383) foci (green) and DAPI (blue) for nuclear staining is shown. Scale bar = 20 µm. (D) The graph shows the number of p-PTEN foci in each nucleus in PTEN-WT transfected PC3 cells following etoposide treatment, either with or without NU7026. Approximately 50 nuclei were measured in each case. Values are the mean ± SD. *p < 0.05. (E) PC3 cells were either treated with etoposide in the presence or absence of NU7026 after being transfected with the PTEN-WT plasmid. After that, cells were ready to be subjected to a FACS analyzer for p-PTEN (S380/T382/383) signal analysis. (F) The quantification of FACS data is shown in the graph. The normalization of a PTEN-WT transfected PC3 cell (control) was 1. Values are the mean ± SD. *p < 0.05.*

3.3.9 Wild-type PTEN directly associated with the C terminal site of DNA-PKcs

Protein-Protein Docking

To study interactions at three separate binding sites, protein-peptide docking calculations were performed using HADDOCK. HADDOCK generated clusters with varied binding orientations, and the top cluster was selected using both Z Scores and HADDOCK. The protein-peptide complex structure of the top cluster, which was derived via HADDOCK, was further examined using PRODIGY. The dissociation constant (K_d) and binding affinity data

were reported by PRODIGY (Table 3.1). PRODIGY also provides details on interfacial contacts, which are divided into charged-charged, charged-polar, charged-apolar, polar-polar, polar-apolar, and apolar-apolar interactions. As determined by the analysis of binding affinity, Ligand 1 (PTEN-WT) had the best binding with an affinity of -16.6 kcal/mol towards Binding Site 2 in comparison to Ligand 2 (PTEN-C124S) (Figure 3.11).

Table 3.1 *HADDOCK Scores of Ligands 1 and 2 with Binding Sites 1, 2 and 3 respectively.*

Scores	Binding Site 1 Ligand 1	Binding Site 1 Ligand 2	Binding Site 2 Ligand 1	Binding Site 2 Ligand 2	Binding Site 3 Ligand 1	Binding Site 3 Ligand 2
HADDOCK Score	59.9 +/- 15.5	92.6 +/- 3.6	328.5 +/- 25.5	357.4 +/- 42.7	29.4 +/- 21.3	41.9 +/- 19.1
Cluster Size	24	19	5	6	38	17
RMSD	0.3 +/- 0.2	2.3 +/- 0.2	0.7 +/- 0.4	3.2 +/- 0.0	0.4 +/- 0.3	0.7 +/- 0.5
Van der Waals Energy	-82.4 +/- 11.8	-73.5 +/- 5.7	-76.5 +/- 6.8	-57.0 +/- 7.6	-84.2 +/- 2.5	-82.1 +/- 11.2
Electrostatic Energy	-657.0 +/- 41.2	-501.5 +/- 12.0	-624.6 +/- 98.9	-503.4 +/- 102.5	-411.9 +/- 44.5	-363.1 +/- 98.2
Desolvation Energy	22.0 +/- 2.9	10.5 +/- 3.6	8.4 +/- 5.3	7.6 +/- 2.7	-10.1 +/- 1.5	-19.5 +/- 10.0
Restraints Violation Energy	2516.2 +/- 98.9	2558.0 +/- 65.5	5215.7 +/- 223.2	5075.3 +/- 415.9	2060.6 +/- 167.6	2160.9 +/- 147.2
Buried Surface Area	3503.5 +/- 69.7	3269.4 +/- 119.2	3339.8 +/- 287.3	3133.6 +/- 321.9	3223.5 +/- 76.8	2874.7 +/- 194.7
Z-Score	-2.3	-1.4	-2.3	-1.8	-2.3	-2.2
K_d	2.3 E-12	3.2 E-12	6.7 E-13	1.1 E-09	6.0 E-12	8.3 E-11
Binding Affinity	**-15.9**	**-15.3**	**-16.6**	**-12.2**	**-15.3**	**-13.7**

Interaction analysis

To find and analyze the critical interactions that contribute to the stability of the protein-peptide complex formation, the top binding poses predicted by HADDOCK were subjected to additional analysis using PDBSUM and Biovia Discovery Studio.

Binding Site 1: Interactions

A significant binding affinity of -15.9 kcal/mol was demonstrated during the formation of the complex between Ligand 1 and Binding Site 1 of the DNA binding protein. According to an

analysis of the binding interactions, 38 receptor residues linked with 30 ligand residues, forming four salt bridges, fifteen hydrogen bonds, and roughly 229 non-bonded contacts (such as van der Waals forces, etc.).

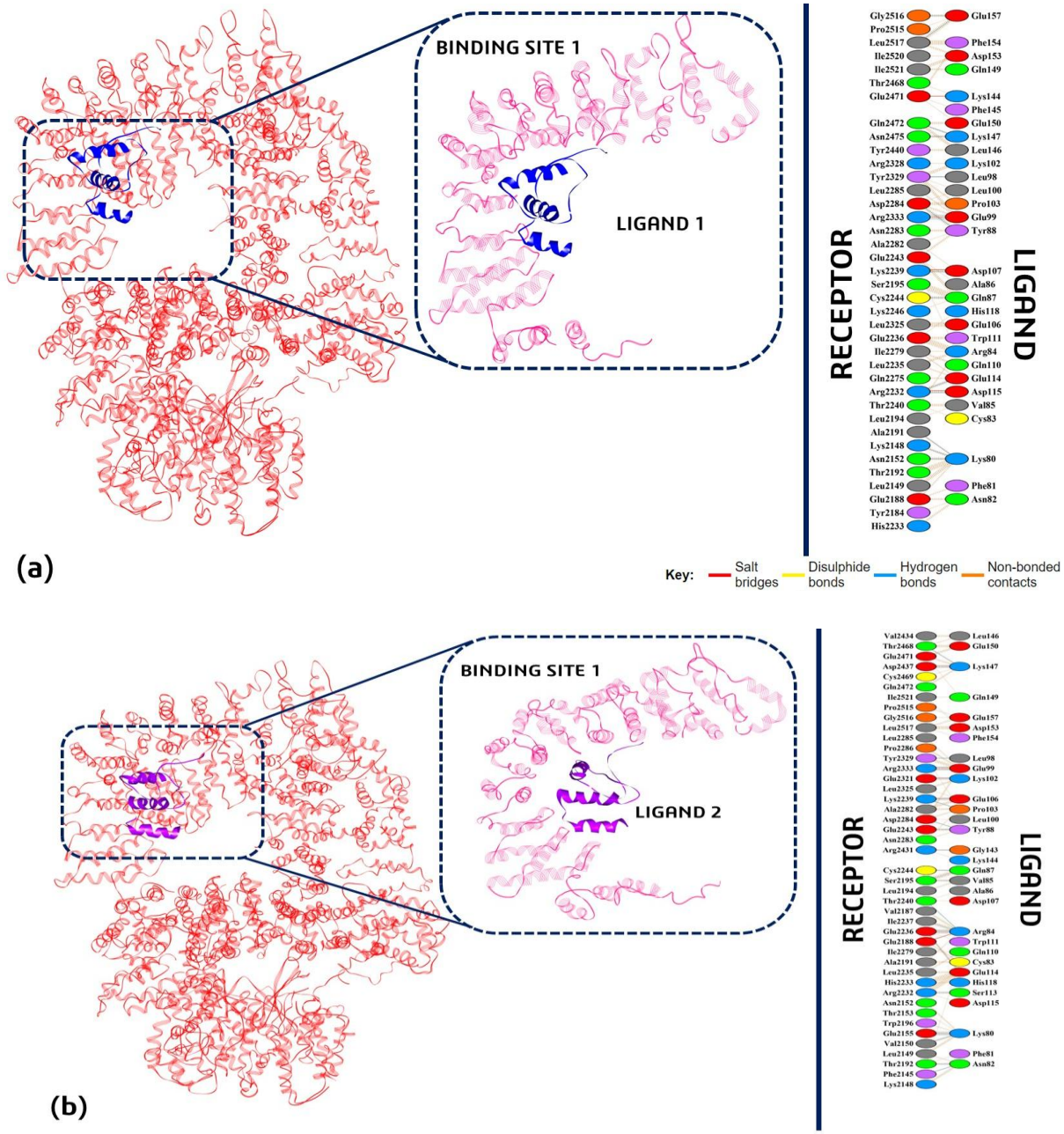
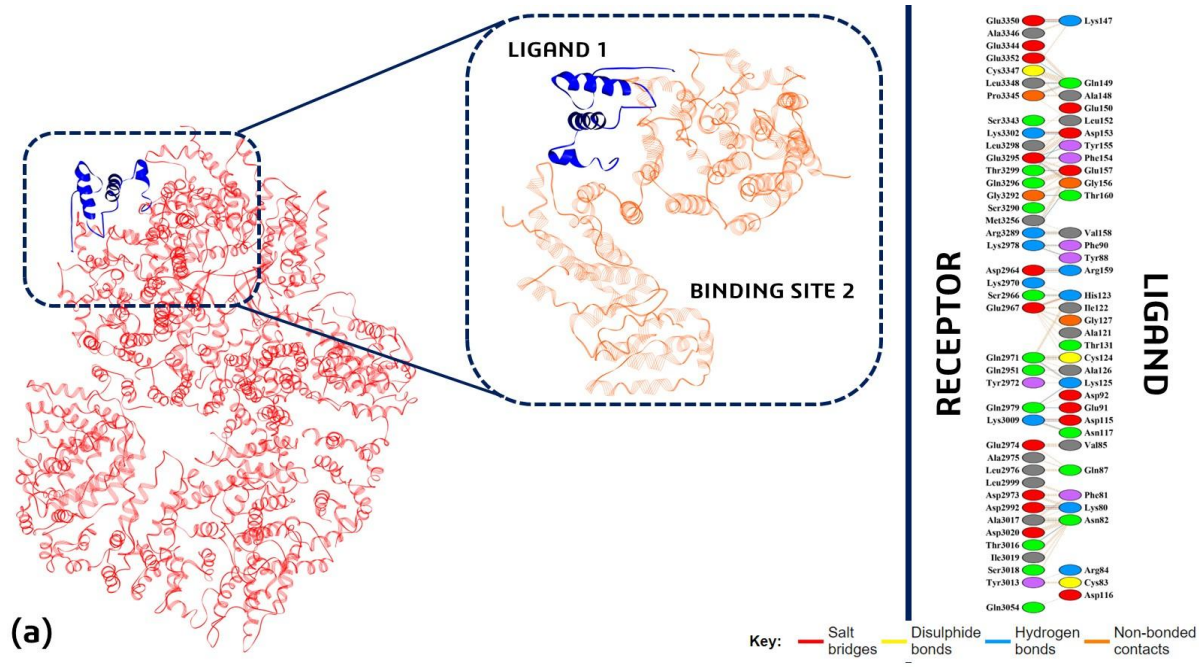


Figure 3.10 *Binding mode and interactions of (a) Ligand 1 and (b) Ligand 2 in Binding Site 1 of the DNA Binding Protein.*

Similar to Ligand 1, Ligand 2 showed a -15.3 kcal/mol binding affinity. The involvement of 44 receptor residues was seen in the binding interactions, resulting in about seven salt bridges, 25 hydrogen bonds, and 263 non-bonded contacts, with Ligand 2 engaging with 31 particular residues.

b) Binding Site 2: Interactions

A strong binding affinity of -16.6 kcal/mol was produced as a result of the interaction between Ligand 1 and Binding Site 2 of the DNA binding protein. The establishment of bonds involving 40 receptor residues and 35 ligand residues was identified through visual inspection of the binding interactions. Six salt bridges, 28 hydrogen bonds, and roughly 302 non-bonded contacts, including van der Waals forces and other intermolecular forces, made up these interactions.



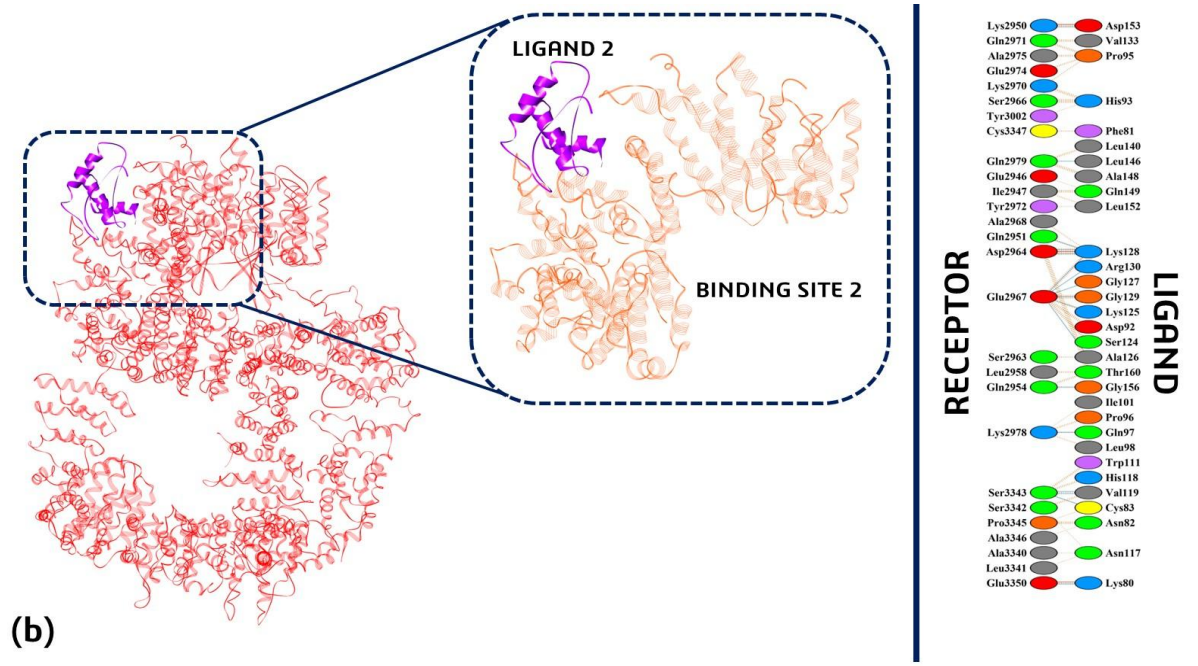


Figure 3.11 *Binding Mode and Interactions of (a) Ligand 1 and (b) Ligand 2 in Binding Site 2 of the DNA Binding Protein.*

Similar to Ligand 1, Ligand 2 displayed a binding affinity of -12.2 kcal/mol. The binding interactions were seen, and it was shown that 27 receptor residues were engaged, allowing for the development of about three salt bridges, 17 hydrogen bonds, and 192 non-bonded contacts with 31 particular residues of Ligand 2.

c) Binding Site 3: Interactions

Ligand 1's interaction with DNA binding protein's Binding Site 3 revealed a binding affinity of -15.3 kcal/mol. When the binding interactions were examined, it was found that 36 receptor residues joined forces with 23 ligand residues. Five salt bridges, 16 hydrogen bonds, and roughly 244 non-bonded contacts were formed as a result of these interactions.

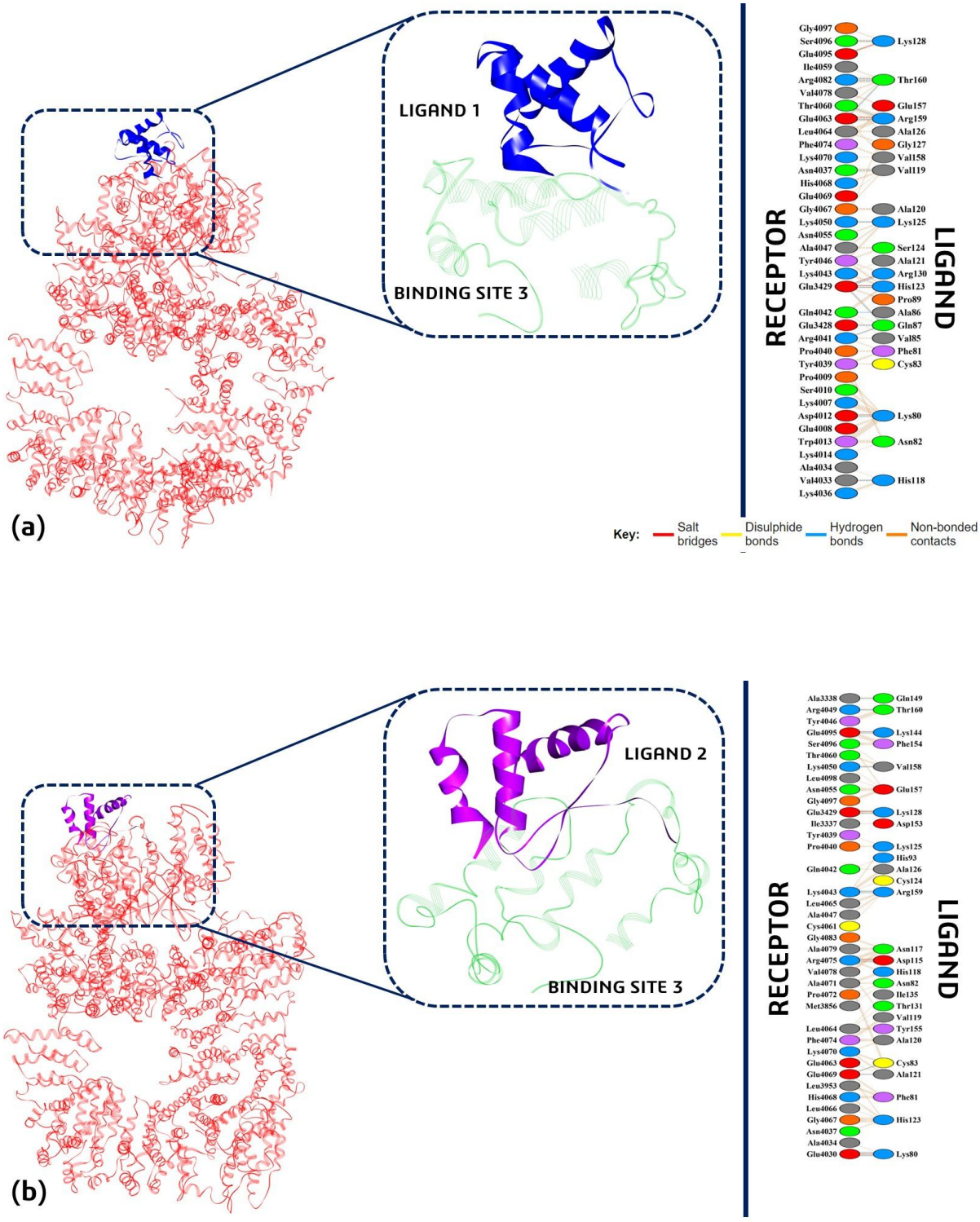


Figure 3.12 *Binding Mode and Interactions of (a) Ligand 1 and (b) Ligand 2 in Binding Site 3 of the DNA Binding Protein.*

Contrarily, Ligand 2 displayed a binding affinity of -13.7 kcal/mol. 38 receptor residues were involved in the binding interactions, producing about four salt bridges, 12 hydrogen bonds, and 200 non-bonded contacts with 27 particular residues of Ligand 2.

DNA-PKcs, a serine/threonine protein kinase that belongs to the PI-3K-like kinase (PIKK) family and plays a crucial role in NHEJ [*Chen et al., 2021*], has its activity closely regulated by changes in phosphorylation. According to several studies, DNA-PKcs is made up of the N-terminal HEAT domain (N-HEAT, residues 1-891), the middle-HEAT domain (M-HEAT, residues 892-2800), the FAT domain (residues 2801-3579), the kinase domain (residues 3580-4099), and the FAT-C domain (residues 4100-4128). Among them, the FAT and FAT-C domains are crucial for DNA-PKcs' kinase activity. Our molecular docking findings indicate that PTEN-WT interacts preferentially with the C terminal region of FAT and the FAT-C domain of DNA-PKcs. Therefore, PTEN might be an important factor in limiting the kinase activity of these two domains.

We aimed to examine whether there is any physical interaction between PTEN and DNA-PKcs using a biochemical study. Thus, we carried out a co-immunoprecipitation assay, which demonstrated the physical interaction between PTEN-WT and DNA-PKcs (Figure 5C). The expression of PTEN-A4, PTEN-C124S, and double mutant transfected cells is drastically altered to a two-fold reduction upon interaction with DNA-PKcs, in contrast to PTEN-WT transfected cells (Figure 3.13.A). Therefore, it may be stated that PTEN's phosphorylation and phosphatase activity are both necessary for its interaction with DNA-PKcs.

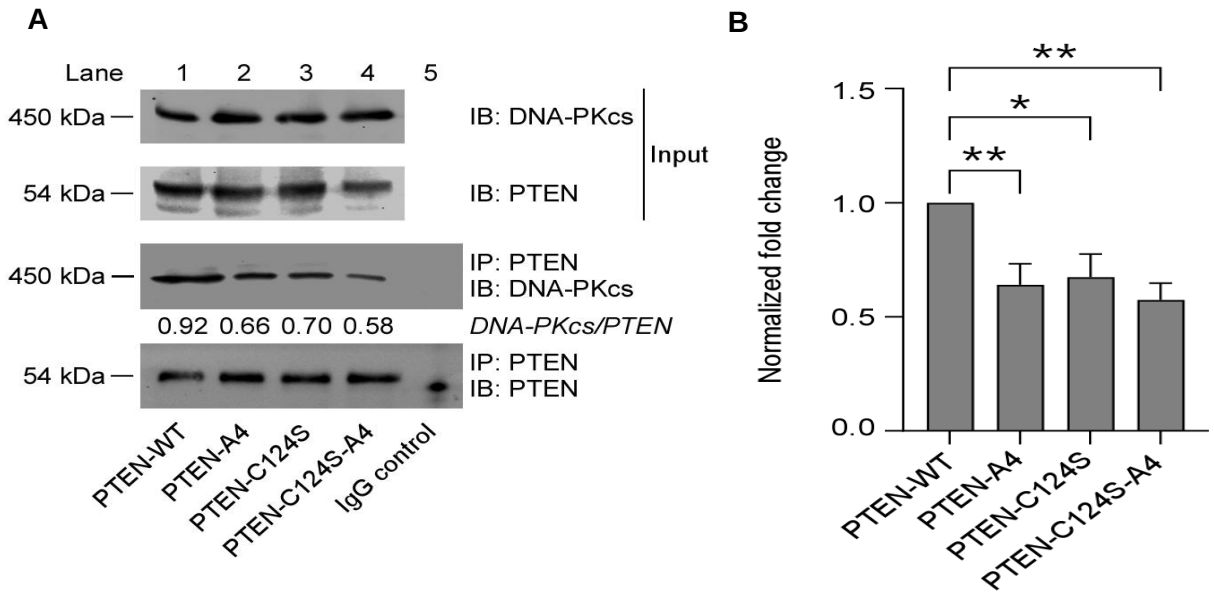


Figure 3.13 *(A) The biochemical data demonstrates that PTEN and DNA-PKcs interact. The indicated PTEN clone was used to transfect PTEN-null PC3 cells. Following etoposide treatment, whole cell lysate was prepared, immune precipitated with anti-PTEN antibody, and immunoblotting with DNA-PKcs antibody demonstrated that PTEN-A4, PTEN-C124S, and PTEN-C124S-A4 could not effectively interact with DNA-PKcs. (B) The fold change is shown on the graph, normalized to PTEN. The transfected PTEN-WT sample has been normalized to 1. Values are the mean ± SD. **p < 0.01, and ***p < 0.001.*

3.3.10 DNA-PKcs phosphorylation is regulated by PTEN phosphatase activity upon DNA damage

DNA damage-induced autophosphorylation of S2056 is a sign of DNA-PKcs activation; nevertheless, the entire effect of phosphorylation needs more research [*Jiang et al., 2019*]. To investigate how PTEN modification affects DNA-PKcs phosphorylation at S2056, various PTEN clones in PTEN null PC3 cells were expressed, damaged with or without etoposide for 3 h, and the cell lysate was then subjected to western blot following a 24 h incubation. In comparison to undamaged cells, we found that etoposide treatment increased DNA-PKcs

phosphorylation (Figure 3.14.A). However, phosphorylation was decreased in the presence of an inhibitor. In comparison to PTEN-WT, DNA-PKcs phosphorylation is increased upon DNA damage when phosphatase-dead and double mutant PTEN are present (Figure 3.14.A). As a result, hyperphosphorylation of this protein persists in those cells since double mutant PTEN and phosphatase-dead PTEN are unable to dephosphorylate DNA-PKcsFurthermore, DNA-PKcs phosphorylation increased in empty-vector transfected PTEN null PC3 cells in the same way as double mutants in the absence of a DNA-PKcs inhibitor. In the presence of a DNA-PKcs inhibitor, damage-induced PTEN null empty-vector transfected cells' DNA-PKcs phosphorylation did not differ noticeably from that of undamaged PTEN null empty-vector cells.

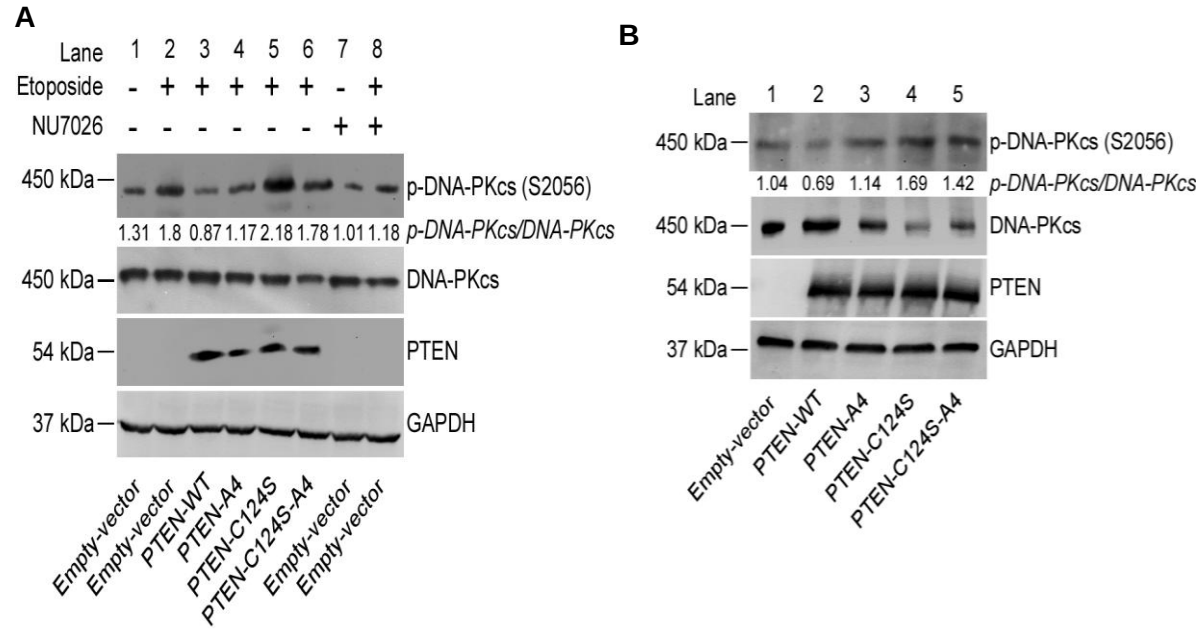


Figure 3.14 *The aforementioned PTEN clones and empty-vectors were transfected into PTEN-null PC3 cells. (A) Cells were treated 24 h after transfection, and (B) mock-treated with etoposide for 3 h. Western blotting was then performed using antibodies against PTEN, GAPDH, DNA-PKcs, and p-DNA-PKcs (S2056). ImageJ software is used to measure band intensities, which are shown here as a ratio of p-DNA-PKcs/DNA-PKcs.*

Next, the formation of p-DNA-PKcs foci upon DNA damage will be assessed in a variety of mutant PTEN transfected cells. It's interesting to note that, compared to PTEN-WT transfected cells, the establishment of foci is greatly hampered in PTEN-A4, PTEN-C124S, and double mutant (PTEN-C124S-A4) transfected cells (Figure 3.15.A). This shows that in mutant PTEN-transfected cells, the DNA repair signaling cascade is severely constrained, leading to the accumulation of DNA damage and impaired DNA repair.

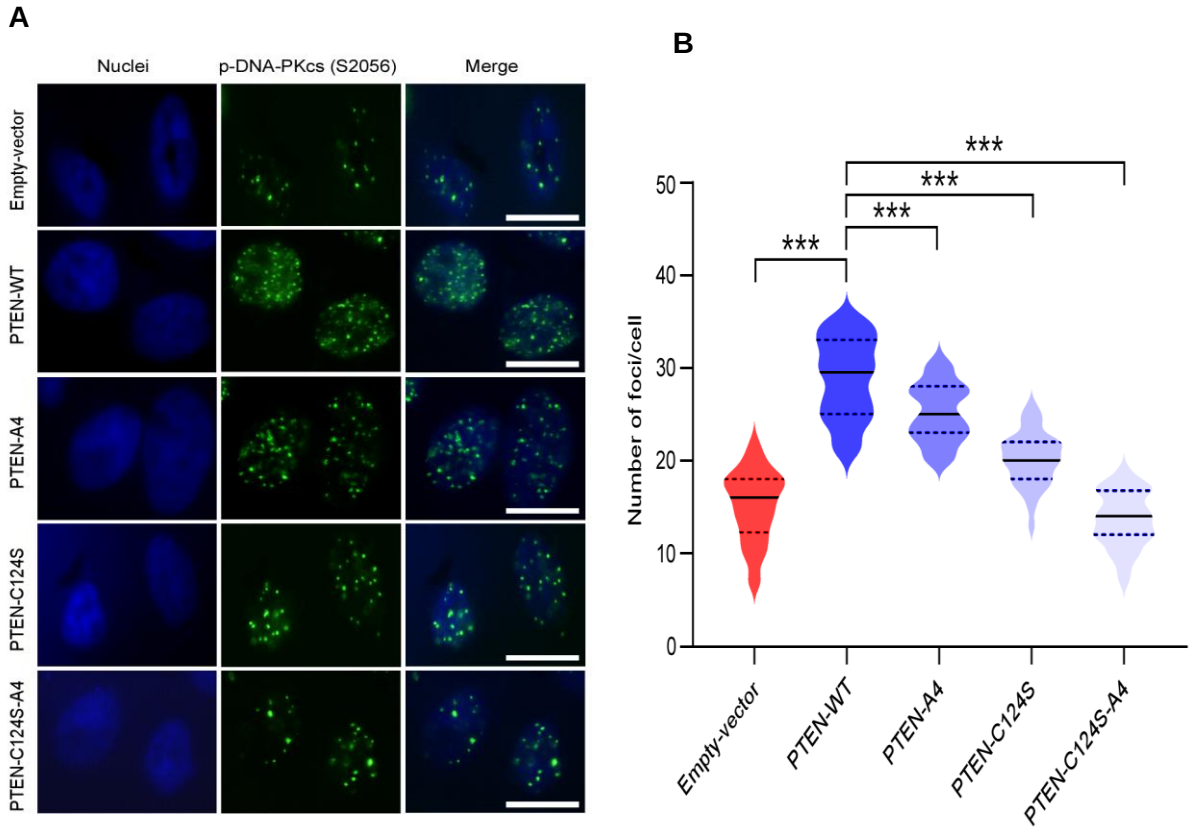


Figure 3.15 *(A) Photomicrograph of p-DNA-PKcs (Ser 2056) foci (green) and DAPI for nuclear staining in cells transfected with the empty vector and mutant PTEN clones. Scale bar = 20 µm. (B) Empty-vector and PTEN mutants-transfected cells were used to quantify the number of p-DNA-PKcs foci per nucleus after etoposide treatment. Each measurement involved about 50 nuclei. Values are the mean ± SD of three independent experiments. ***p < 0.001.*

Next, using either NU7026 or not, we transfected PC3 cells with or without damage using an empty-vector. We found that in the presence of NU7026, the number of p-DNA-PKcs foci is remarkably low in cells without damage, while damaged cells exhibit a modest increase in foci. (Figure 3.16.A).

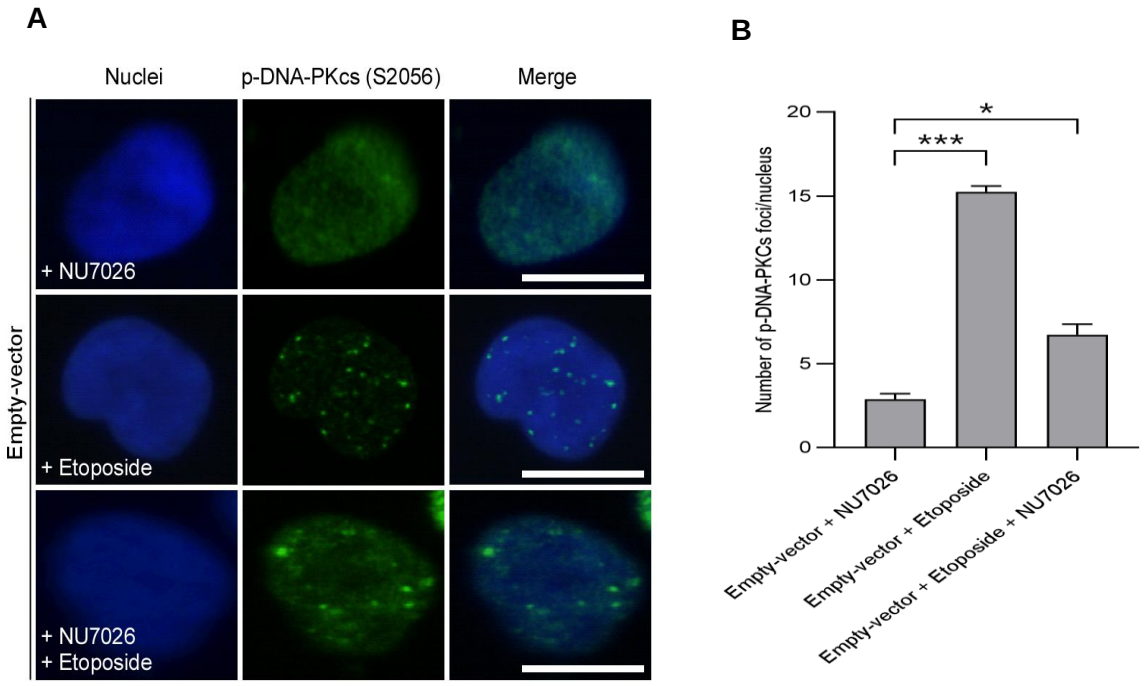


Figure 3.16 *(A) Representative photomicrograph showing DAPI (blue) and p-DNA-PKcs (S2056) foci (green) for nuclear labelling in transfected cells using an empty vector. The cells were either mock-treated or treated with etoposide for 3 h with or without NU7026. Scale bar = 20 µm. (B) The graph represents the quantification of p-DNA-PKcs foci per nucleus. Values are the mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001.*

3.3.11 PTEN promotes stabilization of NHEJ proteins machinery on chromatin depending on C-terminal phosphorylation

Since PTEN phosphorylation is crucial for NHEJ-mediated DSB repair, we sought to determine whether this had an impact on PTEN's chromatin interaction. Cells expressing both

the wild-type and several mutant forms of PTEN were used to prepare the cytoplasmic and nuclear insoluble fractions of chromatin in the presence of etoposide. By using a western blot, the sample was examined. The reduced level of phospho-mutant PTEN as well as double mutant PTEN in the nuclear chromatin fraction in comparison to PTEN-WT indicated its dissociation from chromatin upon etoposide treatment (Figure 3.17.A). However, because the phosphatase dead mutant clone contains an active PTEN phosphorylation site, they may be crucial in the recruitment of PTEN into chromatin during DNA damage. As a result, we conclude that PTEN's phosphorylation is crucial for chromatin association. The association of the proteins DNA-PKcs, XLF, and Ku80 to the chromatin increased roughly two-fold in PTEN-WT transfected cells compared to empty-vector-transfected cells, whereas it significantly decreased in PTEN-A4 and PTEN-C124S-A4 cells (Figure 3.17).

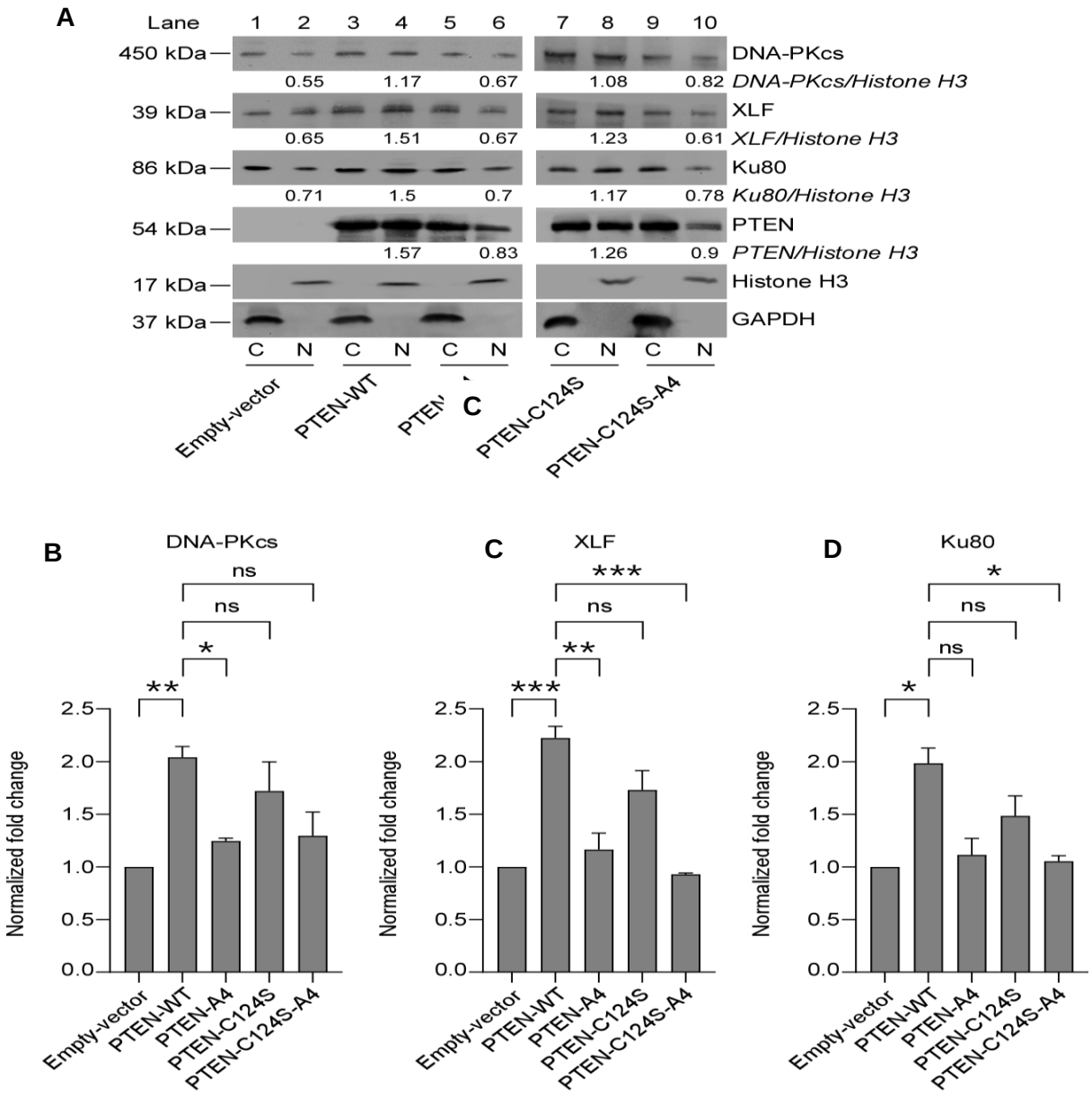


Figure 3.17 *(A) PC3 cells were transfected using an empty vector, along with different PTEN clones. Following a 24 h incubation period, the cytoplasmic and chromatin insoluble fractions were isolated and subjected to immunoblotting using antibodies against DNA-PKcs, Ku80, and XLF. Histone H3 was employed as a loading control for the insoluble fractions. The ratio of the required protein to histone H3 is shown here, based on band intensities determined using ImageJ software. Subcellular fractions are abbreviated as "C" for cytoplasmic fraction and "N" for nuclear insoluble fraction. (B, C, and D) The graph*

*displays the fold change concerning Histone H3. The transfected empty-vector sample was normalized to 1. Values are the mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001.*

3.3.12 Mutant PTEN chemo-sensitized PTEN null cells toward etoposide

To assess the physiological response to etoposide-induced DSBs, we investigate the survival of cells that have been transfected with different mutants of PTEN and empty-vector. We transfected the aforementioned plasmids into PTEN-null PC3 cells individually to ascertain this. Cells were treated with or without etoposide for 3 h following transfection. After allowing the cells to recover in fresh medium for 10 days, they were subjected to a clonogenic assay. In a separate set of studies, treated and mock-treated cells were given fresh medium to recover for 24 h, after which their survival was assessed using the MTT assay. PTEN-WT transfected cells showed a significantly higher percentage of colonies and survival in both circumstances when compared to PTEN-A4, PTEN-C124S, or PTEN-C124S-A4 transfected cells (Figure 3.18.A & B). Perhaps as a result of PI3K's constitutive activation in empty-vector-transfected cells, PTEN-WT transfected cells had higher mortality than empty-vector-transfected cells. Thus, PTEN cells that were double mutant, phospho-dead, or phosphatase-dead were sensitive to etoposide. This phenotypic effect could be caused by diminished development of p-DNA-PKcs foci upon DNA damage in double mutant transfected cells, as well as poor interaction of NHEJ proteins in chromatin. Therefore, a buildup of DNA damage brought on by a malfunctioning NHEJ repair mechanism may contribute to the demise of mutant PTEN-transfected cells.

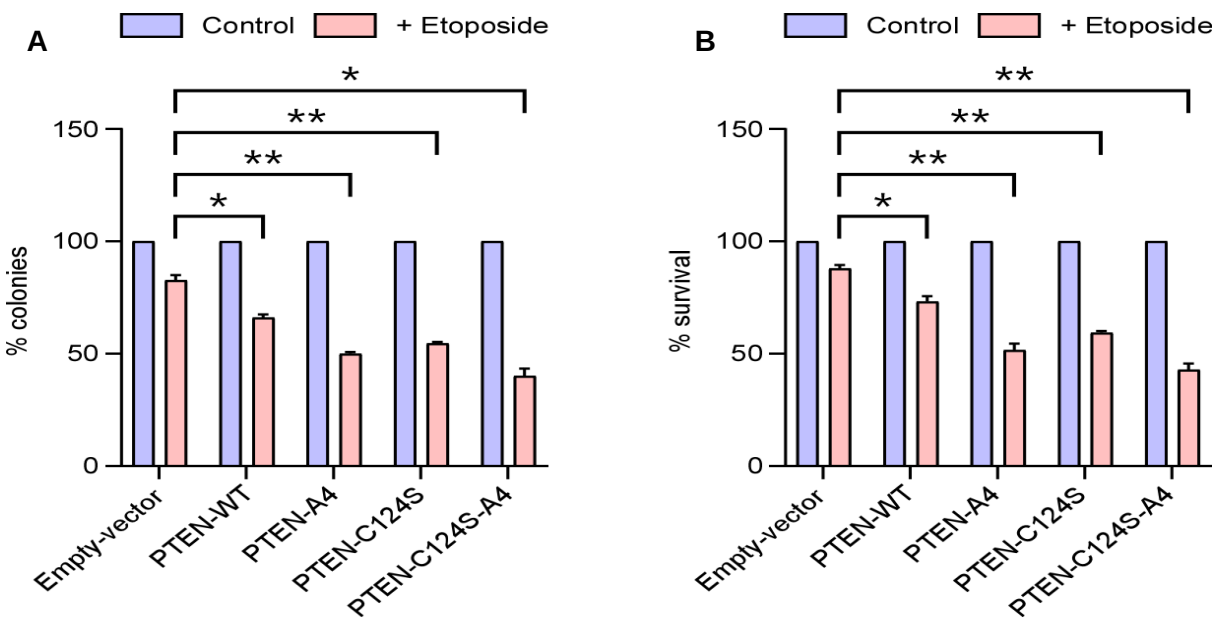


Figure 3.18 *(A) At a concentration of 35 µM etoposide for 3 h, PTEN-WT expressing cells substantially outperformed PTEN-A4 (*p < 0.05) or PTEN-C124S-A4 (***p < 0.001) transfected cells in terms of colony formation efficiency. Empty-vector-transfected etoposide-treated cells were taken with 100% survival. (B) PC3 cells transfected with an empty vector or transiently transfected with PTEN-WT, PTEN-A4, PTEN-C124S, or PTEN-C124S-A4 were treated for 3 h with etoposide at a concentration of 35 µM before being given 24 hours to recover in fresh media. To calculate the percentage of survival, an MTT assay was utilized. Empty-vector-transfected etoposide-treated cells were taken with 100% survival. Values are the mean ± SD of three independent experiments. *p < 0.05, ***p < 0.001.*

3.4 Discussion

In recent years, interest has grown in the investigation of PTEN's non-canonical function to identify unknown tumor suppressive activity. Several pieces of literature have reported PTEN's involvement in DNA repair pathways [*Fan et al., 2020; Ming et al., 2012*]. Although PTEN's role in HR repair is widely acknowledged by numerous research teams, its connection to NHEJ repair is not being thoroughly investigated. According to a recent study,

PTEN disruption impairs HR via lowering RAD51 expression, whereas PTEN knockdown has little effect on the alt-NHEJ and c-NHEJ pathways [*Jiang et al., 2019*]. However, other research indicates that hyperactivated AKT, in the absence of PTEN, phosphorylates XLF, causing XLF to separate from the XRCC4/LIG4 complex and lowering the frequency of NHEJs [*Liu et al., 2015*]. The current study is focused on the non-canonical chromatin modulation-related functions of PTEN. DNA repair is one of the key PTEN actions among its many different roles, and it is intimately related to the protein's function in maintaining genomic integrity [*Ming et al., 2012*]. Apart from its role in DNA repair, post-translational modification of PTEN is thought to be the crucial regulatory event that controls the protein's other functions, including autophagy [*Singh et al., 2011*] and senescence [*González-García et al., 2022*]. Each of the processes that PTEN is exposed to, including phosphorylation, ubiquitination, sumoylation, acetylation, and oxidation, is associated with critical cellular functions. Since PTEN's post-translational modulations heavily influence its functional potential, we looked at the relationship between PTEN's post-translational modulations and the frequency of NHEJ repairs.

The NHEJ pathway appears to be positively regulated by PTEN, according to our *in vitro* reporter-based repair experiment. Furthermore, we discovered that NHEJ repair frequency is dramatically decreased by both phosphorylation and phosphatase-dead PTEN. We also noticed that PTEN null PC3 cells exhibit downregulation of proteins necessary for NHEJ, such as Ku70, Ku80, DNA-PKcs, XRCC4, and XLF. However, phosphorylation and phosphatase-dead PTEN were unable to promote the production of the aforementioned proteins, but PTEN-WT expression dramatically increased their expression. According to a prior study, PTEN may also stimulate the production of XRCC4-like factor (NHEJ1/XLF), which ligates ends of DNA and therefore activates the NHEJ pathway [*Sulkowski et al., 2018*]. According to our research, the instability of repair proteins in PTEN null cells is most

likely the cause of their decreased accumulation. Furthermore, as compared to PTEN-WT-transfected cells, our protein half-life assay reveals that these proteins are significantly more unstable in cells that have been transfected with an empty vector. PTEN, which confers stability on NHEJ repair proteins, may therefore help control this process. However, in empty-vector transfected cells, MG132 inhibiting the proteasome pathway in the presence of CHX results in some rescue of protein expression. Since PTEN phosphorylation is elevated following DNA damage [*Ming et al., 2012*] and is involved in DNA repair, we wanted to assess PTEN phosphorylation upon DNA damage in the presence of DNA-PKcs inhibitor NU7026. Our results demonstrate that NU7026 significantly reduces PTEN C-terminal phosphorylation, which suggests that the defective NHEJ repair in the phospho-mutant transfected cells that we observed. The kinase activity of DNA-PKcs is inhibited by NU7026 because NU7026 is a powerful inhibitor of the ATP-binding pocket of DNA-PKcs. This study suggests that PTEN may be one of DNA-PKcs' targets. The prior study demonstrates that DNA-PKcs's C-terminal site determines its kinase activity [*Yue et al., 2020*]. We found via molecular docking that PTEN-WT may bind to DNA-PKcs' C-terminal region preferentially. Additionally, a physical interaction between PTEN and DNA-PKcs is visible from the co-immunoprecipitation study. Therefore, our next goal was to investigate the p-DNA-PKcs scenario in PTEN mutant transfected cells. An earlier study contends that phosphorylation of DNA-PKcs results in a conformational shift that renders it indistinguishable from the damaged end and encourages DNA end ligation [*Jiang et al., 2012*]. As a result, we discovered that in PTEN-deficient PC3 cells, DNA-PKcs maintained in a hyperphosphorylated state reveal its inactive state. The immune fluorescence assay demonstrated that simultaneously hyperphosphorylated DNA-PKcs could not be recruited on the damage site. PTEN-WT transfection effectively stimulates its recruitment on the damaged region and decreases phosphorylated DNA-PKcs. While phosphatase-dead PTEN has lost its

ability to dephosphorylate DNA-PKcs is unable to do so, which prevents its buildup on the damaged position. As a result, DNA-PKcs' failure to detect DNA damage in PTEN null cells reduces the signal's ability to repair DNA, which suggests that this may be a mechanism by which genomic instability accumulates in these cells. Additionally, our chromatin insoluble fraction demonstrates that PTEN supports the stabilization of the chromatin-bound proteins Ku80, XLF, and DNA-PKcs in a phosphorylation-dependent manner. Although it lessens NHEJ repair, phosphatase dead PTEN transfected cells have an increase of NHEJ machinery on chromatin. Our research therefore suggests that PTEN causes NHEJ machinery recruitment to chromatin in a C-terminal phosphorylation-dependent manner and that its phosphatase activity is necessary for DNA-PKcs dephosphorylation. To prevent DNA-PKcs from becoming phosphorylated and to preserve its presence at the location of the damage, p-PTEN binds to NHEJ proteins in chromatin in response to DNA damage. Consequently, it has more time to phosphorylate its target protein, such as Artemis, LIG4, etc., before it undergoes autophosphorylation and is released from the chromatin. Phospho mutant PTEN can load the NHEJ machinery on chromatin and hence shows reduced repair frequency. Though phosphatase-dead PTEN can recruit Ku80 protein on chromatin, NHEJ repair cannot take place efficiently as DNA-PKcs exist in hyper-phosphorylated form. In double mutant PTEN, DNA-PKcs also exist in a hyperphosphorylated state and Ku80 protein cannot be recruited in chromatin. Consequently, the related NHEJ protein is unable to bind to chromatin, which reduces the effectiveness of NHEJ repair. As our survival assay demonstrated, an accumulation of DNA damage may lead to the sensitization of mutant PTEN-transfected cells to etoposide. These results open up a new mechanistic perspective on PTEN's non-canonical role in DNA repair.

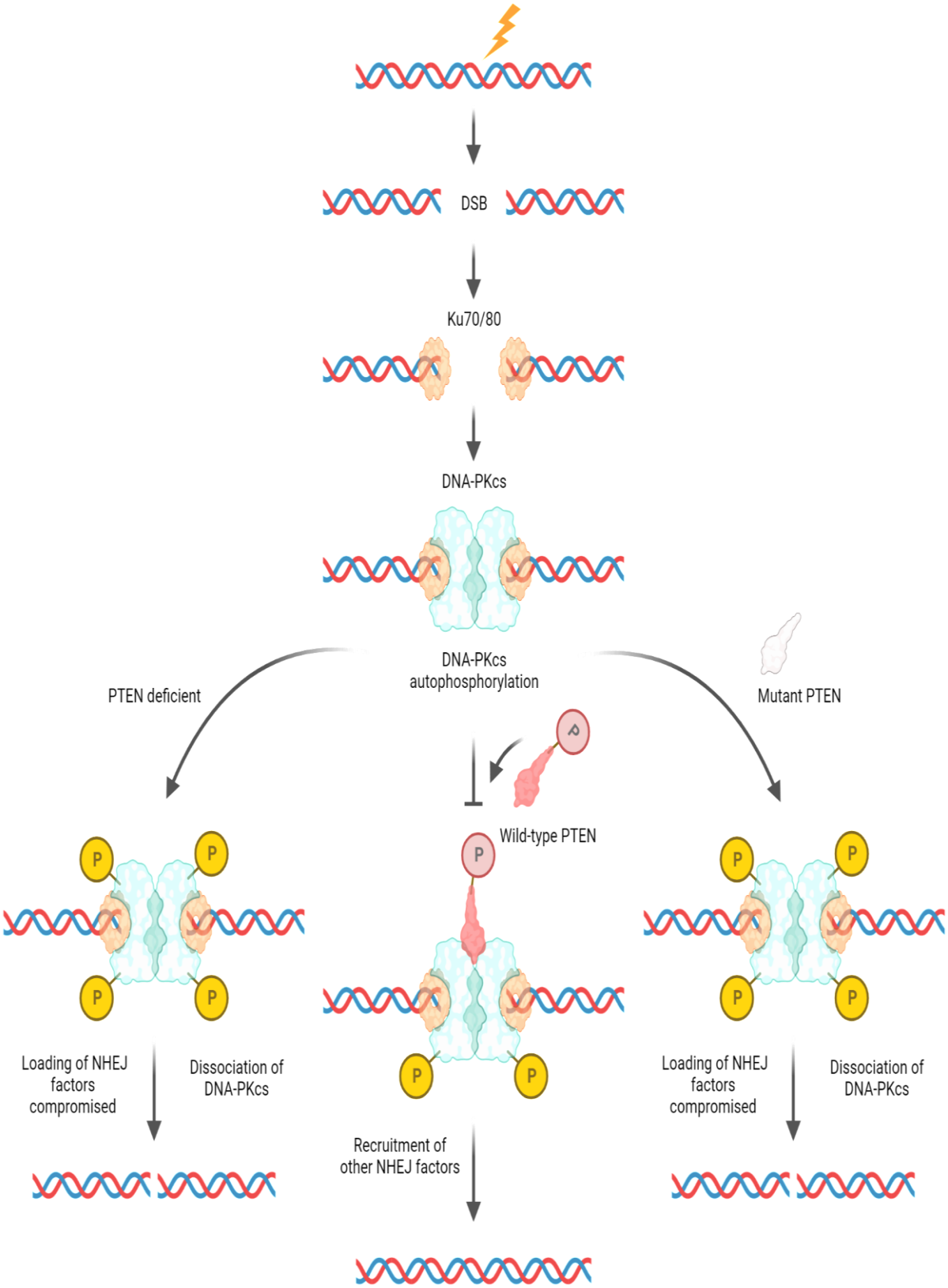


Figure 3.19 *Schematic representation of the hypothesis of our work.*

3.5 Materials & Methods

3.5.1 Chemicals and antibodies

The following chemicals were purchased from HiMedia: RPMI 1640, DMEM, PBS, Paraformaldehyde, Tris base, Glycine, SDS, Penicillin/Streptomycin, Amphotericin-B. FBS was from Gibco. MTT, Tween-20, Low Melting Point Agarose, Triton X-100, EDTA, NaCl, EGTA, Sodium Metavanadate, PMSF, NP-40, Acrylamide/bis-acrylamide, 5% non-fat dry milk, and Tween-20 were from SRL. DAPI from Vector Laboratories. The protease and Phosphatase inhibitor cocktail were from Abcam. BSA, Bradford's reagent, Etoposide, and DMSO were from Sigma Aldrich. NU7026 was from Tocris. Ku80 (2180S), Ku70 (4588S), XRCC4 (23908), XLF (2854S), DNA-PKcs (4602S), p-PTEN (S380/T382/383) (9554S), and GAPDH (2118S) were from Cell Signaling Technology. p-DNA-PKcs (S2056) (ab18192), PTEN (ab32199), Histone H3 (ab8898) were from Abcam. Anti-Mouse or anti-Rabbit IgG conjugated with HRP (1:10000, SRL), and anti-Rabbit IgG conjugated with Alexa fluor 488 (1:200, Invitrogen Molecular Probes) were used as secondary antibodies.

3.5.2 Cell lines and Culture conditions

Penicillin/Streptomycin (100 U/ml, HiMedia), 10% heat-inactivated FBS (Gibco), and Amphotericin-B (HiMedia) were added as supplements to WI-38 and PTEN-null PC3 cells, which were purchased from the National Center for Cell Science in Pune, India. They were kept at 37°C, 5% CO2, and 95% relative humidity (RH) in DMEM, and RPMI 1640 media, respectively.

3.5.3 Site-Directed Mutagenesis

Hemagglutinin-tagged wild-type PTEN protein is encoded by the gene construct pSG5L-HA-PTEN-WT (HA-PTEN-WT), while phospho-dead pSG5L-HA-PTEN-A4 (Ser380Ala,

Thr382Ala, Thr383Ala, Ser385Ala) generates hemagglutinin-tagged mutant PTEN protein. As a control, empty vectors pSG5L-HA were employed. Dr. W.R. Sellers of Harvard Medical School gave this plasmid as a royal gift.

Phosphatase-dead (pSG5L-HA-PTEN-C124S) and double mutant (pSG5L-HA-PTEN-C124S-A4) PTEN were produced using an Agilent site-directed mutagenesis kit by the manufacturer's instructions and were validated by sequencing. For the phosphatase dead mutant PTEN, the following primer was used:

Forward:5´GTTGCAGCAATTCACTCTAAAGCTGGAAAGGG3´

Reverse:5´CCCTTTCCAGCTTTAGAGTGAATTGCTGCAAC 3´

3.5.4 Cellular Transfection

PC3 cells were plated in an antibiotic-free media 18 h before transfection. For transient transfection, Lipofectamine 3000 (Invitrogen) was employed according to manufacturer's instructions.

3.5.5 NHEJ Assay System

With the aid of the Mlu1 restriction enzyme, the cDNA of GFP from the eGFPC1 plasmid with the promoter (pCMV) was sub-cloned in the DsRed vector. Agarose gel electrophoresis and the expression of both fluorescent proteins after transfection served to verify the successful ligation. The name of this hybrid plasmid was DsGFP. With the help of the restriction enzyme (Sbf1) found in the DsRed gene, this plasmid was linearized. Using a nucleofector in a 60 mm plate, 2.5 µg of linearized DsGFP plasmid and 2.5 µg of PTEN-WT or various mutant clones (1:1) were used to co-transfect PC3 cells after the linear segment was obtained using the agarose gel elution procedure. After incubation for 48 h, the cells

were ready to be analyzed using a FACS analyzer to determine the proportion of DsRed cells in the GFP-positive population.

3.5.6 Cell lysis and immunoblotting

Cell pellets were collected following transfection with different PTEN clones, and cell lysates were made using cell lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and protease-phosphatase inhibitor). The lysate was boiled with a protein loading dye concentration of 1X before resolution by 10% SDS-PAGE. The gel was subsequently moved to a PVDF membrane that had been activated with methanol. The membrane was incubated with primary antibodies for an overnight period at 4°C after being blocked for an hour at RT with TBS containing 0.1% (w/v) Tween-20 and 5% (w/v) non-fat milk. The membrane was treated with 1X TBST the next day before being incubated for an hour at RT in the dark with HRP-conjugated anti-rabbit or anti-mouse antibodies. ECL reagents (Abcam) were used to identify the bands.

3.5.7 Protein Half-life Assay

PC3 cells were transfected using the PTEN plasmid and its various mutations. Following a 24 h transfection, 100 g/ml cycloheximide was added to the plate, and as stated, the cycloheximide treatment was stopped at the indicated time points of 0, 8, and 24 h. Protein concentration was calculated after preparing whole-cell lysates. For each sample, 100 µg of total protein was immunoblotted with anti-PTEN, anti-Ku70, anti-Ku80, anti-DNA-PKcs, and anti-XLF antibodies. The quantification process was carried out using ImageJ software and normalized to GAPDH.

3.5.8 Proteasomal Pathway Inhibition Assay

The PC3 cells were transfected using the empty-vector plasmid. Following transfection, cells were subjected to 100 µg/ml of cycloheximide for 0, 8, 16, and 24 h, as indicated by the

presence or absence of 10 µM MG132. Following the preparation of cell lysates, the concentration of proteins was determined. The total protein of each sample was then measured by immunoblotting with antibodies against XLF, Ku70, Ku80, and DNA-PKcs. The ImageJ software was utilized to quantify and normalize protein levels of GAPDH.

3.5.9 Protein-Protein Docking

The Guru interface of the HADDOCK Docking web server was used to perform the protein-protein docking computations [*Gopi et al., 2023*]. A complete collection of over 500 characteristics designed for DNA, RNA, and protein-protein docking are included in the Guru interface [*Khan et al., 2021*]. The receptor protein, DNA-PKcs was used from the PDB (5W1R) for the docking simulations [*Sharif et al., 2017*]. Three different binding sites in the receptor protein's active residues were identified: binding site 1, which includes residues 2000 to 2800, binding site 2, which includes residues 2801 to 3600, and binding site 3, which includes residues 4000 to 4200. Using the online Expasy translate tool, the PTEN-WT protein and PTEN-C124S protein sequences were deduced from gene sequences. Using the homology model (MOE 2009 tool), the tertiary structures of the PTEN-WT and PTEN-C124S proteins were identified. For docking investigations, the proteins PTEN-WT and PTEN-C124S were employed as ligands 1 and 2, respectively.

The active site residues for the different proteins were entered into the docking calculations along with the default input and docking settings. Multiple docked poses were created using the HADDOCK Docking web server and then organized into clusters. HADDOCK supplied a variety of useful measures for each cluster, including Z-scores, RMSD values, Van der Waals, electrostatic energies, and desolvation energies.

The following equation was used to calculate the HADDOCK score, which served as the foundation for cluster ranking [21]:

HADDOCK score = 1.0 * Evdw + 0.2 * Eelec + 1.0 * Edesol + 0.1 * Eair

Here, Evdw represents the intermolecular Van der Waals energy, Eelec represents the intermolecular electrostatic energy, Edesol represents the empirical desolvation energy, and Eair represents the restraint energy.

The dissociation constant (K_d) values were computed using the PROtein BinDing EnerGY Prediction (PRODIGY) server to assess the binding affinities of the protein-protein docked poses [*Xue et al., 2016*]. Lower K_d values indicate stronger interactions, and they are frequently used to measure the strength of interactions in complex macromolecular binding [*Landry et al., 2013*]. The docked protein structure was uploaded to the PDBSUM Generate server to confirm the computed number of interactions based on the top models produced by HADDOCK [*EMBL-EBI, 2013*]. This stage made sure that all interactions created inside the docked complexes were thoroughly evaluated. Additionally, Biovia Discovery Studio was used for the visual examination of the important binding interactions [*BIOVIA, 2019*].

3.5.10 Indirect Immunofluorescence

On 18 mm sterile glass coverslips, PC3 cells were seeded and incubated for the night at 37ºC in a CO_2 incubator. A newly made 4% paraformaldehyde solution was used to fix the cells for 15 min after they had received an etoposide treatment (35 M for 3 h). Following this, the cell was permeabilized with 0.2% Triton X-100 on ice for 15 min before being washed with 1X PBS. Fixed cells were first pre-incubated for 1 h at RT in blocking solution (1% BSA in 1 PBS), and then they were left in wash buffer (0.1% BSA and 0.05% Tween 20 in 1 PBS) overnight at 4°C with primary antibodies (p-PTEN 1:100, Cell Signaling Technology; p-DNA-PKcs 1:200, Abcam). Following a wash with wash buffer, they were probed for 1 h at RT with Alexa fluor 488 conjugated anti-rabbit antibody 1:200 (Invitrogen Molecular

Probes). After thoroughly washing the cells, they were put in a mounting solution containing DAPI (Vector Laboratories) and observed via a fluorescence microscope (Leica).

3.5.11 Flow Cytometry

PTEN-WT plasmid was transfected into PC3 cells, and the cells were then subjected to etoposide treatment at a concentration of 35 µM for 3 h in both the presence and the absence of NU7026. The cells were fixed by adding 4% formaldehyde directly to the centrifuged pellets after being centrifuged the treated and untreated cells. Before being rinsed with 1X PBS, cells were fixed for 15 min at RT. Following vigorous vortexing in 1 ml of ice-cold methanol for a 10 min incubation period at 4°C, the cells were permeabilized. After that, cells were kept overnight at -20°C in methanol. The next day the cells were twice rinsed with a staining medium (1X PBS containing 0.5% BSA) to remove the methanol. Following a resuspension in diluted primary antibody (p-PTEN 1:200, Cell Signaling Technology), the cells were incubated for 1 h at RT. For 30 min at RT in the dark, cells were suspended in diluted fluorochrome-conjugated secondary antibody (1:200) after being rinsed in staining solution. The cells were pelleted after being washed with the staining medium. Samples were then resuspended in 500 µl of 1X PBS and analyzed using flow cytometry.

3.5.12 Immunoprecipitation assay

PTEN-WT, corresponding mutants, and empty vector were transfected into PC3 cells. For 3 h, the cells were treated with a 35 µM dose of etoposide. Protease and phosphatase inhibitors were added to the lysis buffer used to lyse the cells in preparation for the immunoprecipitation assay. Whole-cell lysates were obtained by centrifugation and subsequently incubated at 4°C for overnight while being rotated continuously with anti-PTEN antibody and protein A/G plus Agarose. The immunocomplexes were washed three times in lysis buffer, boiled in SDS sample buffer, and then subjected to western blot analysis.

3.5.13 Cellular Fractionation

The transfected cells were pre-treated for 3 h with 35 µM etoposide, then rinsed with 1X PBS and fixed for 20 min at RT in a freshly made 1% formaldehyde solution. After washing with 1X PBS, cell pellets were collected and suspended in SDS lysis buffer containing phosphatase and protease inhibitors. The samples were sonicated for 15 min after being incubated at 4°C for 10 min. After that, 200 µl of solution A [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, protease and phosphatase inhibitors] were added to the supernatant to resuspension it. Following a 10 min incubation on ice and a 5 min centrifugation at 1500 g, the cytoplasmic and nuclear fractions were obtained. The nuclear fraction was twice rinsed with solution A before being incubated on ice for 15 min in 150 µl of solution B (3 mm EDTA, 0.2 mM EGTA, 1 mM DTT, and protease-phosphatase inhibitors). After being separated into nuclear insoluble fractions, the pellets were boiled in 1X Laemmli sample buffer. After that, the samples underwent a western blot analysis.

3.5.14 Clonogenic Assay

PC3 cells were seeded in a 35 mm plate and transfected with empty-vector, PTEN-WT, and the corresponding mutants. After two days of transfection, cells were treated with 35 µM etoposide for 3 h before being cultured for a further 24 h in new media. With periodic medium changes every 72 h, they were maintained for two weeks. After PBS wash, cells were fixed (1:7-acetic acid/methanol) for 5 min at RT. Following a 30 min incubation period and a wash with distilled water, 0.5% crystal violet solution was applied. For counting, the plates were dried by air. In a clonogenic assay, cell viability was measured as a percentage for each transfected PC3 cell in comparison to empty-vector-transfected cells (which are 100% viable).

3.5.15 MTT Assay

After being transfected with different PTEN clones, etoposide was treated, and cells were washed with 1X PBS. Then they were incubated for 3 h at 37°C in a media containing MTT (0.5 mg/ml). The resultant formazan crystals were dissolved in MTT solubilization buffer (40 ml isopropanol, 10 ml Triton X-100, 170 µl HCl), and the absorbance was measured using a UV vis spectrophotometer (Biotek) at a wavelength of 570 nm.

3.5.16 Statistical Analysis

The groups' statistical differences were computed using a student's t-test. Statistical significance was defined as $P < 0.05$ and 0.001. The Origin Pro v.8 software (Origin Lab) was used to examine the data.

Chapter 4

Conclusion & Future Directions

4.1 Concluding remarks

According to COSMIC, PTEN mutations have been found in at least 30 distinct tumor types. PTEN is one of the most often deleted or altered tumor suppressor genes, with loss of PTEN expression being responsible for many more human malignancies. Loss of PTEN is linked to high-grade malignancies, an unfavorable prognosis, and an insufficient therapeutic response in a variety of human cancers. Apart from deletion, post-translational modulation of PTEN plays an important role in controlling its activity. Any deregulation associated with post-translational modulation of PTEN may link with different cancers. In our laboratory, we are engaged in exploring the link between post-translational modulation of PTEN with different cellular activity including DNA repair.

Previously the function of PTEN in DNA metabolism was investigated as PTEN reduces multinucleation, the role of PTEN phosphorylation in replication stress, and also different post-translational modulation of PTEN associated with cytological damages of cells. Here, we discovered that PTEN C-terminal phosphorylation is crucial for its interaction with chromatin and retention of heterochromatin. By up-regulating H3K9me3 and HP1α, PTEN-proficient cells maintain the heterochromatin signature and have better-organized chromatin. PTEN becomes phosphorylated and linked with chromatin as a result of replication stress or DNA damage, and with the aid of phosphatase activity, it dephosphorylates MCM2. PTEN preserves genomic stability in this way.

According to our research, PTEN recruits the NHEJ machinery to the chromatin in a C-terminally phosphorylation-dependent way, and its phosphatase activity is required for DNA-PKcs dephosphorylation. In response to DNA damage, p-PTEN binds to NHEJ proteins in chromatin, preventing DNA-PKcs from being phosphorylated and maintaining its presence at the site of the damage. So, it has more time to phosphorylate its intended protein, such as

Artemis, LIG4, etc., before it goes through autophosphorylation and is freed from the chromatin. DNA-PKcs also exist in a hyperphosphorylated state in double mutant PTEN, and the Ku70/80 protein cannot be bound in chromatin. This decreases the efficiency of NHEJ repair and results in genomic instability because the associated NHEJ protein is unable to link to chromatin.

In addition to these investigations, research in the fields of molecular biology and biochemistry is illuminating the molecular processes of PTEN regulation pertinent to cancer. Further research in these areas will eventually result in the creation of novel therapeutic approaches for the treatment of PTEN-deficient tumors.

4.2 Future directions

The fundamental cause of tumor development is the constitutive activation of oncogenic proteins and mutational inactivation/compromised expression of proteins involved in tumor suppressor activity/DNA repair pathway. Maintaining genomic stability is the fundamental basis for preventing cancer predisposition as neoplastic transformation shows genomic aberration. Since the past few years, PTEN has been emerging as a DNA repair protein and is essential for maintaining genomic stability. PTEN translocates into the nucleus and is associated with chromatin in response to DNA DSB.

DNA DSBs are harmful because, if they are not effectively repaired, they can result in cancer by triggering mutation and chromosomal rearrangements. Both the HR and NHEJ processes, which are primarily responsible for DSB repair, require a large number of proteins. Among these, HR repair follows a complex mechanism and is error-free. According to reports, PTEN participates in both the HR and NHEJ pathways. Even while PTEN's importance in HR has been highlighted in certain publications, a mechanistic understanding of how PTEN controls HR pathways is still elusive. PTEN is also subject to additional post-translational

modifications that control its activity and chromatin interaction, including phosphorylation, SUMOylation, acetylation, and methylation at different locations. Prior research revealed that PTEN phosphorylation is necessary for chromatin interaction and DNA repair. Furthermore, it has been discovered that PTEN deficiency impairs the expression of the heterochromatin proteins HP1α and H3K9me3 and causes chromatin disorder. Chromatin modulation is crucial for HR repair as evidenced by the diminished H3K36me3 expression caused by overexpression of the H3K36 dimethylase KDM4A. Additionally, interactions between the appropriate histone acetyltransferase and the DNA repair complex are facilitated by crosstalk between H3K36me3 and H4K16 ac. Heterochromatic changes are therefore associated with HR repair, either through promoting the recruitment of repair factors or cell cycle checkpoint proteins, followed by HR enzymatic processes. Since PTEN and H1 linker histone work together to inhibit H4 acetylation, PTEN loss causes an increase in the epigenetic marker for chromatin activation histone H4K16. It is anticipated that PTEN will interact with heterochromatin, HP1α, H3, and histone H1 to influence gene expression. Likely post-transcriptional modification of PTEN in chromatin modulation as well as HR pathways is associated because the majority of PTEN's nuclear activity is attributable to its epigenetic regulation. Therefore, it is necessary to investigate how PTEN post-translational modulation and its catalytic activity influence chromatin modulation and HR repair pathways in response to DNA damage. This would assist us in designing a therapeutic method for targeting HR pathways in cancer cells.

4.2.1 Post-translational modulation of PTEN in HR-mediated repair pathway

We previously developed a plasmid-based HR assay system. Our plasmid-based reporter assay suggests that PTEN-WT increases HR repair frequency in PTEN null cells. We will transfect various PTEN mutant clones in PTEN null PC3 cells by nucleofector and after 24 h

of incubation green fluorescence intensity will be measured by FACS. If HR occurs, then the intensity of green fluorescence will be increased. This experiment would give us an idea about the role of different PTEN mutant clones in HR repair. The expression of HR repair proteins will then be examined in cells that will be transfected with the different PTEN clones.

4.2.2 Significance of nuclear distribution of PTEN in determination of PTEN-HR proteins association

The immunofluorescence technique will be used for nuclear distribution patterns of various HR proteins in PTEN-WT and mutant PTEN transfected cells after DNA damage. Colocalization of PTEN with various HR proteins will also be studied by this technique. Here we will use specific primary antibodies and fluorescence-labelled secondary antibodies for visualization under fluorescence microscopy. The nuclear distribution of PTEN clones after DNA damage may directly help us to determine the components of HR factors with which PTEN needs to be associated after DNA damage.

References

References

Abdulkareem IH, Blair M. Phosphatase and tensin homologue deleted on chromosome 10. Niger Med J. 2013;54(2):79-86. doi: 10.4103/0300-1652.110033

Aggarwal V, Kashyap D, Sak K, Tuli HS, Jain A, Chaudhary A, Garg VK, Sethi G, Yerer MB. Molecular Mechanisms of Action of Tocotrienols in Cancer: Recent Trends and Advancements. Int J Mol Sci. 2019;20(3):656. doi: 10.3390/ijms20030656

Agrawal S, Eng C. Differential expression of novel naturally occurring splice variants of PTEN and their functional consequences in Cowden syndrome and sporadic breast cancer. Hum Mol Genet. 2006;15(5):777-87. doi: 10.1093/hmg/ddi492

Allshire RC, Madhani HD. Ten principles of heterochromatin formation and function. Nat Rev Mol Cell Biol. 2018;19(4):229-244. doi: 10.1038/nrm.2017.119

Al-Noman A, Mokbul MI, Hossain N, Rana MS et al. A young female of Cowden syndrome presenting with Lhermitte-Duclos disease: An illustrative case. Surg Neurol Int. 2023;14:296. doi: 10.25259/SNI_325_2023

Álvarez-Garcia V, Tawil Y, Wise HM, Leslie NR. Mechanisms of PTEN loss in cancer: It's all about diversity. Semin Cancer Biol. 2019;59:66-79. doi: 10.1016/j.semcancer.2019.02.001

Amodio N, Scrima M, Palaia L, Salman AN, Quintiero A et al. Oncogenic role of the E3 ubiquitin ligase NEDD4-1, a PTEN negative regulator, in non-small-cell lung carcinomas. Am J Pathol. 2010;177(5):2622-34. doi: 10.2353/ajpath.2010.091075

Bassi C, Ho J, Srikumar T, Dowling RJ, Gorrini C et al. Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress. Science. 2013;341(6144):395-9. doi: 10.1126/science.1236188

Bauer NC, Doetsch PW, Corbett AH. Mechanisms Regulating Protein Localization. Traffic. 2015;16(10):1039-61. doi: 10.1111/tra.12310

Bermúdez Brito M, Goulielmaki E, Papakonstanti EA. Focus on PTEN Regulation. Front Oncol. 2015;5:166. doi: 10.3389/fonc.2015.00166

Bétermier M, Bertrand P, Lopez BS. Is non-homologous end-joining really an inherently error-prone process? PLoS Genet. 2014;10(1):e1004086. doi: 10.1371/journal.pgen.1004086

BIOVIA DS (2019). Discovery Studio Client (v20.1.0.19295)

Bononi A, Bonora M, Marchi S, Missiroli S, Poletti F et al. Identification of PTEN at the ER and MAMs and its regulation of Ca(2+) signaling and apoptosis in a protein phosphatase-dependent manner. Cell Death Differ. 2013;20(12):1631-43. doi: 10.1038/cdd.2013.77

Bononi A, Pinton P. Study of PTEN subcellular localization. Methods. 2015;77-78:92-103. doi: 10.1016/j.ymeth.2014.10.002

Boyer AS, Walter D, Sørensen CS. DNA replication and cancer: From dysfunctional replication origin activities to therapeutic opportunities. Semin Cancer Biol. 2016;37-38:16-25. doi: 10.1016/j.semcancer.2016.01.001

Brandmaier A, Hou SQ, Shen WH. Cell Cycle Control by PTEN. J Mol Biol. 2017;429(15):2265-2277. doi: 10.1016/j.jmb.2017.06.004

Brandsma I, Gent DC. Pathway choice in DNA double strand break repair: observations of a balancing act. Genome Integr. 2012;3(1):9. doi: 10.1186/2041-9414-3-9

Branzei D, Foiani M. The checkpoint response to replication stress. DNA Repair (Amst). 2009;8(9):1038-46. doi: 10.1016/j.dnarep.2009.04.014

Breuksch I, Welter J, Bauer HK, Enklaar T, Frees S et al. In renal cell carcinoma the PTEN splice variant PTEN-Δ shows similar function as the tumor suppressor PTEN itself. Cell Commun Signal. 2018;16(1):35. doi: 10.1186/s12964-018-0247-9

Briu LM, Maric C, Cadoret JC. Replication Stress, Genomic Instability, and Replication Timing: A Complex Relationship. Int J Mol Sci. 2021;22(9):4764. doi: 10.3390/ijms22094764

Cao J, Schulte J, Knight A, Leslie NR, Zagozdzon A, Bronson R et al. Prdx1 inhibits tumorigenesis via regulating PTEN/AKT activity. EMBO J. 2009;28(10):1505-17. doi: 10.1038/emboj.2009.101

Carter BS, Ewing CM, Ward WS, Treiger BF, Aalders TW et al. Allelic loss of chromosomes 16q and 10q in human prostate cancer. Proc Natl Acad Sci U S A. 1990;87(22):8751-5. doi: 10.1073/pnas.87.22.8751

Chae HD, Broxmeyer HE. SIRT1 deficiency downregulates PTEN/JNK/FOXO1 pathway to block reactive oxygen species-induced apoptosis in mouse embryonic stem cells. Stem Cells Dev. 2011;20(7):1277-85. doi: 10.1089/scd.2010.0465

Chalhoub N, Baker SJ. PTEN and the PI3-kinase pathway in cancer. Annu Rev Pathol. 2009;4:127-50. doi: 10.1146/annurev.pathol.4.110807.092311

Chang CJ, Mulholland DJ, Valamehr B, Mosessian S, Sellers WR, Wu H. PTEN nuclear localization is regulated by oxidative stress and mediates p53-dependent tumor suppression. Mol Cell Biol. 2008;28(10):3281-9. doi: 10.1128/MCB.00310-08

Chen CY, Chen J, He L, Stiles BL. PTEN: Tumor Suppressor and Metabolic Regulator. Front Endocrinol (Lausanne). 2018;9:338. doi: 10.3389/fendo.2018.00338

Chen S, Lees-Miller JP, He Y, Lees-Miller SP. Structural insights into the role of DNA-PK as a master regulator in NHEJ. Genome Instab Dis. 2021;2(4):195-210. doi: 10.1007/s42764-021-00047-w

Chen Z, Dempsey DR, Thomas SN, Hayward D et al. Molecular Features of Phosphatase and Tensin Homolog (PTEN) Regulation by C-terminal Phosphorylation. J Biol Chem. 2016;291(27):14160-14169. doi: 10.1074/jbc.M116.728980

Choi BH, Chen Y, Dai W. Chromatin PTEN is involved in DNA damage response partly through regulating Rad52 sumoylation. Cell Cycle. 2013;12(21):3442-7. doi: 10.4161/cc.26465

Choi BH, Pagano M, Dai W. Plk1 protein phosphorylates phosphatase and tensin homolog (PTEN) and regulates its mitotic activity during the cell cycle. J Biol Chem. 2014;289(20):14066-74. doi: 10.1074/jbc.M114.558155

Chu N, Viennet T, Bae H, Salguero A, Boeszoermenyi A et al. The structural determinants of PH domain-mediated regulation of Akt revealed by segmental labeling. Elife. 2020;9:e59151. doi: 10.7554/eLife.59151

Connor KM, Subbaram S, Regan KJ, Nelson KK, Mazurkiewicz JE et al. Mitochondrial H2O2 regulates the angiogenic phenotype via PTEN oxidation. J Biol Chem. 2005;280(17):16916-24. doi: 10.1074/jbc.M410690200

Covey TM, Edes K, Fitzpatrick FA. Akt activation by arachidonic acid metabolism occurs via oxidation and inactivation of PTEN tumor suppressor. Oncogene. 2007;26(39):5784-92. doi: 10.1038/sj.onc.1210391

Davidson D, Amrein L, Panasci L, Aloyz R. Small Molecules, Inhibitors of DNA-PK, Targeting DNA Repair, and Beyond. Front Pharmacol. 2013;4:5. doi: 10.3389/fphar.2013.00005

Davis AJ, Chen BP, Chen DJ. DNA-PK: a dynamic enzyme in a versatile DSB repair pathway. DNA Repair (Amst). 2014 May;17:21-9. doi: 10.1016/j.dnarep.2014.02.020

Davis AJ, Chen DJ. DNA double strand break repair via non-homologous end-joining. Transl Cancer Res. 2013;2(3):130-143. doi: 10.3978/j.issn.2218-676X.2013.04.02

Debela DT, Muzazu SG, Heraro KD, Ndalama MT et al. New approaches and procedures for cancer treatment: Current perspectives. SAGE Open Med. 2021;9:20503121211034366. doi: 10.1177/20503121211034366

Dempsey DR, Viennet T, Iwase R, Park E, Henriquez S et al. The structural basis of PTEN regulation by multi-site phosphorylation. Nat Struct Mol Biol. 2021;28(10):858-868. doi: 10.1038/s41594-021-00668-5

Denley A, Gymnopoulos M, Kang S, Mitchell C, Vogt PK. Requirement of phosphatidylinositol(3,4,5)trisphosphate in phosphatidylinositol 3-kinase-induced oncogenic transformation. Mol Cancer Res. 2009;7(7):1132-8. doi: 10.1158/1541-7786.MCR-09-0068

Dillon LM, Miller TW. Therapeutic targeting of cancers with loss of PTEN function. Curr Drug Targets. 2014;15(1):65-79. doi: 10.2174/1389450114666140106100909

Elbakry A, Löbrich M. Homologous Recombination Subpathways: A Tangle to Resolve. Front Genet. 2021;12:723847. doi: 10.3389/fgene.2021.723847

Ellinger J, Kahl P, von der Gathen J, Rogenhofer S, Heukamp LC, Gütgemann I, Walter B, Hofstädter F, Büttner R, Müller SC, Bastian PJ, von Ruecker A. Global levels of histone modifications predict prostate cancer recurrence. Prostate. 2010;70(1):61-9. doi: 10.1002/pros.21038

EMBL-EBI. (2013). PDBsum Generate. http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html

Falcicchio M, Ward JA, Macip S, Doveston RG. Regulation of p53 by the 14-3-3 protein interaction network: new opportunities for drug discovery in cancer. Cell Death Discov. 2020;6(1):126. doi: 10.1038/s41420-020-00362-3

Fan X, Kraynak J, Knisely JPS, Formenti SC, Shen WH. PTEN as a Guardian of the Genome: Pathways and Targets. Cold Spring Harb Perspect Med. 2020;10(9):a036194. doi: 10.1101/cshperspect.a036194

Fata JE, Debnath S, Jenkins EC Jr, Fournier MV. Nongenomic Mechanisms of PTEN Regulation. Int J Cell Biol. 2012;2012:379685. doi: 10.1155/2012/379685

Fedorova O, Parfenyev S, Daks A, Shuvalov O, Barlev NA. The Role of PTEN in Epithelial-Mesenchymal Transition. Cancers (Basel). 2022;14(15):3786. doi: 10.3390/cancers14153786

Fell VL, Schild-Poulter C. The Ku heterodimer: function in DNA repair and beyond. Mutat Res Rev Mutat Res. 2015;763:15-29. doi: 10.1016/j.mrrev.2014.06.002

Feng J, Dang Y, Zhang W, Zhao X, Zhang C, Hou Z et al. PTEN arginine methylation by PRMT6 suppresses PI3K-AKT signaling and modulates pre-mRNA splicing. Proc Natl Acad Sci U S A. 2019;116(14):6868-6877. doi: 10.1073/pnas.1811028116

Fenton TR, Nathanson D, Ponte de Albuquerque C, Kuga D et al. Resistance to EGF receptor inhibitors in glioblastoma mediated by phosphorylation of the PTEN tumor suppressor at tyrosine 240. Proc Natl Acad Sci U S A. 2012;109(35):14164-9. doi: 10.1073/pnas.1211962109

Ferrarelli LK. FGFR inhibitors get to nuclear PTEN. Science Signaling. 2019;12(577): p.eaax6492. doi: 10.1126/scisignal.aax6492

Fouladkou F, Landry T, Kawabe H, Neeb A, Lu C et al. The ubiquitin ligase Nedd4-1 is dispensable for the regulation of PTEN stability and localization. Proc Natl Acad Sci U S A. 2008;105(25):8585-90. doi: 10.1073/pnas.0803233105

Fusco N, Sajjadi E, Venetis K, Gaudioso G, Lopez G, Corti C, Rocco EG, Criscitiello C, Malapelle U, Invernizzi M. PTEN Alterations and Their Role in Cancer Management: Are We Making Headway on Precision Medicine? Genes (Basel). 2020;11(7):719. doi: 10.3390/genes11070719

Gbelcová H, Gergely L, Šišovský V, Straka Ľ, Böhmer D et al. PTEN mutations as predictive marker for the high-grade endometrial cancer development in slovak women. Physiol Res. 2022;71(Suppl 1):S125-S135. doi: 10.33549/physiolres.935030

Georgescu MM, Kirsch KH, Akagi T, Shishido T, Hanafusa H. The tumor-suppressor activity of PTEN is regulated by its carboxyl-terminal region. Proc Natl Acad Sci U S A. 1999;96(18):10182-7. doi: 10.1073/pnas.96.18.10182

Gil A, Andrés-Pons A, Fernández E, Valiente M, Torres J et al. Nuclear localization of PTEN by a Ran-dependent mechanism enhances apoptosis: Involvement of an N-terminal nuclear localization domain and multiple nuclear exclusion motifs. Mol Biol Cell. 2006;17(9):4002-13. doi: 10.1091/mbc.e06-05-0380

Gimm O, Perren A, Weng LP, Marsh DJ, Yeh JJ et al. Differential nuclear and cytoplasmic expression of PTEN in normal thyroid tissue, and benign and malignant epithelial thyroid tumors. Am J Pathol. 2000;156(5):1693-700. doi: 10.1016/S0002-9440(10)65040-7

Gong L, Govan JM, Evans EB, Dai H, Wang E, Lee SW, Lin HK, Lazar AJ, Mills GB, Lin SY. Nuclear PTEN tumor-suppressor functions through maintaining heterochromatin structure. Cell Cycle. 2015;14(14):2323-32. doi: 10.1080/15384101.2015.1044174

González-García A, Garrido A, Carrera AC. Targeting PTEN Regulation by Post Translational Modifications. Cancers (Basel). 2022;14(22):5613. doi: 10.3390/cancers14225613

Gopi P, Gurnani M, Singh S, Sharma P, Pandya P. Structural aspects of SARS-CoV-2 mutations: Implications to plausible infectivity with ACE-2 using computational modeling approach. J Biomol Struct Dyn. 2023;41(14):6518-6533. doi: 10.1080/07391102.2022.2108901

Guan J, Zhao Q, Mao W. Nuclear PTEN interferes with binding of Ku70 at double-strand breaks through post-translational poly(ADP-ribosyl)ation. Biochim Biophys Acta. 2016;1863(12):3106-3115. doi: 10.1016/j.bbamcr.2016.10.003

Gupta A, Leslie NR. Controlling PTEN (Phosphatase and Tensin Homolog) Stability: A DOMINANT ROLE FOR LYSINE 66. J Biol Chem. 2016;291(35):18465-73. doi: 10.1074/jbc.M116.727750

Guzman NA, Guzman DE. Immunoaffinity Capillary Electrophoresis in the Era of Proteoforms, Liquid Biopsy and Preventive Medicine: A Potential Impact in the Diagnosis and Monitoring of Disease Progression. Biomolecules. 2021;11(10):1443. doi: 10.3390/biom11101443

Georgescu MM. PTEN Tumor Suppressor Network in PI3K-Akt Pathway Control. Genes Cancer. 2010;1(12):1170-7. doi: 10.1177/1947601911407325

Grundy MK, Buckanovich RJ, Bernstein KA. Regulation and pharmacological targeting of RAD51 in cancer. NAR Cancer. 2020;2(3):zcaa024. doi: 10.1093/narcan/zcaa024

He J, Huang C, Guo Y, Deng R, Li L, Chen R, Wang Y, Huang J, Zheng J, Zhao X, Yu J. PTEN-mediated dephosphorylation of 53BP1 confers cellular resistance to DNA damage in cancer cells. Mol Oncol. 2023. doi: 10.1002/1878-0261.13563

He J, Kang X, Yin Y, Chao KS, Shen WH. PTEN regulates DNA replication progression and stalled fork recovery. Nat Commun. 2015;6:7620. doi: 10.1038/ncomms8620

Ho J, Cruise ES, Dowling RJO, Stambolic V. PTEN Nuclear Functions. Cold Spring Harb Perspect Med. 2020;10(5):a036079. doi: 10.1101/cshperspect.a036079

Hou B, Xu S, Xu Y, Gao Q, Zhang C, Liu L et al. Grb2 binds to PTEN and regulates its nuclear translocation to maintain the genomic stability in DNA damage response. Cell Death Dis. 2019;10(8):546. doi: 10.1038/s41419-019-1762-3

Hou SQ, Ouyang M, Brandmaier A, Hao H, Shen WH. PTEN in the maintenance of genome integrity: From DNA replication to chromosome segregation. Bioessays. 2017;39(10):10.1002/bies.201700082. doi: 10.1002/bies.201700082

Huang J, Yan J, Zhang J, Zhu S, Wang Y et al. SUMO1 modification of PTEN regulates tumorigenesis by controlling its association with the plasma membrane. Nat Commun. 2012;3:911. doi: 10.1038/ncomms1919

Hu Y, Li Z, Guo L, Wang L, Zhang L et al. MAGI-2 Inhibits cell migration and proliferation via PTEN in human hepatocarcinoma cells. Arch Biochem Biophys. 2007;467(1):1-9. doi: 10.1016/j.abb.2007.07.027

Ho J, Cruise ES, Dowling RJO, Stambolic V. PTEN Nuclear Functions. Cold Spring Harb Perspect Med. 2020;10(5):a036079. doi: 10.1101/cshperspect.a036079

Hollander MC, Blumenthal GM, Dennis PA. PTEN loss in the continuum of common cancers, rare syndromes and mouse models. Nat Rev Cancer. 2011;11(4):289-301. doi: 10.1038/nrc3037

Hopkins BD, Hodakoski C, Barrows D, Mense SM, Parsons RE. PTEN function: the long and the short of it. Trends Biochem Sci. 2014;39(4):183-90. doi: 10.1016/j.tibs.2014.02.006

Hou SQ, Ouyang M, Brandmaier A, Hao H, Shen WH. PTEN in the maintenance of genome integrity: From DNA replication to chromosome segregation. Bioessays. 2017;39(10):10.1002/bies.201700082. doi: 10.1002/bies.201700082

Inoue K, Fry EA, Taneja P. Recent progress in mouse models for tumor suppressor genes and its implications in human cancer. Clin Med Insights Oncol. 2013;7:103-22. doi: 10.4137/CMO.S10358

Jang H, Smith IN, Eng C, Nussinov R. The mechanism of full activation of tumor suppressor PTEN at the phosphoinositide-enriched membrane. iScience. 2021;24(5):102438. doi: 10.1016/j.isci.2021.102438

Jiang W, Crowe JL, Liu X, Nakajima S, Wang Y, Li C, Lee BJ, Dubois RL, Liu C, Yu X, Lan L, Zha S. Differential phosphorylation of DNA-PKcs regulates the interplay between end-processing and end-ligation during nonhomologous end-joining. Mol Cell. 2015;58(1):172-85. doi: 10.1016/j.molcel.2015.02.024

Jiang W, Estes VM, Wang XS, Shao Z, Lee BJ, Lin X, Crowe JL, Zha S. Phosphorylation at S2053 in Murine (S2056 in Human) DNA-PKcs Is Dispensable for Lymphocyte Development and Class Switch Recombination. J Immunol. 2019;203(1):178-187. doi: 10.4049/jimmunol.1801657

Kelley MR, Logsdon D, Fishel ML. Targeting DNA repair pathways for cancer treatment: what's new? Future Oncol. 2014;10(7):1215-37. doi: 10.2217/fon.14.60

Khan A, Zia T, Suleman M, Khan T, Ali SS, Abbasi AA, Mohammad A, Wei DQ. Higher infectivity of the SARS-CoV-2 new variants is associated with K417N/T, E484K, and N501Y mutants: An insight from structural data. J Cell Physiol. 2021;236(10):7045-7057. doi: 10.1002/jcp.30367

Kotelevets L, Trifault B, Chastre E, Scott MGH. Posttranslational Regulation and Conformational Plasticity of PTEN. Cold Spring Harb Perspect Med. 2020 Jul;10(7):a036095. doi: 10.1101/cshperspect.a036095

Kulke MH, Odze RD, Thakore KS, Thomas G et al. Allelic loss of 10q23, the PTEN tumour suppressor gene locus, in Barrett's oesophagus-associated adenocarcinoma. Br J Cancer. 2001;84(6):748-53. doi: 10.1054/bjoc.2000.1660

Landry JP, Fei Y, Zhu X, Ke Y, Yu G, Lee P. Discovering small molecule ligands of vascular endothelial growth factor that block VEGF-KDR binding using label-free microarray-based assays. Assay Drug Dev Technol. 2013;11(5):326-32. doi: 10.1089/adt.2012.485

Lee EY, Muller WJ. Oncogenes and tumor suppressor genes. Cold Spring Harb Perspect Biol. 2010;2(10):a003236. doi: 10.1101/cshperspect.a003236

Lee JT. RFP-mediated ubiquitination of PTEN modulates its effect on AKT activation. Cell Res. 2013;23(4): p. 552-64. doi: 10.1038/cr.2013.27

Legartová S, Lochmanová G, Zdráhal Z, Kozubek S, Šponer J, Krepl M, Pokorná P, Bártová E. DNA Damage Changes Distribution Pattern and Levels of HP1 Protein Isoforms in the Nucleolus and Increases Phosphorylation of HP1β-Ser88. Cells. 2019;8(9):1097. doi: 10.3390/cells8091097

Leslie NR, Downes CP. PTEN function: how normal cells control it and tumour cells lose it. Biochem J. 2004;382(Pt 1):1-11. doi: 10.1042/BJ20040825

Li DM, Sun H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. Cancer Res. 1997;57(11):2124-9. PMID: 9187108.

Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem. 2010;79:181-211. doi: 10.1146/annurev.biochem.052308.093131

Li J, Poi MJ, Tsai MD. Regulatory mechanisms of tumor suppressor P16(INK4A) and their relevance to cancer. Biochemistry. 2011;50(25):5566-82. doi: 10.1021/bi200642e

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science. 1997;275(5308):1943-7. doi: 10.1126/science.275.5308.1943

Liu A, Zhu Y, Chen W, Merlino G, Yu Y. PTEN Dual Lipid- and Protein-Phosphatase Function in Tumor Progression. Cancers (Basel). 2022;14(15):3666. doi: 10.3390/cancers14153666

Liu F, Wagner S, Campbell RB, Nickerson JA, Schiffer CA, Ross AH. PTEN enters the nucleus by diffusion. J Cell Biochem. 2005;96(2):221-34. doi: 10.1002/jcb.20525

Liu L, Dai X, Yin S, Liu P, Hill EG, Wei W, Gan W. DNA-PK promotes activation of the survival kinase AKT in response to DNA damage through an mTORC2-ECT2 pathway. Sci Signal. 2022;15(715):eabh2290. doi: 10.1126/scisignal.abh2290

Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov. 2009;8(8):627-44. doi: 10.1038/nrd2926

Liu P, Gan W, Guo C, Xie A, Gao D, Guo J, Zhang J, Willis N, Su A, Asara JM, Scully R, Wei W. Akt-mediated phosphorylation of XLF impairs non-homologous end-joining DNA repair. Mol Cell. 2015;57(4):648-661. doi: 10.1016/j.molcel.2015.01.005

Liu T, Wang Y, Wang Y, Chan AM. Multifaceted Regulation of PTEN Subcellular Distributions and Biological Functions. Cancers (Basel). 2019;11(9):1247. doi: 10.3390/cancers11091247

Li Z, Li J, Bi P, Lu Y, Burcham G, Elzey BD, Ratliff T, Konieczny SF, Ahmad N, Kuang S, Liu X. Plk1 phosphorylation of PTEN causes a tumor-promoting metabolic state. Mol Cell Biol. 2014;34(19):3642-61. doi: 10.1128/MCB.00814-14

Luongo F, Colonna F, Calapà F, Vitale S, Fiori ME, De Maria R. PTEN Tumor-Suppressor: The Dam of Stemness in Cancer. Cancers (Basel). 2019 Jul;11(8):1076. doi: 10.3390/cancers11081076

Lu XX, Cao LY, Chen X, Xiao J, Zou Y, Chen Q. PTEN Inhibits Cell Proliferation, Promotes Cell Apoptosis, and Induces Cell Cycle Arrest via Downregulating the PI3K/AKT/hTERT Pathway in Lung Adenocarcinoma A549 Cells. Biomed Res Int. 2016;2016:2476842. doi: 10.1155/2016/2476842

Lu Y, Yu Q, Liu JH, Zhang J, Wang H, Koul D et al. Src family protein-tyrosine kinases alter the function of PTEN to regulate phosphatidylinositol 3-kinase/AKT cascades. J Biol Chem. 2003;278(41):40057-66. doi: 10.1074/jbc.M303621200

Maccario H, Perera NM, Davidson L, Downes CP, Leslie NR. PTEN is destabilized by phosphorylation on Thr366. Biochem J. 2007;405(3):439-44. doi: 10.1042/BJ20061837

Macoska JA, Xu J, Ziemnicka D, Schwab TS, Rubin MA, Kotula L. Loss of expression of human spectrin src homology domain binding protein 1 is associated with 10p loss in human prostatic adenocarcinoma. Neoplasia. 2001;3(2):99-104. doi: 10.1038/sj.neo.7900145

Malaney P, Palumbo E, Semidey-Hurtado J, Hardee J, Stanford K et al. PTEN Physically Interacts with and Regulates E2F1-mediated Transcription in Lung Cancer. Cell Cycle. 2018;17(8):947-962. doi: 10.1080/15384101.2017.1388970

Malaney P, Uversky VN, Davé V. PTEN proteoforms in biology and disease. Cell Mol Life Sci. 2017;74(15):2783-2794. doi: 10.1007/s00018-017-2500-6

Mansour WY, Tennstedt P, Volquardsen J, Oing C, Kluth M, Hube-Magg C, Borgmann K, Simon R, Petersen C, Dikomey E, Rothkamm K. Loss of PTEN-assisted G2/M checkpoint impedes homologous recombination repair and enhances radio-curability and PARP inhibitor treatment response in prostate cancer. Sci Rep. 2018;8(1):3947. doi: 10.1038/s41598-018-22289-7

Martin LJ. DNA damage and repair: relevance to mechanisms of neurodegeneration. J Neuropathol Exp Neurol. 2008;67(5):377-87. doi: 10.1097/NEN.0b013e31816ff780

Masson GR, Williams RL. Structural Mechanisms of PTEN Regulation. Cold Spring Harb Perspect Med. 2020;10(3):a036152. doi: 10.1101/cshperspect.a036152

Mayo LD, Dixon JE, Durden DL, Tonks NK, Donner DB. PTEN protects p53 from Mdm2 and sensitizes cancer cells to chemotherapy. J Biol Chem. 2002;277(7):5484-9. doi: 10.1074/jbc.M108302200

McClendon AK, Osheroff N. DNA topoisomerase II, genotoxicity, and cancer. Mutat Res. 2007;623(1-2):83-97. doi: 10.1016/j.mrfmmm.2007.06.009

Meng Z, Jia LF, Gan YH. PTEN activation through K163 acetylation by inhibiting HDAC6 contributes to tumour inhibition. Oncogene. 2016;35(18):2333-44. doi: 10.1038/onc.2015.293

Milella M, Falcone I, Conciatori F, Cesta Incani U, Del Curatolo A et al. PTEN: Multiple Functions in Human Malignant Tumors. Front Oncol. 2015;5:24. doi: 10.3389/fonc.2015.00024

Miller SJ, Lou DY, Seldin DC, Lane WS, Neel BG. Direct identification of PTEN phosphorylation sites. FEBS Lett. 2002;528(1-3):145-53. doi: 10.1016/s0014-5793(02)03274-x

Minaguchi T, Waite KA, Eng C. Nuclear localization of PTEN is regulated by Ca(2+) through a tyrosil phosphorylation-independent conformational modification in major vault protein. Cancer Res. 2006 Dec;66(24):11677-82. doi: 10.1158/0008-5472.CAN-06-2240

Ming M, He YY. PTEN in DNA damage repair. Cancer Lett. 2012;319(2):125-129. doi: 10.1016/j.canlet.2012.01.003

Ming M, He YY. PTEN: new insights into its regulation and function in skin cancer. J Invest Dermatol. 2009;129(9):2109-12. doi: 10.1038/jid.2009.79

Misra S, Chowdhury SG, Ghosh G, Mukherjee A, Karmakar P. Both phosphorylation and phosphatase activity of PTEN are required to prevent replication fork progression during stress by inducing heterochromatin. Mutat Res. 2022;825:111800. doi: 10.1016/j.mrfmmm.2022.111800

Misra S, Ghosh G, Chowdhury SG, Karmakar P. Non-canonical function of nuclear PTEN and its implication on tumorigenesis. DNA Repair (Amst). 2021;107:103197. doi: 10.1016/j.dnarep.2021.103197

Misra S, Mukherjee A, Karmakar P. Phosphorylation of PTEN at STT motif is associated with DNA damage response. Mutat Res. 2014 Dec;770:112-9. doi: 10.1016/j.mrfmmm.2014.08.008. Epub 2014 Sep 4. Erratum in: Mutat Res. 2019;816-818:111676

Moiseeva TN, Bakkenist CJ. Regulation of the initiation of DNA replication in human cells. DNA Repair (Amst). 2018;72:99-106. doi: 10.1016/j.dnarep.2018.09.003

Mondal SK, Sen MK. Loss of phosphatase activity in PTEN (phosphatase and tensin homolog deleted on chromosome ten) results in endometrial carcinoma in humans: An in-silico study. Heliyon. 2020 Jan;6(1):e03106. doi: 10.1016/j.heliyon.2019.e03106

Mund T, Pelham HR. Regulation of PTEN/Akt and MAP kinase signaling pathways by the ubiquitin ligase activators Ndfip1 and Ndfip2. Proc Natl Acad Sci U S A. 2010;107(25):11429-34. doi: 10.1073/pnas.0911714107

Mutter GL. Pten, a protean tumor suppressor. Am J Pathol. 2001;158(6):1895-8. doi: 10.1016/S0002-9440(10)64656-1

Nowosielska A, Marinus MG. DNA mismatch repair-induced double-strand breaks. DNA Repair (Amst). 2008;7(1):48-56. doi: 10.1016/j.dnarep.2007.07.015

Odriozola L, Singh G, Hoang T, Chan AM. Regulation of PTEN activity by its carboxyl-terminal autoinhibitory domain. J Biol Chem. 2007;282(32):23306-15. doi: 10.1074/jbc.M611240200

Ogino M, Ichimura M, Nakano N, Minami A, Kitagishi Y, Matsuda S. Roles of PTEN with DNA Repair in Parkinson's Disease. Int J Mol Sci. 2016;17(6):954. doi: 10.3390/ijms17060954

Okahara F, Ikawa H, Kanaho Y, Maehama T. Regulation of PTEN phosphorylation and stability by a tumor suppressor candidate protein. J Biol Chem. 2004;279(44):45300-3. doi: 10.1074/jbc.C400377200

Okumura K, Mendoza M, Bachoo RM, DePinho RA, Cavenee WK, Furnari FB. PCAF modulates PTEN activity. J Biol Chem. 2006;281(36):26562-8. doi: 10.1074/jbc.M605391200

Orhan E, Velazquez C, Tabet I, Sardet C, Theillet C. Regulation of RAD51 at the Transcriptional and Functional Levels: What Prospects for Cancer Therapy? Cancers (Basel). 2021;13(12):2930. doi: 10.3390/cancers13122930

Pei X, Mladenov E, Soni A, Li F, Stuschke M, Iliakis G. PTEN Loss Enhances Error-Prone DSB Processing and Tumor Cell Radiosensitivity by Suppressing RAD51 Expression and Homologous Recombination. Int J Mol Sci. 2022;23(21):12876. doi: 10.3390/ijms232112876

Petermann E, Woodcock M, Helleday T. Chk1 promotes replication fork progression by controlling replication initiation. Proc Natl Acad Sci U S A. 2010 Sep;107(37):16090-5. doi: 10.1073/pnas.1005031107

Planchon SM, Waite KA, Eng C. The nuclear affairs of PTEN. J Cell Sci. 2008;121(Pt 3):249-53. doi: 10.1242/jcs.022459

Puc J, Keniry M, Li HS, Pandita TK, Choudhury AD et al. (2005). Lack of PTEN sequesters CHK1 and initiates genetic instability. Cancer cell. 2005;7(2), 193-204. doi: 10.1016/j.ccr.2005.01.009

Puc J, Parsons R. PTEN loss inhibits CHK1 to cause double stranded-DNA breaks in cells. Cell Cycle. 2005;4(7):927-9. doi: 10.4161/cc.4.7.1795

Radu A, Neubauer V, Akagi T, Hanafusa H, Georgescu MM. PTEN induces cell cycle arrest by decreasing the level and nuclear localization of cyclin D1. Mol Cell Biol. 2003;23(17):6139-49. doi: 10.1128/MCB.23.17.6139-6149.2003

Rahdar M, Inoue T, Meyer T, Zhang J, Vazquez F, Devreotes PN. A phosphorylation-dependent intramolecular interaction regulates the membrane association and activity of the tumor suppressor PTEN. Proc Natl Acad Sci U S A. 2009;106(2):480-5. doi: 10.1073/pnas.0811212106

Ramazi S, Zahiri J. Posttranslational modifications in proteins: resources, tools and prediction methods. Database (Oxford). 2021;2021:baab012. doi: 10.1093/database/baab012

Rawla P. Epidemiology of Prostate Cancer. World J Oncol. 2019;10(2):63-89. doi: 10.14740/wjon1191

Rivera C, Saavedra F, Alvarez F, Díaz-Celis C, Ugalde V, Li J, Forné I, Gurard-Levin ZA, Almouzni G, Imhof A, Loyola A. Methylation of histone H3 lysine 9 occurs during translation. Nucleic Acids Res. 2015;43(19):9097-106. doi: 10.1093/nar/gkv929

Rydell-Törmänen K, Johnson JR. The Applicability of Mouse Models to the Study of Human Disease. Methods Mol Biol. 2019;1940:3-22. doi: 10.1007/978-1-4939-9086-3_1

Salmaninejad A, Ilkhani K, Marzban H, Navashenaq JG, Rahimirad S, Radnia F, Yousefi M, Bahmanpour Z, Azhdari S, Sahebkar A. Genomic Instability in Cancer: Molecular Mechanisms and Therapeutic Potentials. Curr Pharm Des. 2021;27(28):3161-3169. doi: 10.2174/1381612827666210426100206

Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 2017;168(6):960-976. doi: 10.1016/j.cell.2017.02.004

Schwertassek U, Haque A, Krishnan N, Greiner R, Weingarten L et al. Reactivation of oxidized PTP1B and PTEN by thioredoxin 1. FEBS J. 2014;281(16):3545-58. doi: 10.1111/febs.12898

Sellars E, Gabra M, Salmena L. The Complex Landscape of PTEN mRNA Regulation. Cold Spring Harb Perspect Med. 2020;10(6):a036236. doi: 10.1101/cshperspect.a036236

Sharif H, Li Y, Dong Y, Dong L, Wang WL, Mao Y, Wu H. Cryo-EM structure of the DNA-PK holoenzyme. Proc Natl Acad Sci U S A. 2017;114(28):7367-7372. doi: 10.1073/pnas.1707386114

Shen WH, Balajee AS, Wang J, Wu H, Eng C et al. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell. 2007 Jan;128(1):157-70. doi: 10.1016/j.cell.2006.11.042

Shin LJ, Seok KH, Bok KY, Cheol LM, Soo PC. Expression of PTEN in renal cell carcinoma and its relation to tumor behavior and growth. J Surg Oncol. 2003;84(3):166-72. doi: 10.1002/jso.10302

Shi Y, Paluch BE, Wang X, Jiang X. PTEN at a glance. J Cell Sci. 2012;125(Pt 20):4687-92. doi: 10.1242/jcs.093765

Singh G, Chan AM. Post-translational modifications of PTEN and their potential therapeutic implications. Curr Cancer Drug Targets. 2011;11(5):536-47. doi: 10.2174/156800911795655930

Sishc BJ, Davis AJ. The Role of the Core Non-Homologous End Joining Factors in Carcinogenesis and Cancer. Cancers (Basel). 2017;9(7):81. doi: 10.3390/cancers9070081

Song MS, Carracedo A, Salmena L, Song SJ, Egia A et al. Nuclear PTEN regulates the APC-CDH1 tumor-suppressive complex in a phosphatase-independent manner. Cell. 2011;144(2):187-99. doi: 10.1016/j.cell.2010.12.020

Srinivas US, Tan BWQ, Vellayappan BA, Jeyasekharan AD. ROS and the DNA damage response in cancer. Redox Biol. 2019;25:101084. doi: 10.1016/j.redox.2018.101084

Steck PA, Pershouse MA, Jasser SA, Yung WK et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet. 1997;15(4):356-62. doi: 10.1038/ng0497-356

Stumpf M, Blokzijl-Franke S, den Hertog J. Fine-Tuning of Pten Localization and Phosphatase Activity Is Essential for Zebrafish Angiogenesis. PLoS One. 2016;11(5):e0154771. doi: 10.1371/journal.pone.0154771

Sulkowski PL, Scanlon SE, Oeck S, Glazer PM. PTEN Regulates Nonhomologous End Joining By Epigenetic Induction of NHEJ1/XLF. Mol Cancer Res. 2018;16(8):1241-1254. doi: 10.1158/1541-7786.MCR-17-0581

Sun L, Zhang H, Gao P. Metabolic reprogramming and epigenetic modifications on the path to cancer. Protein Cell. 2022;13(12):877-919. doi: 10.1007/s13238-021-00846-7

Sun Z, Huang C, He J, Lamb KL, Kang X, Gu T, Shen WH, Yin Y. PTEN C-terminal deletion causes genomic instability and tumor development. Cell Rep. 2014;6(5):844-54. doi: 10.1016/j.celrep.2014.01.030

Svobodová Kovaříková A, Legartová S, Krejčí J, Bártová E. H3K9me3 and H4K20me3 represent the epigenetic landscape for 53BP1 binding to DNA lesions. Aging (Albany NY). 2018;10(10):2585-2605. doi: 10.18632/aging.101572

Tamura M, Gu J, Takino T, Yamada KM. Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. Cancer Res. 1999;59(2):442-9. PMID: 9927060.

Técher H, Koundrioukoff S, Nicolas A, Debatisse M. The impact of replication stress on replication dynamics and DNA damage in vertebrate cells. Nat Rev Genet. 2017;18(9):535-550. doi: 10.1038/nrg.2017.46

Teng Y, Fan Y, Ma J, Lu W, Liu N, Chen Y, Pan W, Tao X. The PI3K/Akt Pathway: Emerging Roles in Skin Homeostasis and a Group of Non-Malignant Skin Disorders. Cells. 2021;10(5):1219. doi: 10.3390/cells10051219

Thomas KJ, Cookson MR. The role of PTEN-induced kinase 1 in mitochondrial dysfunction and dynamics. Int J Biochem Cell Biol. 2009;41(10):2025-35. doi: 10.1016/j.biocel.2009.02.018

Tian T, Nan KJ, Wang SH, Liang X, Lu CX et al. PTEN regulates angiogenesis and VEGF expression through phosphatase-dependent and -independent mechanisms in HepG2 cells. Carcinogenesis. 2010;31(7):1211-9. doi: 10.1093/carcin/bgq085

Tu T, Chen J, Chen L, Stiles BL. Dual-Specific Protein and Lipid Phosphatase PTEN and Its Biological Functions. Cold Spring Harb Perspect Med. 2020;10(1):a036301. doi: 10.1101/cshperspect.a036301

Tzani I, Ivanov IP, Andreev DE, Dmitriev RI et al. Systematic analysis of the PTEN 5' leader identifies a major AUU initiated proteoform. Open Biol. 2016;6(5):150203. doi: 10.1098/rsob.150203

Tzivion G, Dobson M, Ramakrishnan G. FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. Biochim Biophys Acta. 2011;1813(11):1938-45. doi: 10.1016/j.bbamcr.2011.06.002

Vazquez F, Grossman SR, Takahashi Y, Rokas MV, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. J Biol Chem. 2001;276(52):48627-30. doi: 10.1074/jbc.C100556200

Vazquez F, Matsuoka S, Sellers WR, Yanagida T, Ueda M, Devreotes PN. Tumor suppressor PTEN acts through dynamic interaction with the plasma membrane. Proc Natl Acad Sci U S A. 2006;103(10):3633-8. doi: 10.1073/pnas.0510570103

Vazquez F, Ramaswamy S, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail regulates protein stability and function. Mol Cell Biol. 2000;20(14):5010-8. doi: 10.1128/MCB.20.14.5010-5018.2000

von Stein W, Ramrath A, Grimm A, Müller-Borg M, Wodarz A. Direct association of Bazooka/PAR-3 with the lipid phosphatase PTEN reveals a link between the PAR/aPKC complex and phosphoinositide signaling. Development. 2005;132(7):1675-86. doi: 10.1242/dev.01720

Vuono EA, Mukherjee A, Vierra DA, Adroved MM, Hodson C, Deans AJ, Howlett NG. The PTEN phosphatase functions cooperatively with the Fanconi anemia proteins in DNA crosslink repair. Sci Rep. 2016 Nov;6:36439. doi: 10.1038/srep36439

Wang DS, Rieger-Christ K, Latini JM, Moinzadeh A et al. Molecular analysis of PTEN and MXI1 in primary bladder carcinoma. Int J Cancer. 2000;88(4):620-5. doi: 10.1002/1097-0215(20001115)88:4<620::aid-ijc16>3.0.co;2-z

Wang G, Li Y, Wang P, Liang H, Cui M, Zhu M et al. PTEN regulates RPA1 and protects DNA replication forks. Cell Res. 2015;25(11):1189-204. doi: 10.1038/cr.2015.115

Wang Q, Wang J, Xiang H, Ding P, Wu T, Ji G. The biochemical and clinical implications of phosphatase and tensin homolog deleted on chromosome ten in different cancers. Am J Cancer Res. 2021;11(12):5833-5855. PMID: 35018228

Wei F, Hao P, Zhang X, Hu H, Jiang D, Yin A, Wen L, Zheng L, He JZ, Mei W, Zeng H, Tang D. Etoposide-induced DNA damage affects multiple cellular pathways in addition to DNA damage response. Oncotarget. 2018;9(35):24122-24139. doi: 10.18632/oncotarget.24517

Weng LP, Smith WM, Dahia PL, Ziebold U, Gil E et al. PTEN suppresses breast cancer cell growth by phosphatase activity-dependent G1 arrest followed by cell death. Cancer Res. 1999;59(22):5808-14. PMID: 10582703

Wu X, Hepner K, Castelino-Prabhu S, Do D, Kaye MB et al. Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. Proc Natl Acad Sci U S A. 2000;97(8):4233-8. doi: 10.1073/pnas.97.8.4233

Wu Y, Zhou H, Wu K, Lee S, Li R, Liu X. PTEN phosphorylation and nuclear export mediate free fatty acid-induced oxidative stress. Antioxid Redox Signal. 2014;20(9):1382-95. doi: 10.1089/ars.2013.5498

Wu Z, Cho H, Hampton GM, Theodorescu D. Cdc6 and cyclin E2 are PTEN-regulated genes associated with human prostate cancer metastasis. Neoplasia. 2009;11(1):66-76. doi: 10.1593/neo.81048

Xia Q, Ali S, Liu L, Li Y, Liu X et al. Role of Ubiquitination in PTEN Cellular Homeostasis and Its Implications in GB Drug Resistance. Front Oncol. 2020;10:1569. doi: 10.3389/fonc.2020.01569

Xue LC, Rodrigues JP, Kastritis PL, Bonvin AM, Vangone A. PRODIGY: a web server for predicting the binding affinity of protein-protein complexes. Bioinformatics. 2016;32(23):3676-3678. doi: 10.1093/bioinformatics/btw514

You H, Pellegrini M, Tsuchihara K, Yamamoto K, Hacker G et al. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. J Exp Med. 2006;203(7):1657-63. doi: 10.1084/jem.20060353

Yuan L, Lv Y, Li H, Gao H, Song S, Zhang Y et al. Deubiquitylase OTUD3 regulates PTEN stability and suppresses tumorigenesis. Nat Cell Biol. 2015;17(9):1169-81. doi: 10.1038/ncb3218

Yue X, Bai C, Xie D, Ma T, Zhou PK. DNA-PKcs: A Multi-Faceted Player in DNA Damage Response. Front Genet. 2020;11:607428. doi: 10.3389/fgene.2020.607428

Zahid S, Seif El Dahan M, Iehl F, Fernandez-Varela P, Le Du MH, Ropars V, Charbonnier JB. The Multifaceted Roles of Ku70/80. Int J Mol Sci. 2021;22(8):4134. doi: 10.3390/ijms22084134

Zeman MK, Cimprich KA. Causes and consequences of replication stress. Nat Cell Biol. 2014;16(1):2-9. doi: 10.1038/ncb2897

Zhang H, Sun L, Wang K, Wu D, Trappio M, Witting C, Cao K. Loss of H3K9me3 Correlates with ATM Activation and Histone H2AX Phosphorylation Deficiencies in Hutchinson-Gilford Progeria Syndrome. PLoS One. 2016;11(12):e0167454. doi: 10.1371/journal.pone.0167454

Zhang X, Tang N, Hadden TJ, Rishi AK. Akt, FoxO and regulation of apoptosis. Biochim Biophys Acta. 2011;1813(11):1978-86. doi: 10.1016/j.bbamcr.2011.03.010

Zhang Z, Hou SQ, He J, Gu T, Yin Y, Shen WH. PTEN regulates PLK1 and controls chromosomal stability during cell division. Cell Cycle. 2016;15(18):2476-85. doi: 10.1080/15384101.2016.1203493

Zhao Q, Guan J, Zhang Z, Lv J, Wang Y, Liu L, Zhou Q, Mao W. Inhibition of Rad51 sensitizes breast cancer cells with wild-type PTEN to olaparib. Biomed Pharmacother. 2017;94:165-168. doi: 10.1016/j.biopha.2017.07.090

Zheng Y, Lu Z. Regulation of tumor cell migration by protein tyrosine phosphatase (PTP)-proline-, glutamate-, serine-,and threonine-rich sequence (PEST). Chin J Cancer. 2013;32(2):75-83. doi: 10.5732/cjc.012.10084

Zhou H, Huang S. The complexes of mammalian target of rapamycin. Curr Protein Pept Sci. 2010;11(6):409-24. doi: 10.2174/138920310791824093

Zhou M, Yan JQ, Chen QX, Yang YZ, Li YL, Ren YX, Weng ZJ, Zhang XF, Guan JX, Tang LY, Ren ZF. Association of H3K9me3 with breast cancer prognosis by estrogen receptor status. Clin Epigenetics. 2022;14(1):135. doi: 10.1186/s13148-022-01363-y

Zhu Y, Hoell P, Ahlemeyer B, Krieglstein J. PTEN: a crucial mediator of mitochondria-dependent apoptosis. Apoptosis. 2006;11(2):197-207. doi: 10.1007/s10495-006-3714-5

Zu L, Zheng X, Wang B, Parajuli N, Steenbergen C et al. Ischemic preconditioning attenuates mitochondrial localization of PTEN induced by ischemia-reperfusion. Am J Physiol Heart Circ Physiol. 2011;300(6):H2177-86. doi: 10.1152/ajpheart.01138.2010

List of Publications

A. Thesis related:

1. **Chowdhury SG,** Misra S, Ghosh G, Mukherjee A, Gopi P, Pandya P, Islam Md, Karmakar P. NHEJ is promoted by the phosphorylation and phosphatase activity of PTEN via regulation of DNA-PKcs. (Communicated)

2. Misra S, **Chowdhury SG**, Ghosh G, Mukherjee A, Karmakar P. Both phosphorylation and phosphatase activity of PTEN are required to prevent replication fork progression during stress by inducing heterochromatin. Mutat Res. 2022;825:111800. doi: 10.1016/j.mrfmmm.2022.111800

3. Misra S, Ghosh G, **Chowdhury SG**, Karmakar P. Non-canonical function of nuclear PTEN and its implication on tumorigenesis. DNA Repair (Amst). 2021;107:103197. doi: 10.1016/j.dnarep.2021.103197

4. Ghosh G, Misra S, Ray R, **Chowdhury SG**, Karmakar P. Phospho PTEN mediated dephosphorylation of mitotic kinase PLK1 and Aurora Kinase A prevents aneuploidy and preserves genomic stability. Med Oncol. 2023;40(4):119. doi: 10.1007/s12032-023-01985-z

B. Others:

1. **Chowdhury SG**, Ray R, Karmakar P. Relating aging and autophagy – A new perspective towards the welfare of human health. EXCLI J. 2023;22:732-748. doi: 10.17179/excli2023-6300

2. **Chowdhury SG**, Misra S, Karmakar P. Understanding the Impact of Obesity on Ageing in the Radiance of DNA Metabolism. J Nutr Health Aging. 2023;27(5):314-328. doi: 10.1007/s1260.-023-1912-1

3. **Chowdhury SG**, Ray R, Bhattacharya D, Karmakar P. DNA damage induced cellular senescence and it's PTEN-armed exosomes-the warriors against prostate carcinoma cells. Med Oncol. 2022;39(3):34. doi: 10.1007/s12032-021-01614-7

4. **Chowdhury SG**, Bhattacharya D, Karmakar P. Exosomal long noncoding RNAs - the lead thespian behind the regulation, cause and cure of autophagy-related diseases. Mol Biol Rep. 2022;49(7):7013-7024. doi: 10.1007/s11033-022-07514-x

5. **Chowdhury SG**, Ray R, Karmakar P. Exosomal miRNAs-a diagnostic biomarker acting as a guiding light in the diagnosis of prostate cancer. Funct Integr Genomics. 2022;23(1):23. doi: 10.1007/s10142-022-00951-8

6. Ray R, **Chowdhury SG**, Karmakar P. A vivid outline demonstrating the benefits of exosome-mediated drug delivery in CNS-associated disease environments. Arch Biochem Biophys. 2024 Jan 23;753:109906. doi: 10.1016/j.abb.2024.109906

7. Sarkar S, **Chowdhury SG**, Karmakar P. Drugging non-coding RNAs-A new light of hope in senescence-related cancer therapy. Chem Biol Drug Des. 2022; doi: 10.1111/cbdd.14200

8. Ghorai P, **Chowdhury SG**, Pal K, Mandal J, Karmakar P, Franconetti A, Frontera A, Blasco S, García-España E, Parui PP, Saha A. Aza-Crown-Based Macrocyclic Probe Design for "PET-off" Multi-Cu^{2+} Responsive and "CHEF-on" Multi-Zn2+ Sensor: Application in Biological Cell Imaging and Theoretical Studies. Inorg Chem. 2022;61(4):1982-1996. doi: 10.1021/acs.inorgchem.1c03141

9. Khatun M, Ghorai P, Mandal J, **Chowdhury SG**, Karmakar P, Saha A. Aza-phenol Based Macrocyclic Probes Design for “CHEF-on” Multi Analytes Sensor: Crystal Structure Elucidation and Application in Biological Cell Imaging. ACS Omega. 2023;8:7479–7491. doi:10.1021/acsomega.2c06549.

10. Mandal J, Jana NC, **Chowdhury SG**, Karmakar P, Saha A. Two pyridoxal derived Schiff base chemosensors design for fluorescence sensing of Zn^{2+} ion in aqueous medium. doi: 10.1016/j.inoche.2023.111217

11. Khatun M, Ghorai P, Mandal J, **Chowdhury SG**, Karmakar P, Saha A. Design and synthesis of a hydrazinopthalazine derived chemosensor to detect metal ions Zn^{2+}, Al^{3+} via CHEF effect with biological study and theoretical calculation. doi: 10.1016/j.jphotochem.2023.115145

12. Khatun M, Mandal J, Tamang VW, **Chowdhury SG**, Karmakar P, Saha A. A pyridoxal based bio-compatible fluorometric chemosensor for recognition of Zn^{2+} ions: Theoretical approaches and application in live cell imaging. doi: 10.1016/j.jphotochem.2023.115231

13. Pramanik S, Hossain A, Pathak S, **Chowdhury SG**, Karmakar P, Frontera A, Mukhopadhyay S. Revealing the supramolecular features of two Zn(II) complexes derived from a new hydrazone ligand: a combined crystallographic, theoretical and antibacterial study. CrystEngComm, 2023;25, 866-876. doi: 10.1039/D2CE01445A

14. Mandal J, Pal K, **Chowdhury SG**, Karmakar P, Panja A, Banerjee S, Saha A. Two rhodamine-azo based fluorescent probes for recognition of trivalent metal ions: crystal structure elucidation and biological applications. Dalton Trans. 2022;51(40):15555-15570. doi: 10.1039/d2dt00399f

15. Masum AA, Pal K, Saha I, Ghosh D, Roy S, **Chowdhury SG**, Islam MM, Karmakar P. Facile synthesis of antibiotic encapsulated biopolymeric okra mucilage nanoparticles: molecular docking, in vitro stability and functional evaluation. Adv. Nat. Sci: Nanosci. Nanotechnol. 2020. 11 025020. doi: 10.1088/2043-6254/ab9195

16. Banerjee S, Patra R, Ghorai P, Brandao P, **Chowdhury SG**, Karmakar P, Saha A. Syntheses, crystal structures, DNA binding, DNA cleavage and DFT study of Co(III) complexes involving azo-appended Schiff base ligands. New J. Chem. 2018;42, 16571-16582. doi: 10.1039/C8NJ02235F

Oral/Poster Presentations

1. Presented an oral presentation at 2nd International Conference on "Environment, Agriculture, Human, and Animal Health", organized by Voice of Indian Concern for the Environment (VOICE); Department of Botany and Geology, Govt of Maharashtra's Rajaram College, Kolhapur, Maharashtra, India; Somnogen Canada Inc., Toronto, Canada; and All India Institute of Training and Education (AIITE), New Delhi, India during 20th-21st July 2023.

2. Presented a poster presentation at 4th International Conference on "ENVIRONMENTAL, AGRICULTURAL, CHEMICAL AND BIOLOGICAL SCIENCES", organized by Voice of Indian Concern for the Environment (VOICE); Department of Biotechnology, Srimad Andavan Arts and Science College, Trichy, Tamilnadu, India; Somnogen Canada Inc., Toronto, Canada; and All India Institute of Training and Education (AIITE), New Delhi, India during 13th-14th May 2023.

3. Presented a poster at UGC-SAP (DRS II) sponsored National symposium on "Stress Biology: Recent Advances in Biochemical and Biophysical Research", organized by Department of Biochemistry and Biophysics, University of Kalyani, West Bengal during 22nd-24th March 2023.

4. Presented a poster at International Conference on "Signaling in Disease Management and Diagnostics", organized by CSIR-Indian Institute of Chemical Biology, Kolkata, India during 16th-18th March 2023.

5. Presented an oral presentation at UKIERI-SPARC symposium on "Genome Regulation in Development and Disease" organized by The University of Manchester, United Kingdom on 26th February 2021.

6. Presented a poster at International Virtual Conference on "Frontiers in Biological Research", organized by Department of Botany, St. Joseph's College (Autonomous), Tiruchirappalli & The Biomics, Bengaluru during 15th-21st February 2021.

7. Presented an oral presentation at India International Science Festival-2020 on "Frontiers Area of Research-Life Sciences", organized by the Ministry of Science and Technology, Ministry of Earth Sciences, and Ministry of Health and Family Welfare, Govt. of India in collaboration with Vijnana Bharati (VIBHA) during 22nd-24th December 2020.

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Both phosphorylation and phosphatase activity of PTEN are required to prevent replication fork progression during stress by inducing heterochromatin

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Heterochromatin

ABSTRACT

PTEN is a tumor suppressor protein frequently altered in various cancers. PTEN-null cells have a characteristic of rapid proliferation with an unstable genome. Replication stress is one of the causes of the accumulation of genomic instability if not sensed by the cellular signaling. Though PTEN-null cells have shown to be impaired in replication progression and stalled fork recovery, the association between the catalytic function of PTEN regulated by posttranslational modulation and cellular response to replication stress has not been studied explicitly. To understand molecular mechanism, we find that PTEN-null cells display unrestrained replication fork progression with accumulation of damaged DNA after treatment with aphidicolin which can be rescued by ectopic expression of full-length PTEN, as evident from DNA fiber assay. Moreover, the C-terminal phosphorylation (Ser 380, Thr 382/383) of PTEN is essential for its chromatin association and sensing replication stress that, in response, induce cell cycle arrest. Further, we observed that PTEN induces HP1α expression and H3K9me3 foci formation in a C-terminal phosphorylation-dependent manner. However, phosphatase dead PTEN cannot sense replication stress though it can be associated with chromatin. Together, our results suggest that DNA replication perturbation by aphidicolin enables chromatin association of PTEN through C-terminal phosphorylation, induces heterochromatin formation by stabilizing and up-regulating H3K9me3 foci and augments CHK1 activation. Thereby, PTEN prevents DNA replication fork elongation and simultaneously causes G1-S phase cell cycle arrest to limit cell proliferation in stress conditions. Thus PTEN act as stress sensing protein during replication arrest to maintain genomic stability.

1. Introduction

Understanding the function of PTEN associated with its various tumor suppressor activities has been gaining interest since the last decades. PTEN is mutated or inactivated in many tumor lineages, including glioblastoma, breast, prostate, hepatocellular carcinoma, melanoma, and endometrium carcinoma [1–8]. PTEN promotes its tumor suppressor activity by functioning not only in the cytoplasm by down regulating PI3K-Akt pathway but also in the nucleus by guiding and preserving genomic stability [9]. It emerges as guardian of the genome by controlling multiple cellular obligatory pathways including cell cycle regulation at both G1 and G2 checkpoint [10,14–16], maintenance of heterochromatin structure through stabilizing HP1α and histone H1 [12,13], HR repair [10,11], proper chromosome segregation [17,18] and DNA replication progression [19–21]. It has been found that PTEN null cells are well adapted to overcome intra-S-phase checkpoint and exhibit premature S-phase exit compared to wild-type cells [19]. Aberrant DNA replication and early S-phase exit render genomic instability in daughter cells and are considered to be potential reasons for promoting malignancy.

Cancer cells acquire aggressive proliferative potential by deregulation of replication origin activation resulting over replicated, fragile, or un-replicated DNA accumulation leading to genomic instability [22]. Any form of DNA damage in the S-phase causes replication stress and has

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emerged as a major source of genomic instability if it becomes un-sensed and checkpoint machinery is compromised [23,24]. Controlling and sensing DNA damage during the S-phase is an "Achilles' heel" and must be tightly regulated, otherwise it causes a serious threat to genomic stability [25]. Replication stress constrains fork progression and transient heterochromatin formation ahead of DNA damage is important for fork stability. Additionally, it is crucial for remodeling the chromatin to create an efficient framework for the functioning of DNA repair machinery [26,27]. During replication stress, run-on replication may cause chromosome fragility, gene loss or mutations. Further unsuccessful transmittance of DNA damage response signals during replication stress due to checkpoint defect cause aneuploidy [28,29]. Recently epigenetic alteration on chromosomes during tumorigenesis is gaining attention for cancer predisposition and early prognosis [30,31]. Interestingly various cancers like breast, brain, colon, thyroid carcinoma show reduced Heterochromatin Protein alpha (HP1 α) expression [32], resulted compromised chromatin organization or condensation. Another histone epigenetic marker, H3K9me3 is also found to be significantly reduced in prostate cancer compared to non malignant prostate tissue [33]**.** But in breast carcinoma, enrichment of H3K9me3 is observed, which may correlate with invasion and metastasis [34]. Defect in any or all heterochromatin-associated proteins could lead to broad epigenetic instability with the potential to impact gene expression profiles and ultimately lead to abnormal growth characteristics [31]**.** Disruption of the epigenetic signature associated with late replicating heterochromatin, e.g.H3K9me3, promotes chromatin accessibility and advancing replication timing [35]. Origin licensing is also closely associated with histone H4K20 monomethylation (H4K20me1), and re-replication occurs with deregulation of H4K20me1 by the PR-Set7 methyltransferase [36]. Thus heterochromatin maintenance is essential for genetic stability in cancer cells, which is closely associated with replication dynamics like initiation, space, time and progression [37]. PTEN is involved in both heterochromatin maintenance and DNA replication regulation [12, 19–21]. Further, it has been found that the DNA repair capacity of PTEN depends on its post translational modulation like sumoylation, phosphorylation, acetylation, methylation at different sites. The C-terminal of PTEN is positively associated with genetic stability [38,39] through sumoylation and methylation at lysine residue 254 and 349, respectively [40,41]. This prompted us to investigate how the different post-translational modulations of PTEN are involved in the DNA replication process. Using the PTEN- null PC3 cells as a model system, we demonstrated that PTEN binds to chromatin when C-terminal is phosphorylated, leading to heterochromatin formation in response to exogenous replication stress. These heterochromatin structure are characterized by enrichment and stabilization of histone H3K4me3 mark. Thus, by heterochromatin formation, PTEN prevents DNA replication fork elongation and simultaneously induces G1-S-phase cell cycle arrest and CHK1 activation to check cell proliferation in stress conditions. Moreover, we observed that the phosphatase activity of PTEN is indispensable for sensing replication stress but not the sumoylation. Therefore, PTEN acts as a stress sensing protein that efficiently prevents replication in stress conditions to limit genomic instability.

2. Materials and methods

2.1. Cell culture, reagent and antibody, and plasmid

PTEN null PC3 cells were purchased from National Centre for Cell Science (Pune, India) and maintained at 37^0 C, 5% CO2, and 95% relative humidity (RH) in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 ×non-essential amino acid, penicillin (100 U/ml) and streptomycin (100 U/ml). Cells were seeded overnight and subsequently treated with aphidicolin(6 μM) in an exponential phase. BrdU, IdU, CldU, and aphidicolin (Aph) were purchased from Sigma-Aldrich. The following antibodies were used: PTEN, HP1α and β-actin, tubulin, and histone H3, CHK1, p-CHK1(Ser 345) antibodies were purchased from Cell Signaling Technology; mouse anti-BrdU (B44) antibody was purchased from BD Biosciences; Rat anti-BrdU BU1/75 and H3K9me3 antibodies were purchased from Abcam. Anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (HRP), anti-rat IgG conjugated with Texas Red and anti mouse IgG conjugate with FITC (Invitrogen) were used as secondary antibody. Gene-constructs pSG5L-HA-Wt-PTEN (HA-PTEN) encodes hemagglutinin tagged wild type PTEN protein; phospho-deficient pSG5L-HA-A4-PTEN (Ser380Ala, Thr382Ala, Thr383Ala, Ser385Ala) and phospho-mimicking pSG5L-HA-E4-PTEN (Ser380Glu, Thr382Glu, Thr383Glu, Ser 385Glu) encode hemagglutinin (HA) tagged mutant PTEN proteins. pSG5L-HA C124S PTEN (phosphatase-dead) and pSG5L-HA- K254R PTEN (sumoylation-dead) were created by site-directed mutagenesis (Stratagene) and confirmed by sequencing (Fig. S1). For phosphatase dead mutant PTEN following primer was used: Forward: 5GTTGCAGCAATTCACTCTAAAG CTGGAAAGGG3′,Reverse:5CCCTTTCCAGCTTTAGA- GTGAATTGCTG-CAAC 3′. For sumoylation-dead mutant PTEN following primer was used: Forward: 5'GTGTGTGGTGATA TCAGAGTA GAGTTCTTCCAC 3′, Reverse: 5GTGGAAGAACTCTACTCTGATATCACCAC ACAC3.

2.2. Cellular transfection

PC3 cells were seeded in the antibiotic-free medium overnight prior to transfection. For transient transfection, Lipofectamine 3000 transfection reagent (Invitrogen) was used as per the manufacturer's instruction.

2.3. BrdU incorporation assay

Cells were seeded on 18 mm sterile coverslips. After Aph (6 μM) treatment, cells were incubated with BrdU for 1 h, fixed in freshly prepared 4% paraformaldehyde solution for 15 min, and permeabilized with 0.2% Triton X-100 on ice for 10 minThen denatured cellular DNA with 2 M HCl, washed with 1 ×PBS, and were pre-incubated in blocking solution (1% BSA in 1 × PBS) followed by overnight incubation with anti-BrdU antibody (1:100) in wash buffer (0.1% BSA and 0.05%Tween 20 in 1 × PBS) at 4^0 C. Cells were then washed six times with wash buffer and probed with FITC conjugated anti-mouse antibody (1:200) for 1 h at RT. After proper washing, cells were mounted in the mounting solution containing DAPI (4–6-Diamidino-2- phenylindole, Vector Laboratories) and examined under a fluorescence microscope (Leica).

2.4. BrdU flow cytometry assay

The un-transfected and transfected cells were pre-treated with 6μMAphfor 3 h and then incubated with 50 μM BrdU immediately for 1 h. After rinsing with (PBS), BrdU-labeled cells were then trypsinized and fixed in 70% ethanol overnight at $-\ 20^0$ C.The next day after washing, cells were incubated with 0.4 ml of 2 N HCl/Triton X-100 for 30 min to denature the DNA. Then cell pellets were collected, washed with PBS, and re-suspend in 0.5 ml of 0.1 M Na2B4O7, pH 8.5 to neutralize the sample. Then cells were incubated with anti-BrdU antibody(1:100) and propidium iodide (PI)(50 μg/ml) and processed for FACS analysis.

2.5. Western blotting

Cell pellets were collected after transfection with various PTEN clones, and cell lysates were prepared by cell lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol, 2 μg/ml aprotinin, 2 μg/ml leupeptin and phosphatase inhibitor). Then cell lysates were boiled with 1 × protein loading dye and resolved by 10% SDS–PAGE and then were transferred to methanol-activated PVDF membrane. After blocking the membrane for 1 h at room temperature (RT) with TBS containing 0.1% (w/v) Tween-20% and 5% (w/v) non-fat milk, it was incubated with primary antibodies at 4^0 C of overnight. The next day followed by washing with 1 ×TBST, membrane was incubated

with HRP-conjugated anti-rabbit or anti- mouse antibody for 1 h at RT. Detection was carried out using ECL reagents (Santa Cruz Biotechnology, Inc.).

2.6. DNA fiber assay

Approximately 5 × 10^5 cells were plated in a 35 mm tissue culture plate. Then the cells were transfected with various PTEN clones. After overnight incubation,cells were pulse-labeled with 100 μM CIdU for 30 min, washed with PBS, and co-incubated with Aph and 100 μM ldU for 3 h. Then cells were harvested and re- suspended in 50 μl of PBS. Cell suspensions (2.5 μl) were mixed with 7.5 μl of lysis buffer (0.5% sodium dodecyl sulfate, 200mMTris-HCl (pH 7.4), 50 mM EDTA). Each mixture was dropped on the top of an uncoated glass slide and waited for 10 min to lyse the cells in situ. Slides were inclined at 45^0 angle to spread the suspension on the glass. Once dried, DNA spreads were fixed by incubation for 10 min in methanol- acetic acid solution (3:1). After the PBS wash, DNA was denatured with 2.5 N HCl for 50 min at 37^0 C. The slides were rinsed several times in PBS and incubated with the following antibodies: mouse anti-BrdU antibody (1:40) and rat anti-CldU (1:300) diluted in 1% BSA. After incubation in a humid chamber for 1 h at 37∘C, slides were washed in PBS containing 0.1% Triton X-100. Then slides were incubated with anti- mouse Alexa Fluor 488 (1:1000) and anti-rat TR diluted in 1% BSA for 1 h at 37^0 C. Slides were washed three times for 3 min in PBS with 0.1% Triton X-100 and mounted by using Vectashield (Vector Laboratories) mounting medium and examined under a fluorescence microscope (Leica).

2.7. Fluorescence microscopy

Cells were plated on 18 mm sterile glass coverslips. After treatment, cells were fixed in freshly prepared 4% paraformaldehyde solution for 15 min and permeabilized with 0.2% Triton X-100 on ice for 10 min. Fixed cells were pre-incubated in blocking solution (1% BSA in 1 × PBS), followed by overnight incubation with anti H3K9me3 antibody in wash buffer (0.1% BSA and 0.05%Tween 20 in 1 × PBS) at 4^0 C. Cells were then washed six times with wash buffer and probed with Alexa Fluor 488 conjugated anti-mouse antibody (1:200) for 1 h at RT. After proper washing, cells were mounted in the mounting solution containing DAPI (4–6- Diamidino-2-phenylindole, Vector Laboratories) and examined under a fluorescence microscope (Leica).

2.8. Cellular fractionation

Cells (2 ×10^6) were washed with 1 × PBS and resuspended in 200 μl of solution A [10 mM HEPES (pH 7.9), 10 mMKCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, protease and phosphatase inhibitors]. After incubation on ice for 10 min, the cytoplasmic and nuclear fractions were harvested by centrifugation at 1500 ***g*** for 5 min. Next, the nuclear fraction was washed twice in solution A, incubated in 150 μl solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease and phosphatase inhibitors) on ice for 15 min. Then soluble nuclear fractions were separated by centrifugation at 1700 ***g*** for 4 min. Then pellet is incubated in 300 μl of high salt buffer(10 mM Tris pH 7.4, 2 mM MgCl, 2 mM EGTA, 0.1% Triton X-100, 600 Mm NaCl and rotate at 4^0 C for 30 min. Then lysate were centrifuged at 13,200 rpm for 10 min. The pallet was discarded, and isolated chromatin fraction was boiled with 1 ×Laemmli sample buffer. Cytoplasmic and nuclear high salt fraction fractions were subjected to western blot analysis.

2.9. Double thymidine Block

WI-38 cells were plated at 30 – 40% confluence in a 10 cm culture dish in a DMEM medium, and 2 mM thymidine-containing medium was added after overnight incubation at 37^0 C. After 18 h of incubation, thymidine-containing medium was discarded, washed with PBS, and incubated in fresh medium for 9 h. Then again, 2 mM thymidine containing medium was added and incubated for another 18 h at CO2 incubator. Then cells were subjected to immune-fluorescence assay.

2.10. Statistical analysis

A Student's t-test was used to calculate the statistical differences between the groups. $P < 0.05$ and 0.005 were considered statistically significant. Data analysis was performed using the Graph Pad prism software.

3. Results

3.1. Phospho-PTEN accumulates in the nucleus after S-phase arrest by double thymidine block

Previously we and another group reported that phosphorylated PTEN accumulates in nucleus after DNA damage induced by etoposide and IR, respectively [42,43]. However, when we synchronized Wi-38 cells at S-phase by double thymidine block, we observed a very similar observation for the phosphorylated PTEN as we found in etoposide-treated cells, whereas the total PTEN was distributed throughout the cells. These results indicate that phosphorylated PTEN may be linked with DNA replication (Fig. 1).

3.2. Replication arrest and cell cycle progression after aphidicolin treatment depend on post-translational modulation of PTEN

As we found phospho-PTEN accumulation in the nucleus of S-phase cells, we then want to assess how posttranslational modulations of PTEN are associated with DNA replication and cell cycle progression. Initially, we developed the PTEN isogenic cell line models by over expression of the plasmids containing HA fusion constructs with PTEN wild type (PTEN-WT), PTEN C-terminal phosphorylation-dead mutant (PTEN-A4), PTEN C-terminal phosphorylation-mimetic mutant (PTEN-E4), PTEN phosphatase-dead mutant (PTENC124S) and PTEN sumoylation-dead mutant (PTEN-K254R) in PTEN-negative PC3 cells. The efficiency of the expression of the respective plasmids was validated by western blot analysis. Next, the above cells were subjected to BrdU labeling (Fig. 2A) after release from replication stress induced by DNA replication inhibitor aphidicolin (Aph) and analyzed the frequency of BrdU positive cells (Fig. 2B). The expression of wild-type PTEN significantly reduced the percentage of BrdU positive cells (17.9%) in comparison to PTEN-null cells (42.25%). Similarly, the cells expressing sumoylation-mutant plasmids displayed a lower percentage of BrdU positive cells. Conversely, the cells expressing phosphorylation-dead, phosphorylation-mimetic and phosphatase-dead PTEN mutants demonstrated a significantly higher percentage of cells with BrdU signal (30.66%, 31.44%, and 29.81%, respectively) in comparison to the wild type PTEN expressing cells. Additionally, in the similar experimental setup described above, cell cycle was analyzed by flow cytometry (Fig. 2C). The PTEN-null cells displayed a higher percentage of the S-phase population (41.50%) than wild-type PTEN expressing cells (16.06%) after aphidicolin treatment. The S phase population in the sumoylation-mutants was 20.66%, which is very parallel to the wild-type PTEN expressing cells. However, PTEN-A4 (30.44%), PTEN- E4 (28.11%), and phosphatase-dead (32.11%) mutants expressing cells displayed an increase in the S-phase population upon aphidicolin treatment (Fig. 2D). These results indicate that replication stress-induced checkpoint could be facilitated by wild-type PTEN expression in the PTEN-null cells resulting in G1-S-phase cell cycle arrest, whereas sumoylation has no significant effect on this process. Alternatively, premature S-phase exits of cells are prevented by PTEN depending on its phosphatase activity and C-terminal phosphorylation. Thus, the carboxyl-terminal phosphorylation cluster of PTEN and phosphatase activity may have a role in the activation of replication checkpoint.

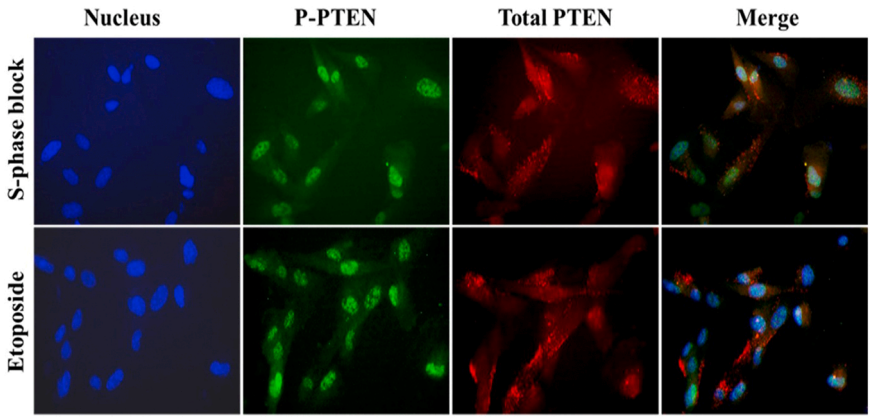

Fig. 1. Representative photomicrographs of S-phase arrested control, and etoposide treated Wi-38 cells immune labeled with PTEN-Ser380 (green) or total PTEN (red).

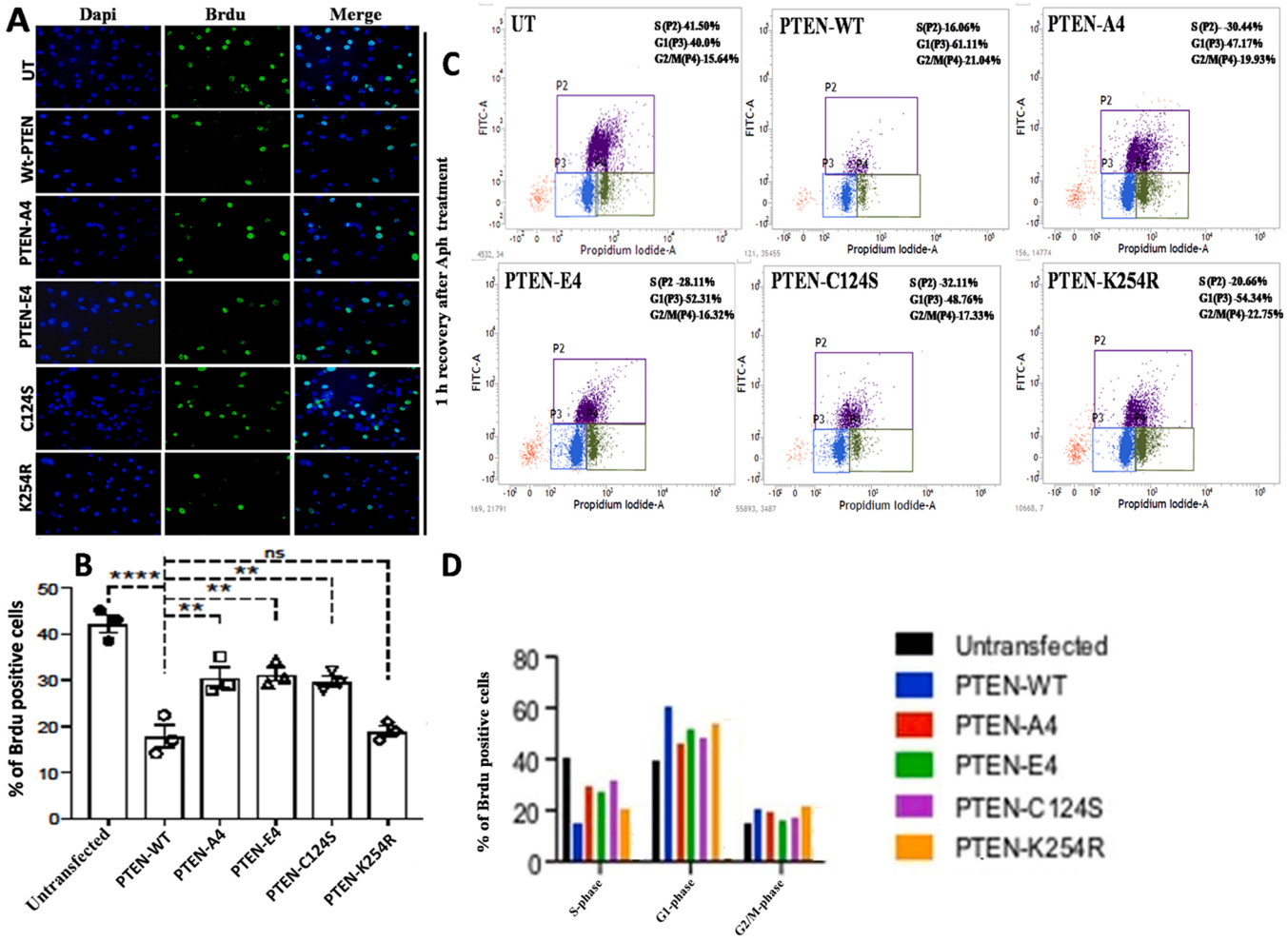

Fig. 2. (A).Immunofluorescence image of BrdU positive cells. PTEN null PC3 cells were transiently transfected with various PTEN clones. After overnight incubation cells were treated with Aph (6 μm for 3 h) and subsequently labeled with BrdU for 1 h after washing with 1 × PBS. (B). Graph represents percentages of BrdU positive cells containing various PTEN clones transfected cells. Values are the mean ± SD of three independent experiments.(C). PC3 cells were transfected with mentioned PTEN clones and after overnight incubation, cells were treated with 6 μm Aph for 3 h prior to BrdU labeling and propidium iodide (PI) staining for flow cytometric analysis for cell cycle distribution. Two experiments were performed obtaining similar results. A typical experiment is displayed." (D) Graph represents quantification of cell cycle analysis.

3.3. Loss of C-terminal phosphorylation and phosphatase activity of PTEN causes unrestrained DNA replication during aphidicolin treatment

Next, we performed a DNA fiber assay to observe how post-translational modulation of PTEN affects replication fork dynamics at a single-molecule resolution in the presence of aphidicolin. In this assay, we ectopically expressed different PTEN clones in PTEN-null cells and labeled them with CldU for 30 min. After washing, we incubated the cells in the IdU-containing medium with or without Aph for 3 h (Fig. 3A). Then cells were subjected to DNA fiber analysis. To visualize individual DNA fibers, CldU and IdU were probed with the secondary antibodies anti-rat Texas Red (red) and anti-mouse Alexa Fluor 488 (green), respectively (Fig. 3B). The quantification of continuous forks is measured by observing cells where both the labels are incorporate. Our data suggests that cells expressing wild-type PTEN and the sumoylation-PTEN mutants showed a significantly lower percentage of continuous forks progression (22.83% and 25.93% respectively) with aphidicolin treatment in comparison to the PTEN null cells. On the contrary, expression of phospho-dead, phospho-mimetic and phosphatase-dead PTEN displayed a significant increase in the percentage of the continuous fork (36.69% for A4 and 34.96% for E4) with aphidicolin treatment in comparison to the wild-type PTEN expressing cells. Interestingly, the distribution of the DNA fiber tract length for wild type, and the sumoylation were followed the same pattern; however, for two phospho mutants (PTEN-A4 and PTEN-E4) and the phosphatase-dead, it was shifted towards longer tract length. We found 62% of fiber has IdU tract length in PTEN transfected cells in between 1 and 2 μm and 29.7% fiber within 2–3 μm, whereas PTEN-null cells show 50.3%, 25.4%, and 14.5% of the fiber in 1–2 μm, 2–3 μm, and 3–4 μm, respectively. In the case of A4 and E4 PTEN, 46.3% and 58.1% of fiber show IdU tract length, respectively in between 2 and 3 μm, which is significantly higher than wild-type PTEN. Phosphatase dead PTEN transfected cells shows over 40% increase in IdU tract length in 2–3 μm length categories compared to wild type PTEN. This data suggest that PTEN can sense Aph-induced replication fork arrest and slows down the fork progression to cooperate with the recovery process, and its phosphatase activity and C-terminal phosphorylation may have an essential role in these events.

3.4. Elevated DNA damage co-exists with CHK1 inactivation in phosphomutant and phosphtase dead PTEN transfected cells

To check if replication restrain with APH is associated with persistence of DNA damage, we transfect various clone of PTEN in PC3 cells and γH2AX foci was measured after Aph treatment of 3 h. We found that compared to untransfected cells, PTEN transfected cells shows significantly less intense γH2AX foci. Again compared to Wt-PTEN transfected cells, phospho and phosphatased dead PTEN transfected cell exhibit higher intense of gamma γH2AX foci. These results indicate that mutant PTEN or untransfected cells continue to replicate with damaged DNA (Fig. 4A). To check if accumulation of damaged DNA is associated with compromised CHK1 activation, as activated CHK1 can confers replication fork stability [44], we evaluate CHK1 phosphorylation status (Ser 345) in various PTEN clone transfected cell. We found that CHK1 activation is compromised in A4, E4 and phosphatase dead transfected cells (Fig. 4B). Thus this data indicate that due to compromised CHK1 activation and hence defective functional checkpoint functionary in PTEN null and mutant transfected cells are responsible of unrestrained replication in stress conditions with damaged DNA.

3.5. Chromatin association of PTEN depends on its C- terminal phosphorylation

Since phosphorylation of PTEN at C-terminal sense the aphidicolin mediated replication arrest, thereby we wanted to evaluate whether the same could affect chromatin association of PTEN. The cytoplasmic and the nuclear high salt fractions were prepared from the cells expressing the wild type and the mutant PTEN in the presence of aphidicolin followed by western blotting. The reduced level of phosphomutant-PTEN (probed with anti-HA antibody) in the nuclear high salt chromatin fractions compared to wild-type PTEN indicated its disassociation from

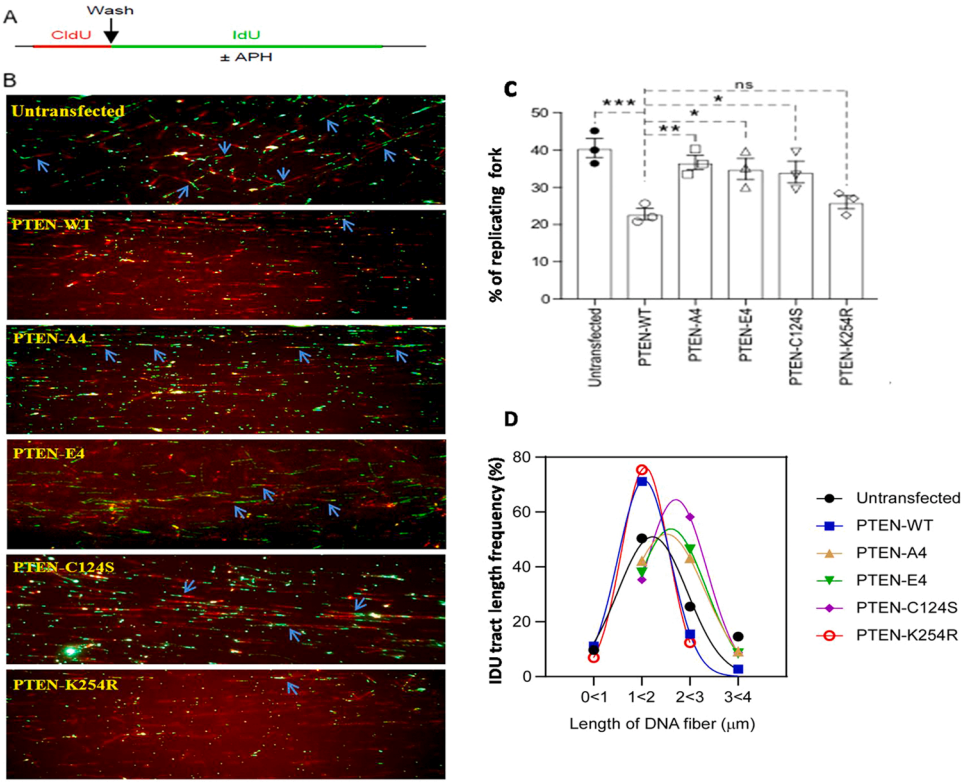

Fig. 3. Experimental design of DNA fiber assay. (A).Cells were labeled with CldU(red) for 45 min and then labeled with IdU(green) in the presence of Aph for 3 h. Representative photomicrograph of DNA fiber stretch. PTEN null cells were transfected with various PTEN clones and subjected to DNA fiber analysis. Red tract represents before treatment, and green tract represent during treatment.(B) Approx 120 well- defined fibers are counted for each sample and data was represented as percentages of replicating fork (green+red) for 3 independent experiments.(C). Representation of IdU (green) tract length (μm) distribution pattern of each PTEN clone transfected cells.

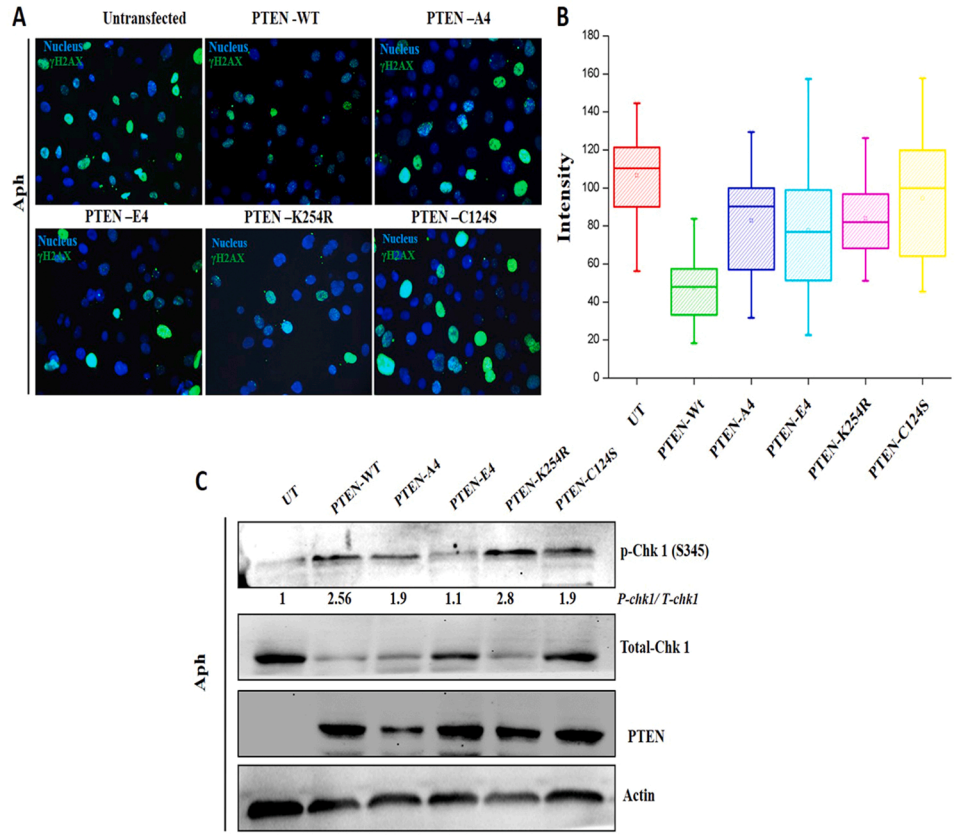

Fig. 4. (A) Representative photomicrograph of γH_2AX foci (green) in untransfected, and mutant PTEN clones transfected cells. (B) Quantification of γH2AX intensity per nucleus after APH treatment in PTEN null and PTEN mutants transfected cells. (C) PTEN-null PC3 cells were transfected with mentioned PTEN clones. After 24 h of incubation, they were treated with APH for 3 h. After that whole-cell lysate was prepared and subsequently western blotting was performed with anti-CHK1, anti- p-CHK1(Ser 345) and anti-PTEN antibodies. β-actin was used as loading control. Band intensities were measured by ImageJ software. Here we represent the ratio of intensity of p-CHK1 and total CHK1 (Ser 345). Untransfected control sample was normalized to 1.

chromatin upon aphidicolin treatment (Fig. 5A). At the same time, a slight increase in the level of phosphatase and sumoylation dead-PTEN in aphidicolin-treated cells suggested their possible role in the recruitment of PTEN into chromatin during replication stress.

3.6. *Heterochromatin maintenance is regulated by PTEN post-translational modulation*

Methylation of lysine 9 of histone H3 (H3K9me3) marks constitutive

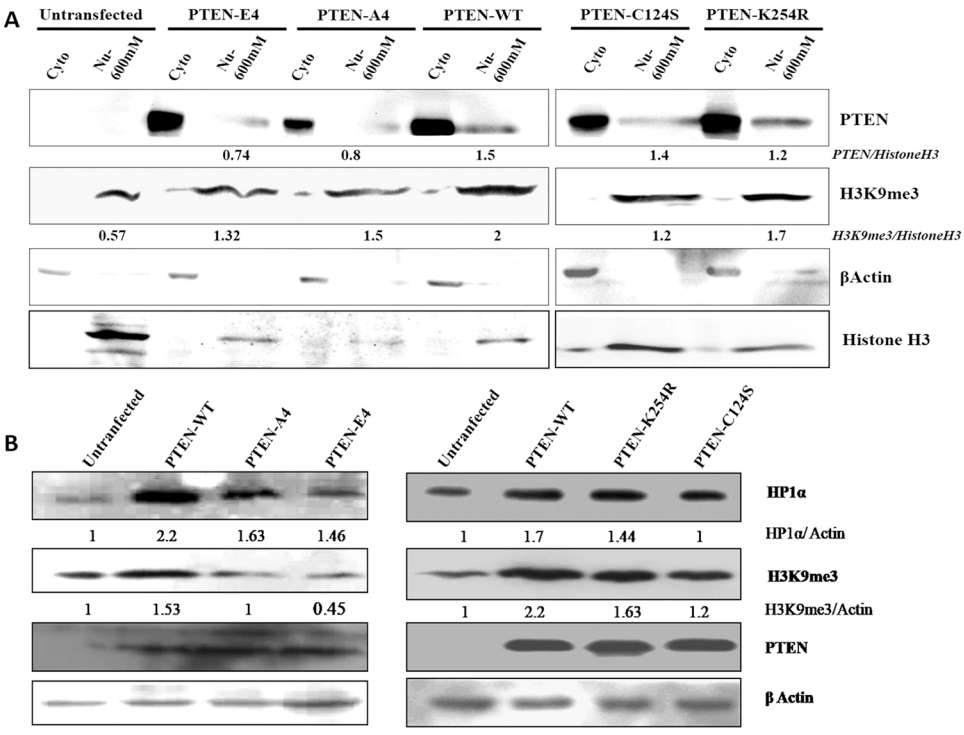

Fig. 5. (A). PTEN-null PC3 cells were transfected with mentioned PTEN clones. After 24 h of incubation, nuclear high salt fraction was prepared. Then cytoplasmic and nuclear high salt fractions were immune blotted with anti-HA, and anti-H3K9me3 antibodies. histone H3 were used as loading control.(C) PTEN-null PC3 cells were transfected with mentioned PTEN clones. After 24 h of incubation, whole-cell lysate was prepared and subsequent western blotting was performed with anti-HP1α, anti-H3K9me3, and anti-PTEN antibodies. β-actin was used as loading control. Band intensities were measured by ImageJ software. Untransfected control was normalized to 1. (B).

heterochromatin, the regions of high- density repetitive DNA elements that control genomic integrity by preventing chromosome mis-segregation, recombination, and DNA replication [45]. Since earlier report demonstrated that destabilization of heterochromatin occurred in the absence of PTEN [13], therefore we wanted to evaluate how post-translational modification of PTEN affects the same. Here, PTEN null PC3 cells showed reduced expression of H3K9me3 and HP1α, a marker of transcriptional inactive heterochromatin in comparison to wild type PTEN expressing cells, which is corroborated the previous study [13]. Further analysis has revealed that cells expressing two phospho-mutants and the phosphatase-dead PTEN have a significantly lower percentage of H3K9me3 protein level in chromatin than the wild type PTEN expressing cells. However, in sumoylation- dead PTEN expressing cells, chromatin H3K9me3 protein levels are similar to wild type-PTEN. HP1α expression is also decreased in phospho-mutant and phosphatase dead PTEN (Fig. 5B). We further evaluate heterochromatin foci formation of H3K9me3 upon transfection of various PTEN clones (Fig. 6). We found that 41% of untransfected cells show $>$ 5 foci/cells whereas 60.33% of PTEN transfected cells show $>$ 5 foci. The count was significantly lower in the case of phospho-mutant, and phosphatase-dead PTEN transfected cells. However, in the case of sumoylation-deficient PTEN, H3K9me3, foci count was nearly the same as wild-type PTEN. Thus this open chromatin maintenance could be associated with unperturbed replication during replication arrest leading to defective replication by overcoming the checkpoint barrier, which is a hallmark of cancer predisposition. Thereby, PTEN helps to sense replication arrest through maintaining heterochromatin, or it may retain heterochromatin state by sensing replication arrest via phosphorylation at C-terminal and phosphatase-dependent manner.

4. Discussion

Aberrant DNA replication timing appears to affect mutagenesis directly [46]. Cancer cells are in increased replication stress rendering accumulation of genomic instability [47]. Failures in the transmittance of cellular response to replication stress, like those caused by dysfunctional DNA-damage checkpoint, or DNA repair are tumorigenic, as seen in mouse and various human syndromes. Thus preserving replication fork is key to prevent the accumulation of genomic instability [24]. The role of PTEN in DNA repair is well documented in various literatures [48]. It was also reported that both heterochromatin marker HP1 α and H3K9me3 are down-regulated in PTEN-null cells [13]. Further replication origins are largely present on the heterochromatinized chromosomes [49]. Thus an increased expression of HP1α and H3K9me3 in PTEN positive cells may avert unrestricted ORC accession. Replication stress caused by DNA damage or poor growing conditions can increase the usage of flexible origins or activate a neighboring dormant origin to complete DNA replication, but that may be error-prone [50,51]. In this study, we explored that replicating fork progression in PTEN null cells is deregulated on replication stress induced by aphidicolin which may due to reduce compactness of chromosome. Previously it was shown that upon DNA damage, the HP1/KAP1/ Suv39h1axis leads to the trimethylation of H3K9 over tens of kilobase flanking double-strand breaks (DSBs) to induce chromatin compaction [27] and prevent fork progression. Another report suggests that upon hydroxyurea treatment, H3K9 is trimethylated, and H2BK33 is de-acetylated surrounding stalling forks causing higher level of chromatin compaction in these regions and stabilized stalled fork [26]. Here we observed that Wt- PTEN transfected cells shows elevated expression and chromatin association of HP1α and H3K9me3 depending on C terminal phosphorylation as both phospho-dead and phospho-mimetic PTEN expressing cells display compromised heterochromatin. Thereby, PTEN may act as a silencing factor during replication stress by stabilizing heterochromatin foci and by preventing replication fork progression as revealed by our DNA fiber assay. As expected, we found the replication fork progression are mostly heterogeneous in these populations. Moreover, the global loss of H3K9me3 mark causes impaired DNA damage response in progeroid cells by compromising ATM activation and reduced formation of γH2AX foci [52], which may be a cue for the aberrant DNA damage signal in the PTEN-null cells too. We previously demonstrated that C-terminal phosphorylation of PTEN was increased and phosphorylated PTEN developed nuclear foci upon genotoxic stress [42]. Accordingly, in this study, we found that phosphorylated PTEN is also necessary for heterochromatin maintenance. Our results also indicate that replication stress-induced checkpoint could be rescued by wild-type PTEN expression in the PTEN-null cells resulting in G1-S-phase cell cycle arrest, whereas sumoylation of PTEN has no significant effect on this process. Alternatively, premature S-phase exits of cells are prevented by PTEN

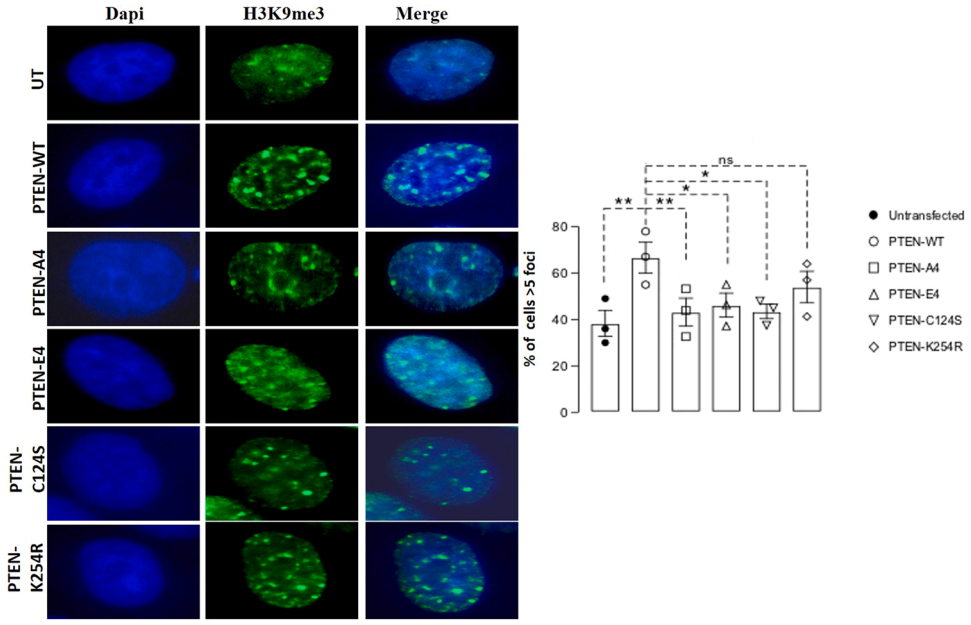

Fig. 6. (A). Representative photomicrograph of H3K9me3 foci (green) in untransfected, and mutant PTEN clones transfected cells. DAPI represents nucleus.(B). Quantification of data represents % of cells those have $>$ 5 no. of foci.

depending on its both phosphatase activity and C-terminal phosphorylation status. Thus, the carboxyl-terminal phosphorylation cluster of PTEN and phosphatase activity may have a role in the activation of replication checkpoint. Chk 1 is an important kinase responsible for fork stability and suppresses origin firing in response to replication blocks [53]. Previous report suggests that PTEN loss inhibit CHK1 activation which causes accumulation of DNA DSB [54]. Our study also suggested that p-CHK1 is greatly diminished in PTEN null cells. Wt-PTEN transfection restore CHK1 activation upon replication stress and prevent DNA damage in PTEN null cells while CHK1 is less activated in A4 and E4 transfected cells. Interestingly in phosphatase dead transtested cells, CHK1 activation is elevated but not as strong as Wt-PTEN transfected cells. PTEN also dephosphorylates MCM2 in response to replication stress that prevents fork progression and maintains genomic stability [21]. Likewise, we uncovered the phosphatase activity of PTEN could become essential for sensing replication stress. A strong association of phosphatase-dead PTEN with chromatin (Fig. 4) indicates phosphorylation of PTEN stabilized it on chromatin by preventing it from ubiquitin-mediated degradation and phosphatase activity is critical for dephosphorylation of MCM2 [21]. As phospho-PTEN shows reduced phosphatase activity [55], it seems it is tightly regulated by phosphorylation during replication stress. Although phospho-mutant PTEN has constitutive phosphatase activity, lack of stability and reduced chromatin association may be the reasons for its inability to sense aphidicolin mediated replication arrest. At least during replication arrest, sumoylation-mutant PTEN expressing cells, having both phosphorylation sites and phosphatase activity, can function like wild type PTEN. This observation may correlate with previous report that chromatin association of PTEN is sumoylation independent in mitomycin C-induced DNA inter strand crosslinks [56]. We reported previously that in PTEN null cells, simultaneous up-regulation of phospho-Akt and the reduced RAD51 expression lead to increase growth potential with genomic instability [57]. Therefore, Akt hyper-activation, diminished RAD51 expression, compromised heterochromatin maintenance and diminished CHK1 activation, may result in the rapid proliferation of PTEN mutant carcinoma cells with an unstable genome. As phosphorylation of C-terminal Ser/Thr residues of PTEN reduces its phosphatase function, a probable explanation could be that phospho-PTEN is a stable form, and prerequisite stability is required for the functioning of PTEN in a phosphatase-dependent manner [58]. Recent studies indicate the phosphorylation of PTEN inhibits its ubiquitination in vivo and in vitro by NEDD4–1 and WWP-1 ubiquitin ligase [59,60], whereas polyubiquitination of PTEN greatly reduces its phosphatase activity [61]. This conflicting data suggests that PTEN phosphorylation is tightly and delicately regulates PTEN phosphatase activity and maintains homeostasis in vivo, perhaps through ubiquitination. Further phosphatase activity of PTEN is required for its own de-phosphorylation at C-terminal tail [62,63] which may transiently activate phosphates activity. Overall, our current understating implies a fine regulation between the phosphatase activity and the phosphorylation of PTEN for its proper functioning in the canonical axis and the non-canonical nuclear functions implicated in maintaining genomic stability.

Conflict of Interests

All authors declare that there is no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mrfmmm.2022.111800.

References

[1] T. Sano, H. Lin, X. Chen, L.A. Langford, D. Koul, M.L. Bondy, K.R. Hess, J.N. Myers, Y.K. Hong, W.K. Yung, et al., Differential expression of MMAC/PTEN in glioblastoma multiforme: Relationship to localization and prognosis, Cancer Res. 59 (1999) 1820–1824.
[2] S.I. Wang, J. Puc, J. Li, J.N. Bruce, P. Cairns, D. Sidransky, R. Parsons, Somatic mutations of PTEN in glioblastoma multiforme, Cancer Res. 57 (1997) 4183–4186.
[3] H.Y. Zhang, F. Liang, Z.L. Jia, S.T. Song, Z.F. Jiang, PTEN mutation, methylation and expression in breast cancer patients, Oncol. Lett. 6 (2013) 161–168.
[4] G. Pourmand, A.A. Ziaee, A.R. Abedi, A. Mehrsai, H.A. Alavi, A. Ahmadi, H. R. Saadati, Role of PTEN gene in progression of prostate cancer, Urol. J. 4 (2007) 95–100.
[5] M.S. Geybels, M. Fang, J.L. Wright, X. Qu, M. Bibikova, B. Klotzle, J.B. Fan, Z. Feng, E.A. Ostrander, P.S. Nelson, et al., PTEN loss is associated with prostate cancer recurrence and alterations in tumor DNA methylation profiles, Oncotarget 8 (2017) 84338–84348.
[6] L.D. Dong, Z.X. Ran, C.X. Rong, Expression and significance of new tumor suppressor gene PTEN in primary liver cancer, J. Cell. Mol. Med 7 (2003) 67–71.
[7] H. Wu, V. Goel, F.G. Haluska, PTEN signaling pathways in melanoma, Oncogene 22 (20) (2003) 3113–3122.
[8] I.U. Ali, L.M. Schriml, M. Dean, Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity, J. Natl. Cancer Inst. 91 (22) (1999) 1922–1932.
[9] J. Ho, E.S. Cruise, R.J.O. Dowling, V. Stambolic, PTEN Nuclear Functions, Cold Spring HarbPerspect Med 10 (5) (2020), a036079.
[10] W.Y. Mansour, P. Tennstedt, J. Volquardsen, et al., Loss of PTEN-assisted G2/M checkpoint impedes homologous recombination repair and enhances radiocurability and PARP inhibitor treatment response in prostate cancer, Sci. Rep. 8 (1) (2018) 3947.
[11] B. McEllin, C.V. Camacho, B. Mukherjee, et al., PTEN loss compromises homologous recombination repair in astrocytes: implications for glioblastoma therapy with temozolomide or poly(ADP-ribose) polymerase inhibitors, Cancer Res 70 (13) (2010) 5457–5464.
[12] Z.H. Chen, M. Zhu, J. Yang, et al., PTEN interacts with histone H1 and controls chromatin condensation, Cell Rep. 8 (6) (2014) 2003–2014.
[13] L. Gong, J.M. Govan, E.B. Evans, H. Dai, E. Wang, S.W. Lee, H.K. Lin, A.J. Lazar, G. B. Mills, S.Y. Lin, Nuclear PTEN tumor-suppressor functions through maintaining heterochromatin structure, Cell Cycle 14 (14) (2015) 2323–2332.
[14] A.R. Ottschalk, D. Basila, M. Wong, et al., p27Kip1 is required for PTEN-induced G1 growth arrest, Cancer Res 61 (5) (2001) 2105–2111.
[15] A. Radu, V. Neubauer, T. Akagi, H. Hanafusa, M.M. Georgescu, PTEN induces cell cycle arrest by decreasing the level and nuclear localization of cyclin D1, Mol. Cell Biol. 23 (17) (2003) 6139–6149.
[16] J. Puc, M. Keniry, H.S. Li, et al., Lack of PTEN sequesters CHK1 and initiates genetic instability, *Cancer* Cell 7 (2) (2005) 193–204.
[17] Z. Sun, J. Lu, M. Wu, M. Li, L. Bai, Z. Shi, L. Hao, Y. Wu, Deficiency of PTEN leads to aberrant chromosome segregation through downregulation of MAD2, Mol. Med Rep. 20 (5) (2019) 4235–4243.
[18] J. He, Z. Zhang, M. Ouyang, F. Yang, H. Hao, K.L. Lamb, J. Yang, Y. Yin, W. H. Shen, PTEN regulates EG5 to control spindle architecture and chromosome congression during mitosis, Nat. Commun. 7 (2016) 12355.
[19] J. He, X. Kang, Y. Yin, K.S. Chao, W.H. Shen, PTEN regulates DNA replication progression and stalled fork recovery, Nat. Commun. 6 (2015) 7620.
[20] G. Wang, Y. Li, P. Wang, et al., PTEN regulates RPA1 and protects DNA replication forks, Cell Res 25 (11) (2015) 1189–1204.
[21] J. Feng, J. Liang, J. Li, et al., PTEN Controls the DNA Replication Process through MCM2 in Response to Replicative Stress, Cell Rep. 13 (7) (2015) 1295–1303.
[22] L.M. Briu, C. Maric, J.C. Cadoret, Replication stress, genomic instability, and replication timing: a complex relationship, Int J. Mol. Sci. 22 (9) (2021) 4764.
[23] H. Kitao, M. Iimori, Y. Kataoka, T. Wakasa, E. Tokunaga, H. Saeki, E. Oki, Y. Maehara, DNA replication stress and cancer chemotherapy, Cancer Sci. 109 (2) (2018) 264–271.
[24] H. Gaillard, T.G. Muse, A. Aguilera, Replication stress and cancer, Nat. Rev. Cancer 15 (5) (2015) 276–289.
[25] N. Willis, N. Rhind, Regulation of DNA replication by the S-phase DNA damage checkpoint, Cell Div. 4 (2009) 13.
[26] G. Feng, Y. Yuan, Z. Li, L. Wang, B. Zhang, J. Luo, J. Ji, D. Kong, Replication fork stalling elicits chromatin compaction for the stability of stalling replication forks, Proc. Natl. Acad. Sci. USA 116 (29) (2019) 14563–14572.
[27] M.K. Ayrapetov, O.G. Yuzugullu, C. Xu, Y. Xu, B.D. Price, DNA double-strand breaks promote methylation of histone H3 on lysine 9 and transient formation of repressive chromatin, Proc. Natl. Acad. Sci. USA 111 (25) (2014) 9169–9174.
[28] R. Jossen, R. Bermejo, The DNA damage checkpoint response to replication stress: A Game of Forks, Front Genet 4 (2013) 26.
[29] T. Wilhelm, M. Said, V. Naim, DNA replication stress and chromosomal instability: dangerous liaisons, Genes (Basel) 11 (6) (2020) 642.
[30] Y. Chervona, M. Costa, Histone modifications and cancer: biomarkers of prognosis? Am. J. Cancer Res 2 (5) (2012) 589–597.
[31] D.M. Carone, J.B. Lawrence, Heterochromatin instability in cancer: from the Barr body to satellites and the nuclear periphery, Semin Cancer Biol. 23 (2) (2013) 99–108.

[32] G.K. Dialynas, M.W. Vitalini, L.L. Wallrath, Linking Heterochromatin Protein 1 (HP1) to cancer progression, Mutat. Res 647 (1–2) (2008) 13–20.
[33] J. Ellinger, P. Kahl, J.V.D. Gathen, S. Rogenhofer, L.C. Heukamp, I. Gütgemann, B. Walter, F. Hofstädter, R. Büttner, S.C. Müller, P.J. Bastian, A.V. Ruecker, Global levels of histone modifications predict prostate cancer recurrence, Prostate 70 (1) (2010) 61–69.
[34] Y. Okoyama, M. Hieda, Y. Nishioka, A. Matsumoto, S. Higashi, H. Kimura, H. Yamamoto, M. Mori, S. Matsuura, N. Matsuura, Cancer-associated upregulation of histone H3 lysine 9 trimethylation promotes cell motility in vitro and drives tumor formation in vivo, Cancer Sci. 104 (7) (2013) 889–895.
[35] R. Wu, P.B. Singh, D.M. Gilbert, Uncoupling global and fine-tuning replication timing determinants for mouse pericentric heterochromatin, J. Cell Biol. 174 (2) (2006) 185–194.
[36] J. Rustel, N. Kirstein, F. Izard, C. Grimaud, P. Prorok, C. Cayrou, G. Schotta, A. F. Abdelsamie, J. Déjardin, M. Méchali, G. Baldacci, C. Sardet, J.C. Cadoret, A. Schepers, E. Julien, Histone H4K20 tri-methylation at latefiring origins ensures timely heterochromatin replication, EMBO J. 36 (18) (2017) 2726–2741.
[37] N. Donley, M.J. Thayer, DNA replication timing, genome stability and cancer: late and/or delayed DNA replication timing is associated with increased genomic instability, Semin Cancer Biol. 23 (2) (2013) 80–89.
[38] Z. Sun, C. Huang, J. He, K.L. Lamb, X. Kang, T. Gu, W.H. Shen, Y. Yin, PTEN C-terminal deletion causes genomic instability and tumor development, Cell Rep. 6 (5) (2014) 844–854.
[39] W.H. Shen, A.S. Balajee, J. Wang, H. Wu, C. Eng, P.P. Pandolfi, Y. Yin, Essential role for nuclear PTEN in maintaining chromosomal integrity, Cell 128 (1) (2007) 157–170.
[40] C. Bassi, J. Ho, T. Srikumar, R.J. Dowling, C. Gorrini, S.J. Miller, T.W. Mak, B. G. Neel, B. Raught, V. Stambolic, Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress, Science 341 (6144) (2013) 395–399.
[41] J. Hang, Y.R. Lee, F. Dang, W. Gan, A.V. Menon, J.M. Katon, C.H. Hsu, J.M. Asara, P. Tibarewal, N.R. Leslie, Y. Shi, P.P. Pandolfi, W. Wei, PTEN Methylation by NSD2 Controls Cellular Sensitivity to DNA Damage, Cancer Disco 9 (9) (2019) 1306–1323.
[42] S. Misra, A. Mukherjee, P. Karmakar, Phosphorylation of PTEN at STT motif is associated with DNA damage response, Mutat. Res 770 (2014) 112–119.
[43] L. Zhao, R. Li, J.Z. Qiu, J.B. Yu, Y. Cao, R.T. Yuan, YY1-mediated PTEN dephosphorylation antagonizes IR-induced DNA repair contributing to tongue squamous cell carcinoma radiosensitization, Mol. Cell Probes 53 (2020), 101577.
[44] R.G. Syljuåsen, C.S. Sørensen, L.T. Hansen, et al., Inhibition of human CHK1 causes increased initiation of DNA replication, phosphorylation of ATR targets, and DNA breakage, Mol. Cell Biol. 25 (9) (2005) 3553–3562.
[45] M. Suzuki, T. Takahashi, Aberrant DNA replication in cancer, Mutat. Res 743–744 (2013) 111–117.
[46] M.J. O'Connor, Targeting the DNA damage response in cancer, Mol. Cell 60 (4) (2015) 547–560.
[47] A. Bowry, R.D.W. Kelly, E. Petermann, Hypertranscription and replication stress in cancer, Trends Cancer 7 (9) (2021) 863–877.
[48] M. Ming, Y.Y. He, PTEN in DNA damage repair, Cancer Lett. 319 (2) (2012) 125–129.
[49] A. Janssen, S.U. Colmenares, G.H. Karpen, Heterochromatin: Guardian of the Genome, Annu Rev. Cell Dev. Biol. 34 (2018) 265–288.
[50] S.G. Prasanth, Z. Shen, K.V. Prasanth, B. Stillman, Human origin recognition complex is essential for HP1 binding to chromatin and heterochromatin organization, Proc. Natl. Acad. Sci. USA 107 (34) (2010) 15093–15098.
[51] M. Fragkos, O. Ganier, P. Coulombe, M. Méchali, DNA replication origin activation in space and time, Nat. Rev. Mol. Cell Biol. 16 (6) (2015) 360–374.
[52] H. Zhang, L. Sun, K. Wang, D. Wu, M. Trappio, C. Witting, K. Cao, Loss of H3K9me3 Correlates with ATM Activation and Histone H2AX Phosphorylation Deficiencies in Hutchinson-Gilford Progeria Syndrome, PLoS One 11 (12) (2016), e0167454.
[53] J. Scorah, C.H. McGowan, Claspin and CHK1 regulate replication fork stability by different mechanisms, Cell Cycle 8 (7) (2009) 1036–1043, https://doi.org/10.4161/cc.8.7.8040.
[54] J. Puc, R. Parsons, PTEN loss inhibits CHK1 to cause double stranded-DNA breaks in cells, Cell Cycle 4 (7) (2005) 927–929.
[55] F. Vazquez, S. Ramaswamy, N. Nakamura, W.R. Sellers, Phosphorylation of the PTEN tail regulates protein stability and function, Mol. Cell Biol. 20 (14) (2000) 5010–5018.
[56] E.A. Vuono, A. Mukherjee, D.A. Vierra, et al., The PTEN phosphatase functions cooperatively with the Fanconi anemia proteins in DNA crosslink repair, Sci. Rep. 6 (2016) 36439, https://doi.org/10.1038/srep36439.
[57] A. Mukherjee, P. Karmakar, Attenuation of PTEN perturbs genomic stability via activation of Akt and down-regulation of RAD51 in human embryonic kidney cells, MolCarcinog 52 (8) (2013) 611–618.
[58] S. Misra, G. Ghosh, S.G. Chowdhury, P. Karmakar, Non-canonical function of nuclear PTEN and its implication on tumorigenesis [published online ahead of print, 2021 Jul 29], DNA Repair (Amst.) 107 (2021), 103197.
[59] X. Wang, L.C. Trotman, T. Koppie, A. Alimonti, Z. Chen, Z. Gao, J. Wang, H. E. Bromage, P. Tempst, C.C. Cardo, P.P. Pandolfi, X. Jiang, NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN, Cell 128 (1) (2007) 129–139.
[60] Y.R. Lee, M. Chen, J.D. Lee, J. Zhang, S.Y. Lin, T.M. Fu, H. Chen, T. Ishikawa, S. Y. Chiang, J. Katon, Y. Zhang, Y.V. Shulga, A.C. Bester, J. Fung, E. Monteleone, L. Wan, C. Shen, C.H. Hsu, A. Papa, J.G. Clohessy, J.T. Feldstein, S. Jain, H. Wu, L. Matesic, R.H. Chen, W. Wei, P.P. Pandolfi, Reactivation of PTEN tumor suppressor for cancer treatment through inhibition of a MYC-WWP1 inhibitory pathway, Science 364 (6441) (2019) eaau0159.
[61] H. Maccario, N.M. Perera, A. Gray, C.P. Downes, N.R. Leslie, Ubiquitination of PTEN (phosphatase and tensin homolog) inhibits phosphatase activity and is enhanced by membrane targeting and hyperosmotic stress, J. Biol. Chem. 285 (17) (2010) 12620–12628.
[62] P. Tibarewal, G. Zilidis, L. Spinelli, et al., PTEN protein phosphatase activity correlates with control of gene expression and invasion, a tumor-suppressing phenotype, but not with AKT activity, Sci. Signal 5 (213) (2012) 18.
[63] X.C. Zhang, A. Piccini, M.P. Myers, L. Van Aelst, N.K. Tonks, Functional analysis of the protein phosphatase activity of PTEN, Biochem J. 444 (3) (2012) 457–464.

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Review Article

Non-canonical function of nuclear PTEN and its implication on tumorigenesis

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ABSTRACT

Suppression of genomic instability is the key to prevent tumor development. PTEN is a unique tumor suppressor protein having both lipid and protein phosphatase activities. Interestingly though it is a cytoplasmic protein, but a significant pool of PTEN can also be localized in nucleus. The function of cytoplasmic PTEN is well defined and extensively studied in various literatures focusing mainly on the negative regulation of oncogenic PI-3Kinase-AKT pathway but functional regulation of nuclear PTEN is less defined and therefore it is a fascinating subject of research in cancer biology. Post-translation modulation of PTEN such as phosphorylation, sumorylation, acetylation and methylation also regulates its cellular localization, protein-protein association and catalytic function. Loss or mutation in PTEN is associated with the development of tumors in various tissues from the brain to prostate. Here we have summarized the role of nuclear PTEN and its epigenetic modulation in various DNA metabolic pathways, for example, DNA damage response, DNA repair, DNA replication, DNA segregation etc. Further, pathways involved in nuclear PTEN degradation are also discussed. Additionally, we also emphasize probable potential targets associated with PTEN pathway for chemotherapeutic purpose.

1. Introduction

Phosphatase and Tensin homolog deleted on chromosome ten (PTEN) is a unique and bona fide tumor suppressor protein that possesses both lipid and protein phosphatase activity. This protein has been identified simultaneously by two research groups in the year 1997 as tumor suppressor genes located at chromosome 10q23 in glioblastoma and prostate cancer cell lines [1,2]. Soon after its discovery, it has been found that the frequency of monoallelic mutations at this locus has been estimated at 50 %–80 % in sporadic endometrial carcinoma, and at 30 %–50 % in breast, colon, and lung carcinoma. Accumulation of PTEN germline mutations develops in a group of autosomal dominant syndromes characterized by various developmental disorder, neurological deficits, multiple hamartomas, and an increased risk of breast, thyroid, and endometrial cancers which are collectively referred to as the PTEN hamartoma tumor syndromes (PHTS), that includes Cowden syndrome, Lhermitte-Duclos disease, Bannayan-Riley-Ruvalcaba syndrome and Proteus and Proteus-like syndromes [3]. The latest list of COSMIC cancer database includes more than 2700 mutations in PTEN in 28 different tumor types, and the cBio portal of The Cancer Genome Atlas (TCGA) lists 1120 mutations in 27 tumor types. Considering the vast mutational diversity of PTEN in different tumor specimens, it is essential to understand the functional regulation of PTEN in cancer.

The crystal structure of PTEN (403 amino acid) revealed that it is a multi domain protein: N terminal phosphatase domain, the C2 domain and the C-terminal tail. C2 domain as well as 6 to 15 amino acid residues of N-terminus are involved in membrane localization through interaction with phospholipid PIP3 [3–5]. The C-terminal tail contains a number of phosphorylation sites at Serine 370, Serine 380, Threonine 382, Threonine 383, and Serine 385 residues. Interestingly phosphorylation of Ser 380, Thr 382, Thr 383 residues (collectively named STT motif) but not Ser 370 or Ser 385, increases the stability of PTEN and at the same time decreases its phosphatase activity [6]. It has been suggested that mutations or dephosphorylation of this STT cluster unwraps the protein conformation, making it less stable, but increases its phosphatase activity [6,7]. Thus STT motif of PTEN regulates its catalytic activity as well as its stability. Apart from C terminal phosphorylation sites, there are also multiple phosphorylation, sumorylation, acetylation and methylation sites which are important for PTEN nuclear functions (See Fig. 1 and Table 1). PTEN specifically dephosphorylates the D3

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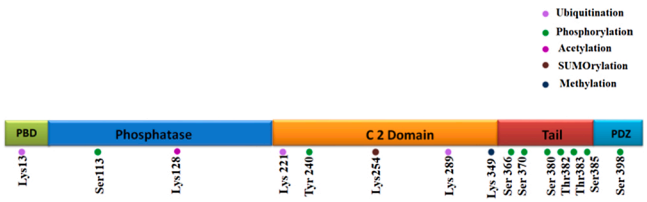

Fig. 1. Domain structure of PTEN with different post translational modulation sites which was reported to be involved in nuclear function.

Table 1
Table indicates different post translational modulation of PTEN which regulate its nuclear localization, chromatin association and DNA repair.

Modification of PTEN	Target site of PTEN	Enzyme responsible for modification	Cellular impact
Phosphorylation	Ser 380, 382, 383	PLK1, CK2, GSK3β	Chromatin associations, DNA repair
	Ser 366 and Thr 370	CK2, GSK3β	Chromatin associations
	Ser 385	PLK 1	Inhibits its nuclear entry
	Tyr 240	FGFR2	Associated with chromatin via Ki67 and recruits RAD51 to promote DNA repair
	Ser113	ATM Ser/Thr kinase	Nuclear accumulation of PTEN and induces autophagy
	Ser 398	ATM Ser/Thr kinase	DNA repair.
SUMOrylation	Lys254	SUMO1	DNA repair via HR Pathway
Methylation	Lys 349	NSD2	Interact with 53bp1 and involve in NHEJ pathway
Ubiquitination	Lys 289, Lys13 (Mono)	NEDD 4−1	Nuclear localization, DNA repair
	Lysine 221(Poly)	FBXO22	PTEN nuclear degradation
Acetylation	Lys128	PCAF	Recruit PCAF and CBP to the promoter of XLF and induces its expression.

position of phosphoinositide-3,4,5-trisphosphate, thus negatively regulates proto-oncogenic PI3K/Akt signaling pathway, which is key for cell survival, proliferation and growth [8]. PTEN significantly contributes in the regulation of cell cycle progression, apoptosis and cell migration. Mechanistically PTEN induces $p27^{kip}$ expression in vivo, which inhibits CDK2 activity and thus inhibits G1-S phase transition [9,10]. In the absence of PTEN, $P27^{kip}$ protein is ubiquitinated by SKP2 of Skp, Cullin, F-box containing complex (SCF^{SKP2}) [11]. Phosphatase activity of PTEN is also necessary for down regulation of cyclin D1 expression, thus preventing G1 transition [12]. Thus PTEN simultaneously down regulates cyclins, which are necessary for cell cycle progression and up-regulates CKI, which are essential for cell cycle arrest, implicating its immense influence on cell cycle control. It has been reported that PTEN interacts with P300 and maintains p53 in acetylated form, which increases PTEN-p53 interaction upon DNA damage. Acetylated p53 is important for G1 arrest [13]. Further PTEN depletion is associated with over expression of cdc6 and cyclin E2 in metastatic human prostate cancer. Decreased expression of cdc6 and cyclin E2 protein levels as a consequence of PTEN expression is associated with G1 arrest [14]. Upon genotoxic stress, PTEN null cell shows premature G2/M exit [15,16]. Mechanistically PTEN null causes hyper phosphorylation of Chk1 at Ser 280 by PI3K/AKT pathway which in turn abrogates G2 cell cycle arrest. Recent studies suggest that a significant pool of PTEN is also localized to nucleus though PTEN lacks classical nuclear localization signal. Partial or complete inactivation of PTEN is associated with radio resistance /chemo resistance attribute of tumor cells [17]. This phenotype is characterized by the ability of PTEN to suppress the formation or accumulation of the γH2AX foci, a well known marker of DNA damage, which may alternatively suggests that PTEN decreases DNA double strand break levels [17]. PTEN deficient cells exhibit an elevated level of chromosomal breaks, gap and aneuploidy either spontaneously or in response to DNA damage induced by various genotoxic agents [18]. Reduced level of ionizing radiation induced DNA repair in PTEN null cells has also been reported [19]. PTEN deficient cells exhibit multinucleated cell formation upon DNA damage which indicates aberrant mitotic entry with damaged DNA or defect in chromosomal segregation, or compromised spindle assembly checkpoint machinery [8]. In the last decade role of PTEN in DNA repair has been extensively studied in various literatures. Here we summarize recent advanced studies emphasizing on the role of PTEN in DNA metabolism.

2. Nuclear translocation of PTEN

As PTEN plays a significant role in DNA repair, a significant pool of PTEN resides in nucleus and loss of nuclear PTEN is closely associated with more aggressive cancers with high rate of genomic instability, making nuclear PTEN a useful prognostic indicator in some cancer types [20]. But there are no specific mechanisms for nuclear translocation of PTEN as it has no classical Nuclear Localization Signal (NLS). So multiple hypotheses exist about the nuclear localization including simple diffusion, protein-protein interaction and post translational modifications [21–24]. It has been reported that oxidative stress induced by H_2O_2 causes nuclear accumulation of phospho PTEN at Ser 380, which in turn interacts with p53 to augment cell cycle arrest. Xenograft studies further suggests that nuclear PTEN, specially p-PTEN at Ser 380, independent of its phosphatase activity, is sufficient to regulate tumorigenesis in vivo [21]. Takeo Minaguchi et al. suggested that PTEN nuclear transport is mediated by tyrosine phosphorylation-independent interaction with Major Vault Protein (MVP) where Ca(2+) concentration is important for PTEN –MVP interaction [22]. The nuclear transport receptor protein importin-11 physically interacts with PTEN, which prevents its degradation by ubiquitin-conjugating enzyme UBE2E1 and helps in nuclear translocation [23]. Reports suggested that monoubiquitination of PTEN at Lys 289 by NEDD 4−1 plays an important role in PTEN nuclear localization as PTEN K289R mutant is unable to enter nucleus and promotes tumorigenesis [24]. Growth factor receptor bound protein 2 (Grb2) binds to PTEN and assists its translocation into nucleus in response to oxidative stress [25]. In endometrial adenocarcinoma, nuclear PTEN accumulation is greatly diminished and aggravated DNA damage marker was observed. Thus it seems that deficient nuclear PTEN induces defective DNA damage response which is associated with tumor development [26]. Bassi et al. reported that the SUMOylation of PTEN at Lys254 is very important for nuclear retention and reduced within hours upon DNA damage. Also Sumo deficient PTEN is unable to repair DNA break as measured by γH2AX foci recovery assay [27]. But arguing with this observation another report shows that PTEN SUMOylation promotes its efficient chromatin removal after DNA damage by MMC, when compared with wild type PTEN [28]. Thus probable explanation may be that upon DNA damage, chromatin PTEN is removed by SUMOylation and accumulates in nucleoplasm. Then it may act as intermediated component of DNA repair pathway. Further studies are required to resolve, how Sumo-PTEN functions in DNA repair pathway. We previously reported that DNA damage induces phosphorylation of PTEN at

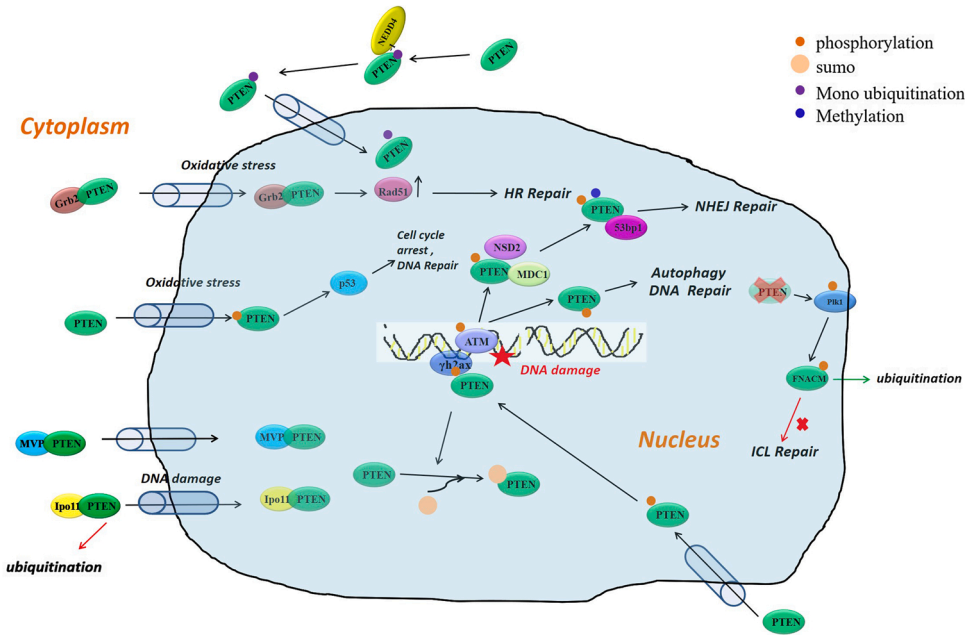

Fig. 2. Proposed different mechanisms of PTEN trafficking in the nucleus. In Oxidative stress PTEN interact with grb 2 or it get phosphorylated at Ser 380 and translocate to nucleus. DNA damage also causes PTEN translocation in nucleus which is governed by Ser 380 phosphorylation. Ipo11 interacts with PTEN and prevents its ubiquitination in cytoplasm and helps in translocation in nucleus. PTEN also get monoubiquitinated at Lys 289 by NEDD4 in cytoplasm and monoubiquitinated PTEN efficiently translocates in nucleus. PTEN also enter in to nucleus by major vault protein mediated interaction. Upon entering into nucleus, PTEN induces the expression of RAD 51, p53 and facilitated cell cycle arrest and HR mediated DNA repair. Phosphorylated PTEN also attached to DNA damage sites alongside with ATM and gamma H2AX, also it get methylated by NSD2 and facilitate 53bp1 mediated NHEJ repair. Sumolrylation also causes PTEN to enter in nucleus and take part in HR repair pathway. Nuclear PTEN also prevent polyubiquitination of FANCM by dephosphorylation and thus involves ICL repair pathway. Thus PTEN nuclear translocation and function depends on its different post translational modifications.

STT motif and which in turn accumulates in nucleus. Further phosphorylation dependent nuclear foci formation of PTEN was also observed after ionizing radiation or etoposide treatment which colocalized with γH2AX [29]. Accordingly, it has been found that PTEN C-terminal (189–403) deletion causes genomic instability and common fragile site re-arrangement in mouse model. Mice heterozygous for PTEN C-terminal deletion also developed multiple spontaneous tumors, and B cell lymphoma [30]. Another report suggested that topotecan (TPT), a topoisomerase I inhibitor, and cisplatin treatment activates ATM Ser/Thr kinase, which induces PTEN phosphorylation at Ser113 residue. This phosphorylation causes nuclear accumulation of PTEN and induces autophagy [31]. ATM mediated PTEN phosphorylation at Ser 398 has also been reported after etoposide induced DNA damage [27]. Going further depth in the molecular mechanism, Jinfang Zhang et al. found that upon DNA damage phospho PTEN forms a ternary complex with MDC1 and NSD2 and promotes NSD2-mediated di-methylation of PTEN at Lys 349. Then methylated PTEN interacts with 53BP1 and recruited to DNA damage sites, initiate DNA repair process partly through de phosphorylating γH2AX via NHEJ pathway [32]. Additionally, it has been reported that PTEN plays an important role in ICL repair. Here PTEN dephosphorylates FANCM and thus prevents it from polyubiquitination. PTEN deficient cells show elevated PLK1 kinase mediated phosphorylation of FANCM, which causes constitutive FANCM polyubiquitination. Thus the assembly of the FA core complex, FANCD2, and FANCI into DNA repair foci is compromised [28]. Therefore PTEN translocation in nucleus is a complex physiologically stimulated phenomenon and largely controlled by post translational modulation. Proposed mechanism PTEN nuclear translocation is summarized in Fig. 2. As in various stress conditions, PTEN accumulates in nucleus, thus it seems that PTEN acts as stress responder which coordinates between external stimulus and in vivo response, aiming to preserve genomic stability.

3. Chromatin association of PTEN depends on its phosphorylation at various sites

As various reports illustrate that PTEN is an important member of DNA repair proteins, so PTEN may be associated with chromatin. Several mechanistic studies have been carried out to focus on the association of PTEN with chromatin. BH Choi and his colleague reported that PTEN became associated with chromatin upon DNA damage and phosphorylation of Ser 366 and Thr 370 of PTEN is essential for this attachment [33]. Further they have found that C2 domain is also responsible for the nuclear translocation of PTEN. After attachment PTEN regulates sumorylation of Rad 52 and thus participates in DNA damage response pathway. Additionally, it has been reported that PLK1 phosphorylates PTEN at Ser 380 in vivo, which is also essential for association of PTEN with chromatin [34–36]. p-PTEN at Ser 380 is important for normal mitotic progression as replacing Ser 380 by Ala resulted in a significant reduction of mitotic cells [36]. As previously reported that PTEN interacts with centromere as well as PLK1 [36,37], thus it may be pertinent to state that phosphorylation of PTEN by PLK1 is an upstream event as C-terminal truncated PTEN is unable to interact with centromere [38]. PTEN can also dephosphorylates PLK1 and suppress polyploid cell populations [34]. Thus phosphorylated PTEN in chromatin causes mitotic arrest whereas soluble PTEN dephosphorylates PLK1 and causes resistance to uncontrolled genome segregation (Fig. 3A). As phosphorylated PTEN has reduced phosphatase activity so in chromatin, it can't dephosphorylates PLK1. So it is tempting to speculate the existence of some mechanisms in cells to dissociate PTEN from chromatin which we will discuss later. But another study suggested that PLK 1 phosphorylates PTEN at Ser 385 and inhibits its nuclear entry [35] rendering promotion of tumor development. Recently Ma et al. also reported that PTEN phosphorylation is associated with chromatin and DNA repair [39,40]. They have identified Tyrosine 240 residue which gets phosphorylated upon IR induced DNA damage by Fibroblast Growth Factor Receptor 2 (FGFR2). Upon phosphorylation, it becomes

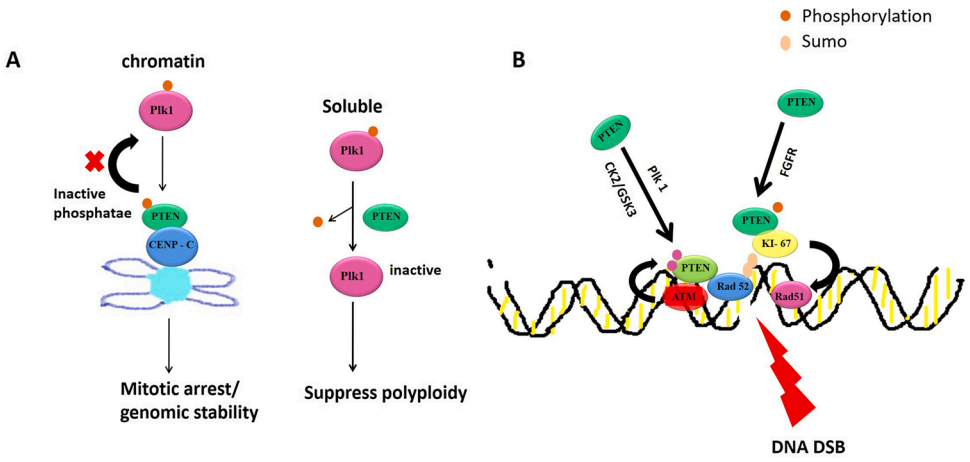

Fig. 3. A. Proposed model of PTEN and PLK 1 functional crosstalk by phosphorylation in maintaining genomic stability. Chromatin PTEN is phosphorylated by phospho-PLK1, associated with CENP-C centromeric protein and maintained genomic stability. As phospho-PTEN is partially inactive phosphatase so its dephosphorylation activity is reduced. In soluble form PTEN dephosphorylates PLK1 and thus inactivate it. Hyper phosphorylation of PLK 1 causes anuploidy. B. PTEN chromatin association is controlled by phosphorylation. Upon DNA damage PTEN gets phosphorylated either by PLK1, CK2, GSK3β or ATM at different phosphorylation site. Then it promotes RAD 52 sumorylation. FGFR also phosphorylated PTEN and in turn helps in RAD51 recruitment at DNA damage site and promotes DNA repair.

associated with chromatin via Ki67 and recruits RAD51 to promote DNA repair in glioblastoma cells and promotes survival (Fig. 3B). Thus inhibition of FGFR 2 may be useful for the radiosensitization of glioblastoma [39,40].

4. PTEN maintained chromatin architecture

Apart from chromatin association, PTEN prevents centromere breakage and anuploidy by stabilizing the centromeric regions. PTEN-centromere interaction maintains chromosomal stability, possibly by modulating homologous recombination [9,28,37,38]. PTEN localizes and physically interacts with CENP-C centromeric protein independent of its phosphatase activity but depends on its C-terminal tail [38]. It has been found that PTEN maintains heterochromatin status of chromatin by interacting and stabilizing HP1α. Further in Mouse Embryonic Fibroblast cells, PTEN knockout led to a significant reduction in H3K9me3 foci intensity which is indicative of de-condensation of the heterochromatin [41]. Again Zhu Hong Chen et al. reported that loss of PTEN leads to dissociation of histone H1 from chromatin and consequently elevated level of histone H4 acetylation at lysine 16, an epigenetic marker for chromatin activation was observed [42]. They have also been found that PTEN physically interacts with histone H1 through their C-terminal domains as truncated PTEN is unable to interact with histone H1. Compromised heterochromatin could lead to broad epigenetic instability, which with a potential impact on gene expression profiles may ultimately lead to abnormal growth characteristics [43]. Disruption of the epigenetic signature such as H3K9me3, which is associated with late replicating heterochromatin, promotes chromatin accessibility, advancing replication timing and leading to re-replication [44]. So heterochromatin maintenance is important for genomic stability of cancer cells which is closely associated with replication initiation. Apart from replication, loss of heterochromatin also positively influences global gene transcription which is another characteristic of PTEN null cells. Thus PTEN maintains chromatin in an inactive condensed form and thus prevents unregulated transcription as well as replication.

5. PTEN involve in both HR and NHEJ

The foremost mechanism that eukaryotic cell carries out to repair its own damaged DNA by either Homologous Recombination (HR) or Non Homologous End Joining (NHEJ). HR is more specific and error less mechanism that occurs predominantly in S phase where NHEJ is error prone and occurs throughout cell cycle. Compromising either or both of these pathways lead to genomic instability and accumulation of cancer driven mutations [45–47]. In vivo plasmid based assay exploit by multiple research groups suggests that PTEN depletion diminished homologous recombination repair frequency. When they ectopically transfect

Table 2
Role of PTEN in DNA double strand break repair.

Homologous recombination	Non Homologous End Joining
1. PTEN physically interact with Rad 51 on chromatin. PTEN also regulates Rad 51 transcription by encompassing Rad 51promoter or by regulating E2F1mediated Rad 51 transcription	1. Acetylated PTEN at Lys 128 localize in NHEJ1 promoter and recruits the histone acetyltransferases, PCAF,CBP. It also induces the expression of XRCC4-like factor (XLF) which bridges and ligates DNA ends by NHEJ. 2.PTEN reduces error prone NHEJ pathway by poly(ADP-ribosyl)ation of Ku70 and prevents binding of Ku 70 to DNA DSB. 3.NSD2-mediated di-methylation of phospho PTEN at Lys 349 causes interaction with 53BP1 thus regulate DNA repair via NHEJ pathway. 4.PTEN increases H3K9me3 expression, thus positively regulate NHEJ by 53BP1 mediated pathway.

PTEN in PTEN null cells, HR frequency significantly up regulated [27,48,49]. Mechanistically PTEN up regulates Rad 51 transcription by encompassing Rad 51 promoter or by modulating chromatin so that E2F1 direct Rad 51 transcription [38]. But another report suggests that PTEN C–terminal phospho mutant physically interacts with E2F1 and represses transcription at the promoter site of various gene [50]. Another report shows that after ionizing radiation induced DNA damage, Rad51 protein expression was significantly up-regulated in Wt-PTEN and phosphatase dead PTEN but not in sumo deficient PTEN transfected cells [51]. Recent studies reveales that PTEN augments NHEJ repair pathway through direct induction of expression of XRCC4-like factor (NHEJ1/XLF) which functions in DNA end bridging and ligation. Again acetylated PTEN at Lys128 interacts and recruits the histone acetyltransferases, PCAF and CBP to the promoter of XLF and induces its expression [52]. But another report suggested that nuclear PTEN reduces error prone NHEJ pathway by poly(ADP-ribosyl)ation of Ku70 and prevents binding of Ku 70 to DNA DSB. Thus DNA PKcs unable to bind DNA broken end to execute repair process [51]. They further shows that phosphatase activity is not required rather sumorylation of PTEN is important for this activity. At Table 2 role of PTEN in HR and NHEJ is summarized. Again H3K9me3 represents epigenetic marker that are important for the function of the 53BP1 protein mediated non-homologous end joining (NHEJ) [53,54]. As PTEN loss causes chromatin disorganization and compromised H3K9me3 protein expression and foci formation, so PTEN may indirectly positively regulate NHEJ by 53BP1 mediated pathway. Thus this PTEN-H3K9-me3−53BP1 axis should be studied elaborately to get insight about the

mechanism. Thus mechanistic role of PTEN and its post translational modulation in regulating NHEJ repair pathway is needed to be explored further which will help in understanding the therapeutic approach for targeting NHEJ pathway in cancer cells.

6. Replicative function of PTEN

Mutation appears in the genome if cells are unable to repair its DNA and replicate with unrepaired DNA due to checkpoint inaccuracy. Thus daughter cells acquire mutations from parental cells. Controlling and sensing DNA damage during replication or replication stress is an Achilles' heel and must be tightly regulated to prevent DNA replication progression, otherwise it causes a serious threat to genomic stability leading to cancer development. De-regulation of DNA replication occurs when cells are unable to overcome stress successfully and may initiate uncontrolled initiation, promoting DNA damage and genomic instability. As PTEN null or PTEN mutant cells shows high growth potential with unstable genome, so PTEN may function in replication machinery to maintain the integrity of genome during replication. There are number of reports suggest that PTEN indeed functions in DNA replication and which may be dependent on its post translational modulation. At first, it has been observed in 2015 by two different research groups that PTEN null cells show higher frequency of anaphase bridges and lagging chromosomes as compared to wild type cells indicating replication defect [55,56]. Further study reveals that PTEN null cells show compromised replication progression which indicates endogenous replication stress. Replication fork restart after aphidicolin/Hydroxyurea treatment is largely impeded in PTEN null cells compared to wild type cells. Molecular iPOND study showed that PTEN itself localized at stalled fork induced by HU and recruits Rad 51 on chromatin to promote its successful recovery [56]. Following replication stress, replication protein A (RPA1) binds to the single-strand DNA adjacent to newly synthesized double-stranded DNA. The purpose of RPA1 loading is to protect single naked strand, halt fork movement and preparing cells to overcome stress [57]. It has been reported that PTEN directly binds to C-terminal domain of RPA1 and promotes RPA1 accumulation on the replication fork. PTEN also recruits the deubiquitinase OTUB1 and prevents RPA1 ubiquitination and thus stabilizes RPA1 [58]. Further phosphatase activity of PTEN is not required to stabilize RPA1 at replication fork. Almost at the same time another report suggested that PTEN is involved in restriction of the replication fork rate movement in response to exogenous replication stress through its phosphatase activity. They have found that PTEN physically associated with and dephosphorylates mini-chromosome maintenance 2 (MCM2) protein at Ser 41 residue and prevents replication fork progression upon HU induced replication stress [59]. Our unpublished Brdu pulse experimental data shows that significantly higher percentage of Brdu positive cells appeared in PTEN null cells compared to Wt–PTEN transfected cells upon aphidicolin induced replication stress, indicating unperturbed DNA replication during Aph treatment in PTEN null cells. Thus PTEN null cells unable to sense replication stress and continue to grow with damaged DNA as we found gamma H2AX foci persists along Brdu foci. PTEN transfection greatly diminished this phenotype. So all these reports suggest that during replication stress PTEN dephosphorylates MCM2 helicase on one hand and recruits RPA1 on ssDNA generated due to uncoupling of polymerase on helicase ahead of replication fork on the other and this is followed by recruitment of RAD 51 for successful fork recovery. RPA-coated ssDNA serves as a common intermediate structure necessary for activation of the intra-S checkpoint [60]. Thus PTEN maintains intra S phase checkpoint integrity. Further when replication stress is removed PTEN promotes stalled fork recovery. Additionallly, PTEN null cell unable to dephosphorylates MCM 2 and RPA1 loading is compromised as a result, replicative DNA damage accumulates that induce genomic instability. We diagrammatically explain the current understanding of functional involvement of PTEN in DNA replication in Fig. 4.

7. PTEN regulate mitotic spindle integrity and Architecture

In eukaryotic organisms, mitosis is an essential part of the cell cycle where replicated chromosomes are segregated into daughter nuclei. Mitosis should be carried out in a continuous and proportionate manner for the successful transmission of genetic informations to the progeny. Mitosis requires a regulatory mechanism in which mitotic spindles play the most essential role [61]. Mitotic spindle is the cytoskeleton structure, comprises of microtubules, microtubule dependent motor proteins and non-motor microtubule binding proteins [62]. As previously mentioned, PTEN null cells show various chromosomal damage including centrosome amplification, aneuploidy, lagging chromosome, anaphase bridge, micronucleus, chromosomal bud [9,28,37,38,52,56]. PTEN–AKT pathway controls the centrosome composition and integrity during mitosis. PTEN is associated with PLK1 [34–36] and γ tubulin at centrosome and maintain centrosome integrity [37]. Knockdown of PTEN reduces centrosomal level of pericentrin in an Akt independent manner. But when Akt is inhibited, centrosomal level of PLK1 and γ tubulin is reduced and recruitment of PTEN at centrosome is compromised, thus increased defect in centrosome number and metaphase separation is observed [37]. PTEN also act as a component of mitotic checkpoint complex which is essential to sense defective kinetochor attachment during metaphase. If microtubule–kinetochore attachment

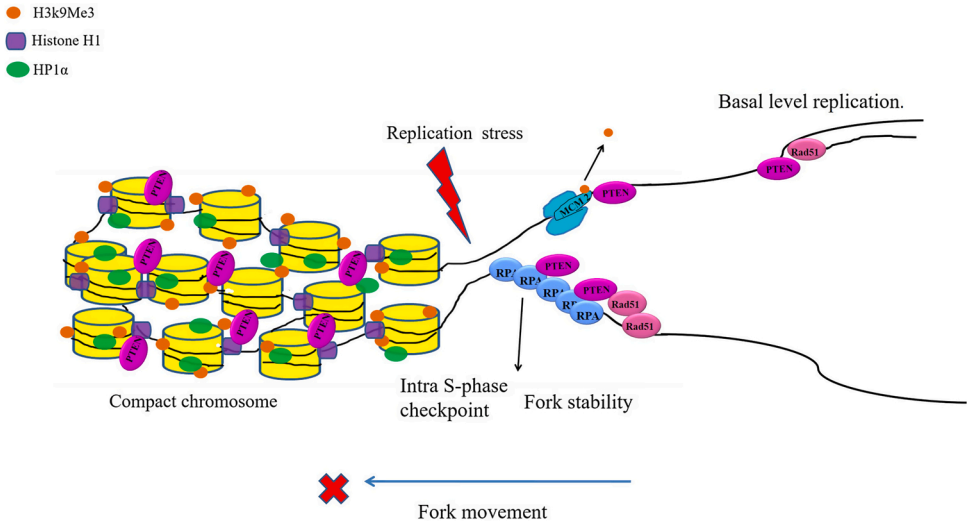

Fig. 4. PTEN localized to replication fork and stabilized it in stress condition. In response to replication stress, PTEN interacts with MCM2 and dephosphorylates it. Thus prevent replication fork progression in stress condition. PTEN also interacts with single-strand DNA (ssDNA) binding protein RPA1 to stabilize DNA replication forks and recruits Rad51 to promote reactivation of stalled forks when stress was relieved. Further PTEN maintain heterochromatin signature of chromatin and it may further prevent fork progression.

is compromised, Spindle Assemble Checkpoint(SAC)/Mitotic Checkpoint Complex(MCC) comes to its function and halts anaphase so that attachment occurs properly. PTEN physically interacts with Mad1 protein and promotes its dimerization, localization in the nuclear envelope and maintains SAC timing [63]. PTEN null cells show significant reduction in M phase compared to WT cells indicating SAC arrest [34, 36]. Another report suggested that PTEN physically interacts with Cdc20 and Mad2, two important components of MCC [64]. PTEN dependent recruitment of a scaffold protein Dlg1 was observed at centrosome as PTEN knockdown impaired Dlg1 recruitment. Mechanistically PLK1 phosphorylates PTEN at C- terminal tail and recruits it to centrosome. This creates docking site for attachment of dlg1 and phospho-EG5 Ser1033, Thr926 (induced by NEK6, Cdk1) [65]. EG5 belongs to the super family of kinesin motor proteins that are responsible for centrosome movement, proper maintenance and assembly of mitotic spindles by rearranging the microtubules [66]. But another report suggests that PTEN deficiency induces aberrant EG5 phosphorylation and abrogates EG5 recruitment to the mitotic spindle apparatus, leading to spindle disorganization. PTEN physically interacts with EG5 at centrosome/spindle pole and dephosphorylates it at Thr 926 residue and helps to station them at spindle pole [67]. These conflicting reports suggests that delicate maintenance of the phosphorylation status of EG 5 is important for the efficient functioning of motor proteins. Thus EG5 phosphorylation must be precisely regulated as erroneous mitosis arises from either hypo or hyper-phosphorylation of EG5. It seems that EG5 phosphorylation is required for the formation of the PTEN-Dlg1-EG5 complex on centrosomes where hyper-phosphorylation of EG5 impairs its PTEN-mediated maintenance of the mitotic spindle length. For example, hyper-phosphorylation of EG5 at Thr 926 in PTEN-deficient cell results in a reduced affinity to spindle microtubules and impairs the spindle architecture [67].

8. Mechanism of the loss of nuclear PTEN

Form the above discussion it is clear that PTEN translocates in to the nucleus to preserve genomic stability by various mechanisms including DNA repair by HR or NHEJ, DNA replication and chromosome segregation. Further various post translational modulation including phosphorylation and sumorylation of PTEN is essential for maintaining the integrity of the genome. It has been reported that in human endometrial tumors, significant nuclear PTEN was lost when compared with normal endometrial tissues [26]. Nuclear PTEN localization is also positively correlated to tumor suppression in primary cutaneous melanoma [68] and colorectal cancer [69]. It is essentially clear that loss of nuclear PTEN causes indigenous DNA damage as well as unsuccessful DNA repair and compromised S phase checkpoint after genotoxic insult which is the initial step of cancer predisposition. Thus the mechanism of degradation of nuclear PTEN and prevention of nuclear entry of PTEN is important to study. Though cytoplasmic PTEN degradation is extensively studied in several literatures but very few studies have been carried out so far regarding nuclear PTEN degradation. One of the four subunits of the ubiquitin protein ligase complex, F-box only protein 22 (FBXO22) is over expressed in various cancer types including colorectal cancer and this induces ubiquitylation of nuclear PTEN at lysine 221 followed by proteasome-mediated degradation of nuclear PTEN [70]. PTEN C–terminal phospho mutant more efficiently interacts with FBXO22 than Wt-PTEN, indicating that though phospho mutant PTEN accumulates in nucleus, but due to degradation it is unable to take its journey towards chromatin. Another report supports this hypothesis where they found that phospho mutant PTEN efficiently interacts with APC–Cdh1 and promotes its degradation. Phosphorylation of PTEN at Ser-380 impaired its interaction with Cdh1, thus positively regulating PTEN stability on chromatin [71]. It has been also reported that phospho-PTEN status is significantly reduced in ATM null cells and promotes its degradation. ATM deficiency resulted in the accumulation of XIAP/p-XIAP levels which ubiquitinated PTEN and Casein kinase 2 [72]. Interestingly we found that p-PTEN co-localized with pATM upon DNA damage indicates close proximity between them. Importin-11 a nuclear import receptor, interacts with PTEN and facilitates its nuclear import. Knockdown of Importin-11 drastically reduces nuclear PTEN. Functionally Importin-11 prevents PTEN degradation by two mechanisms- (1) by sequestering ubiquitinated PTEN that has been primed for cytoplasmic polyubiquitination and degradation and (2) by importing the activated Ub-loaded UBE2E1, a E2 ubiquitin- conjugating enzyme, thus restricting its ability to prime PTEN for degradation in the cytoplasm [23].

9. Conclusion and prospect

Latest understanding of the functional aspect of PTEN reveals that it is not confined in cytoplasm to down regulate PI3-Kinase pathway but maintains genomic stability in nucleus by multiple mode of mechanism as discussed above. Nuclear function of PTEN is also governed by epigenetic regulation e.g phosphorylation, sumorylation, acetylation and ubiquitination at the various sites from N terminal to C-terminal end. Both phosphatase dependent and independent function of PTEN was reported to maintain genomic stability. Phosphorylation of C terminal Ser/Thr residue of PTEN reduces its phosphatase function but simultaneously phosphorylation at this site and other residue increases the affinity of PTEN to chromatin. Probable explanation may be given as phospho PTEN is a stable form and prerequisite stability is required for functioning of PTEN on phosphatase dependent manner [34]. Phosphatase dead PTEN unable to control cell proliferation as it causes up regulation of MCM2 and AKT phosphorylation but able to initiate DNA damage response (Rad 51 mediated) and survive with genomic instability after DNA damage. Thus PTEN null cells show extensive proliferation with unrepaired DNA. Again PTEN phosphorylation mutant unable to attached with chromatin, which may contributes for genomic instability. Thus it may be interesting to observe different epigenetic modification of PTEN and their chromatin association in response to DNA damage. This may additionally reveal how different epigenetic modulation of PTEN is associated with DNA repair-sub pathways. It has recently been reported that the phosphorylated PTEN inhibits its ubiquitination in vivo and in vitro by NEDD4−1 and WWP ubiquitin ligase. But poly ubiquitination of PTEN greatly reduces PTEN phosphatase activity [73]. This conflicting data indicates that PTEN phosphorylation tightly and delicately regulates PTEN phosphatase activity and maintains homeostasis in vivo perhaps through ubiquitination. So there may be fine regulation between PTEN phosphatase activity and PTEN phosphorylation for proper functioning. Further phosphatase activity of PTEN is required for its own de-phosphorylation at C-terminal tail [74, 75] which may transiently activate phosphates activity. PTEN involves in both HR and NHEJ repair pathways but exact mechanism is not clearly understood. In NHEJ pathway, binding to DNA end cause auto-phosphorylation of DNA PKcs at number of sites which causes dissociation of DNA PK complex from DNA end. Phosphorylation of Ku by DNA-PKcs results in the dissociation of the Ku heterodimer from the forked dsDNA and the subsequent free dsDNA ends are available for Exo1-mediated DNA end resection. So instant phosphorylation of this protein must be prevented by some mechanism otherwise NHEJ pathway must be compromised which causes genetic instability. Obviously, by the end of the repair process, either the phosphorylated protein has to be degraded or the phosphate group must be released. Although the (auto) phosphorylation activity of DNA-PK has been shown to be essential for repair of both random double-strand breaks, the corresponding phosphatase has been elusive. As PTEN is phosphatase and involved in DNA repair pathway so it is quite possible that PTEN may be involved in this process. Factors and specific mechanisms that are required for the dissolution of NHEJ complex once repair is completed remain to be studied. Thus the mechanistic role of PTEN and its post translational modulation in regulation NHEJ repair pathway is needed to be explored, which will help in understanding the therapeutic

approach for targeting NHEJ pathway in cancer cells. Further how chromatin modulation in PTEN null cells influence NHEJ repair pathway will also be an interesting aspect of research.

Declaration of Competing Interest

All authors declare that there are no conflicts of interest.

Acknowledgments

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References

[1] J. Li, C. Yen, D. Liaw, et al., PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer, Science 275 (5308) (1997) 1943–1947, https://doi.org/10.1126/science.275. 5308.1943.
[2] D.H. Teng, R. Hu, H. Lin, et al., MMAC1/PTEN mutations in primary tumor specimens and tumorcell lines, Cancer Res. 57 (23) (1997) 5221–5225.
[3] L. Salmena, A. Carracedo, P.P. Pandolfi, Tenets of PTEN tumor suppression, Cell 133 (3) (2008) 403–414, https://doi.org/10.1016/j.cell.2008.04.013.
[4] J.O. Lee, H. Yang, M.M. Georgescu, et al., Crystal structure of the PTEN tumor suppressor:implications for its phosphoinositide phosphatase activity and membrane association, Cell 99 (3) (1999) 323–334, https://doi.org/10.1016/s0092-8674(00)81663-3.
[5] B.D. Hopkins, R.E. Parsons, Molecular pathways: intercellular PTEN and the potential of PTEN restoration therapy, Clin. Cancer Res. 20 (21) (2014) 5379–5383, https://doi.org/10.1158/1078-0432.CCR-13-2661.
[6] F. Vazquez, S. Ramaswamy, N. Nakamura, W.R. Sellers, Phosphorylation of the PTEN tail regulates protein stability and function, Mol. Cell. Biol. 20 (14) (2000) 5010–5018, https://doi.org/10.1128/mcb.20.14.5010-5018.2000.
[7] N.R. Leslie, C.P. Downes, PTEN function: how normal cells control it and tumour cellslose it, Biochem. J. 382 (2004) 1–11, https://doi.org/10.1042/BJ20040825.
[8] A. Mukherjee, S. Misra, N.G. Howlett, P. Karmakar, Multinucleation regulated by theAkt/PTEN signaling pathway is a survival strategy for HepG2 cells, Mutat. Res. 755 (2) (2013) 135–140, https://doi.org/10.1016/j.mrgentox.2013.06.009.
[9] A. Di Cristofano, M. De Acetis, A. Koff, C. Cordon-Cardo, P.P. Pandolfi, Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse, Nat. Genet. 27 (2) (2001) 222–224, https://doi.org/10.1038/84879.
[10] A.R. Ottschalk, D. Basila, M. Wong, et al., p27Kip1 is required for PTEN-induced G1 growth arrest, Cancer Res. 61 (5) (2001) 2105–2111.
[11] J.H. Jonason, N. Gavrilova, M. Wu, H. Zhang, H. Sun, Regulation of SCF(SKP2) ubiquitin E3 ligase assembly and p27(KIP1) proteolysis by the PTEN pathway and cyclin D1, Cell Cycle 6 (8) (2007) 951–961, https://doi.org/10.4161/cc.6.8.4104.
[12] A. Radu, V. Neubauer, T. Akagi, H. Hanafusa, M.M. Georgescu, PTEN induces cell cycle arrest by decreasing the level and nuclear localization of cyclin D1, Mol. Cell. Biol. 23 (17) (2003) 6139–6149, https://doi.org/10.1128/mcb.23.17.6139-6149.2003.
[13] A.G. Li, L.G. Piluso, X. Cai, G. Wei, W.R. Sellers, X. Liu, Mechanistic insights into maintenance of high p53 acetylation by PTEN, Mol. Cell 23 (4) (2006) 575–587, https://doi.org/10.1016/j.molcel.2006.06.028.
[14] Z. Wu, H. Cho, G.M. Hampton, D. Theodorescu, Cdc6 and cyclin E2 are PTEN-regulated genes associated with human prostate cancer metastasis, Neoplasia 11 (1) (2009) 66–76, https://doi.org/10.1593/neo.81048.
[15] J. Puc, M. Keniry, H.S. Li, et al., Lack of PTEN sequesters CHK1 and initiates genetic instability, Cancer Cell 7 (2) (2005) 193–204, https://doi.org/10.1016/j.ccr.2005.01.009.
[16] W.Y. Mansour, P. Tennstedt, J. Volquardsen, et al., Loss of PTEN-assisted G2/M checkpoint impedes homologous recombination repair and enhances radio-curability and PARP inhibitor treatment response in prostate cancer, Sci. Rep. 8 (1) (2018), https://doi.org/10.1038/s41598-018-22289-7, 3947.
[17] G. Pappas, L.A. Zumstein, A. Munshi, M. Hobbs, R.E. Meyn, Adenoviral-mediated PTEN expression radiosensitizes non-small cell lung cancer cells by suppressing DNA repair capacity, Cancer Gene Ther. 14 (6) (2007) 543–549, https://doi.org/10.1038/sj.cgt.7701050.
[18] G.D. Kao, Z. Jiang, A.M. Fernandes, A.K. Gupta, A. Maity, Inhibition of phosphatidylinositol-3-OH kinase/Akt signaling impairs DNA repair in glioblastoma cells following ionizing radiation, J. Biol. Chem. 282 (29) (2007) 21206–21212, https://doi.org/10.1074/jbc.M703042200.
[19] M. Ming, Y.Y. He, PTEN in DNA damage repair, Cancer Lett. 319 (2) (2012) 125–129, https://doi.org/10.1016/j.canlet.2012.01.003.
[20] O. Gimm, A. Perren, L.P. Weng, et al., Differential nuclear and cytoplasmic expression of PTEN in normal thyroid tissue, and benign and malignant epithelial thyroid tumors, Am. J. Pathol. 156 (5) (2000) 1693–1700, https://doi.org/10.1016/S0002-9440(10)65040-7.
[21] C.J. Chang, D.J. Mulholland, B. Valamehr, S. Mosessian, W.R. Sellers, H. Wu, PTEN nuclear localization is regulated by oxidative stress and mediates p53-dependent tumor suppression, Mol. Cell. Biol. 28 (10) (2008) 3281–3289, https://doi.org/10.1128/MCB.00310-08.
[22] T. Minaguchi, K.A. Waite, C. Eng, Nuclear localization of PTEN is regulated by Ca(2+) through a tyrosil phosphorylation-independent conformational modification in major vault protein, Cancer Res. 66 (24) (2006) 11677–11682, https://doi.org/10.1158/0008-5472.CAN-06-2240.
[23] M. Chen, D.G. Nowak, N. Narula, et al., The nuclear transport receptor Importin-11 is a tumor suppressor that maintains PTEN protein, J. Cell Biol. 216 (3) (2017) 641–656, https://doi.org/10.1083/jcb.201604025.
[24] L.C. Trotman, X. Wang, A. Alimonti, et al., Ubiquitination regulates PTEN nuclear import and tumor suppression, Cell 128 (1) (2007) 141–156, https://doi.org/10.1016/j.cell.2006.11.040.
[25] B. Hou, S. Xu, Y. Xu, et al., Grb2 binds to PTEN and regulates its nuclear translocation to maintain the genomic stability in DNA damage response, Cell Death Dis. 10 (8) (2019), https://doi.org/10.1038/s41419-019-1762-1763, 546.
[26] A. Mukherjee, A.L. Patterson, J.W. George, et al., Nuclear PTEN localization contributes to DNA damage response in endometrial adenocarcinoma and could have a diagnostic benefit for therapeutic management of the disease, Mol. Cancer Ther. 17 (9) (2018) 1995–2003, https://doi.org/10.1158/1535-7163.MCT-17-1255.
[27] C. Bassi, J. Ho, T. Srikumar, et al., Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress, Science 341 (6144) (2013) 395–399, https://doi.org/10.1126/science.1236188.
[28] E.A. Vuono, A. Mukherjee, D.A. Vierra, et al., The PTEN phosphatase functions cooperatively with the Fanconi anemia proteins in DNA crosslink repair, Sci. Rep. 6 (2016) 36439, https://doi.org/10.1038/srep36439.
[29] S. Misra, A. Mukherjee, P. Karmakar, Phosphorylation of PTEN at STT motif is associated with DNA damage response, Mutat. Res. 770 (2014) 112–119, https://doi.org/10.1016/j.mrfmmm.2014.08.008. December.
[30] Z. Sun, C. Huang, J. He, et al., PTEN C-terminal deletion causes genomic instability and tumor development, Cell Rep. 6 (5) (2014) 844–854, https://doi.org/10.1016/j.celrep.2014.01.030.
[31] J.H. Chen, P. Zhang, W.D. Chen, et al., ATM-mediated PTEN phosphorylation promotes PTEN nuclear translocation and autophagy in response to DNA-damaging agents in cancer cells, Autophagy 11 (2) (2015) 239–252, https://doi.org/10.1080/15548627.2015.1009767.
[32] J. Zhang, Y.R. Lee, F. Dang, et al., PTEN methylation by NSD2 controls cellular sensitivity to DNA damage, Cancer Discov. 9 (9) (2019) 1306–1323, https://doi.org/10.1158/2159-8290.CD-18-0083.
[33] B.H. Choi, Y. Chen, W. Dai, Chromatin PTEN is involved in DNA damage response partly through regulating Rad52 sumoylation, Cell Cycle 12 (21) (2013) 3442–3447, https://doi.org/10.4161/cc.26465.
[34] Z. Zhang, S.Q. Hou, J. He, T. Gu, Y. Yin, W.H. Shen, PTEN regulates PLK1 and controls chromosomal stability during cell division, Cell Cycle 15 (18) (2016) 2476–2485, https://doi.org/10.1080/15384101.2016.1203493.
[35] Z. Li, J. Li, P. Bi, et al., Plk1 phosphorylation of PTEN causes a tumor-promoting metabolic state, Mol. Cell. Biol. 34 (19) (2014) 3642–3661, https://doi.org/10.1128/MCB.00814-14.
[36] B.H. Choi, M. Pagano, W. Dai, Plk1 protein phosphorylates phosphatase and tensin homolog (PTEN) and regulates its mitotic activity during the cell cycle, J. Biol. Chem. 289 (20) (2014) 14066–14074, https://doi.org/10.1074/jbc.M114.558155.
[37] M.K. Leonard, N.T. Hill, P.A. Bubulya, M.P. Kadakia, The PTEN-Akt pathway impacts the integrity and composition of mitotic centrosomes, Cell Cycle 12 (9) (2013) 1406–1415, https://doi.org/10.4161/cc.24516.
[38] W.H. Shen, A.S. Balajee, J. Wang, et al., Essential role for nuclear PTEN in maintaining chromosomal integrity, Cell 128 (1) (2007) 157–170, https://doi.org/10.1016/j.cell.2006.11.042.
[39] J. Ma, J.A. Benitez, J. Li, et al., Inhibition of nuclear PTEN tyrosine phosphorylation enhances glioma radiation sensitivity through attenuated DNA repair, Cancer Cell 35 (3) (2019) 504–518, https://doi.org/10.1016/j.ccell.2019.01.020.
[40] Y. Yan, Z. Li, S. Zeng, X. Wang, Z. Gong, Z. Xu, FGFR2-mediated phosphorylation of PTEN at tyrosine 240 contributes to the radioresistance of glioma, J. Cell Commun. Signal. 13 (3) (2019) 279–280, https://doi.org/10.1007/s12079-019-00518-6.
[41] L. Gong, J.M. Govan, E.B. Evans, et al., Nuclear PTEN tumor-suppressor functions through maintaining heterochromatin structure, Cell Cycle 14 (14) (2015) 2323–2332, https://doi.org/10.1080/15384101.2015.1044174.
[42] Z.H. Chen, M. Zhu, J. Yang, et al., PTEN interacts with histone H1 and controls chromatin condensation, Cell Rep. 8 (6) (2014) 2003–2014, https://doi.org/10.1016/j.celrep.2014.08.008.
[43] D.M. Carone, J.B. Lawrence, Heterochromatin instability in cancer: from the Barr body to satellites and the nuclear periphery, Semin. Cancer Biol. 23 (2) (2013) 99–108, https://doi.org/10.1016/j.semcancer.2012 .06 .00.
[44] Q. Du, S.A. Bert, N.J. Armstrong, et al., Replication timing and epigenome remodelling are associated with the nature of chromosomal rearrangements in cancer, Nat. Commun. 10 (1) (2019), https://doi.org/10.1038/s41467-019-08302-1, 416.
[45] T. Helleday, E. Petermann, C. Lundin, B. Hodgson, R.A. Sharma, DNA repair pathways as targets for cancer therapy, Nat. Rev. Cancer 8 (3) (2008) 193–204, https://doi.org/10.1038/nrc2342.
[46] J.M. Furgason, M. Bahassi el, Targeting DNA repair mechanisms in cancer, Pharmacol. Ther. 137 (3) (2013) 298–308, https://doi.org/10.1016/j.pharmthera.2012.10.009.
[47] M.R. Kelley, D. Logsdon, M.L. Fishel, Targeting DNA repair pathways for cancer treatment: what's new? Future Oncol. 10 (7) (2014) 1215–1237, https://doi.org/10.2217/fon.14.60.
[48] W.Y. Mansour, P. Tennstedt, J. Volquardsen, et al., Loss of PTEN-assisted G2/M checkpoint impedes homologous recombination repair and enhances radio-curability and PARP inhibitor treatment response in prostate cancer, Sci. Rep. 8 (1) (2018), https://doi.org/10.1038/s41598-018- 22289-7, 3947.

[49] B. McEllin, C.V. Camacho, B. Mukherjee, et al., PTEN loss compromises homologous recombination repair in astrocytes: implications for glioblastoma therapy with temozolomide or poly(ADP-ribose) polymerase inhibitors, Cancer Res. 70 (13) (2010) 5457–5464, https://doi.org/10.1158/0008-5472.CAN-09-4295.
[50] P. Malaney, E. Palumbo, J. Semidey-Hurtado, et al., PTEN physically interacts with and regulates E2F1-mediated transcription in lung cancer, Cell Cycle 17 (8) (2018) 947–962, https://doi.org/10.1080/15384101.2017.138897.
[51] J. Guan, Q. Zhao, W. Mao, Nuclear PTEN interferes with binding of Ku70 at double-strand breaks through post-translational poly(ADP-ribosyl)ation, Biochim. Biophys. Acta 1863 (12) (2016) 3106–3115, https://doi.org/10.1016/j.bbamcr.2016.10.003.
[52] P.L. Sulkowski, S.E. Scanlon, S. Oeck, P.M. Glazer, PTEN regulates nonhomologous end joining by epigenetic induction of NHEJ1/XLF, Mol. Cancer Res. 16 (8) (2018) 1241–1254, https://doi.org/10.1158/1541-7786.MCR-17-0581.
[53] Y. Zhang, J.F. Chang, J. Sun, et al., Histone H3K27 methylation modulates the dynamics of FANCD2 on chromatin to facilitate NHEJ and genome stability, J. Cell. Sci. 131 (12) (2018), https://doi.org/10.1242/jcs.215525 jcs215525.
[54] A. Svobodová Kovaříková, S. Legartová, J. Krejčí, E. Bártová, H3K9me3 and H4K20me3 represent the epigenetic landscape for 53BP1 binding to DNA lesions, Aging (Albany NY) 10 (10) (2018) 2585–2605, https://doi.org/10.18632/aging.101572.
[55] X. Kang, C. Song, X. Du, et al., PTEN stabilizes TOP2A and regulates the DNA decatenation, Sci. Rep. 5 (2015) 17873, https://doi.org/10.1038/srep17873. Published 2015 Dec 10.
[56] J. He, X. Kang, Y. Yin, K.S. Chao, W.H. Shen, PTEN regulates DNA replication progression and stalled fork recovery, Nat. Commun. 6 (2015) 7620, https://doi.org/10.1038/ncomms8620.
[57] S.J. Haring, A.C. Mason, S.K. Binz, M.S. Wold, Cellular functions of human RPA1. Multiple roles of domains in replication, repair, and checkpoints, J. Biol. Chem. 283 (27) (2008) 19095–19111, https://doi.org/10.1074/jbc.M800881200.
[58] G. Wang, Y. Li, P. Wang, et al., PTEN regulates RPA1 and protects DNA replication forks, Cell Res. 25 (11) (2015) 1189–1204, https://doi.org/10.1038/cr.2015.115.
[59] J. Feng, J. Liang, J. Li, et al., PTEN controls the DNA replication process through MCM2 in response to replicative stress, Cell Rep. 13 (7) (2015) 1295–1303, https://doi.org/10.1016/j.celrep.2015.10.01.
[60] Y. Zou, Y. Liu, X. Wu, S.M. Shell, Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses, J. Cell. Physiol. 208 (2) (2006) 267–273, https://doi.org/10.1002/jcp.20622.
[61] J.R. McIntosh, Mitosis, Cold Spring Harb. Perspect. Biol. 8 (9) (2016), https://doi.org/10.1101/cshperspect.a023218 a023218.
[62] R. Fraschini, Factors that control mitotic spindle dynamics, Adv. Exp. Med. Biol. 925 (2017) 89–101, https://doi.org/10.1007/5584_2016_74.
[63] Y. Liu, X. Du, S. Zhang, et al., PTEN regulates spindle assembly checkpoint timing through MAD1 in interphase, Oncotarget 8 (58) (2017) 98040–98050, https://doi.org/10.18632/oncotarget.20532.
[64] B.H. Choi, S. Xie, W. Dai, PTEN is a negative regulator of mitotic checkpoint complex during the cell cycle, Exp. Hematol. Oncol. 6 (2017) 19, https://doi.org/10.1186/s40164-017-0079-0.
[65] J.H. Van Ree, H.J. Nam, K.B. Jeganathan, A. Kanakkanthara, J.M. van Deursen, Pten regulates spindle pole movement through Dlg1-mediated recruitment of Eg5 to centrosomes, Nat. Cell Biol. 18 (7) (2016) 814–821, https://doi.org/10.1038/ncb3369.
[66] M.T. Valentine, P.M. Fordyce, S.M. Block, Eg5 steps it up!, Cell Div. 1 (2006) 31, https://doi.org/10.1186/1747-1028-1-31.
[67] J. He, Z. Zhang, M. Ouyang, et al., PTEN regulates EG5 to control spindle architecture and chromosome congression during mitosis, Nat. Commun. 7 (2016) 12355, https://doi.org/10.1038/ncomms12355.
[68] D.C. Whiteman, X.P. Zhou, M.C. Cummings, S. Pavey, N.K. Hayward, C. Eng, Nuclear PTEN expression and clinicopathologic features in a population-based series of primary cutaneous melanoma, Int. J. Cancer 99 (1) (2002) 63–67, https://doi.org/10.1002/ijc.10294.
[69] X.P. Zhou, A. Loukola, R. Salovaara, et al., PTEN mutational spectra, expression levels, and subcellular localization in microsatellite stable and unstable colorectal cancers, Am. J. Pathol. 161 (2) (2002) 439–447, https://doi.org/10.1016/S0002-9440(10)64200-9.
[70] M.K. Ge, N. Zhang, L. Xia, et al., FBXO22 degrades nuclear PTEN to promote tumorigenesis, Nat. Commun. 11 (1) (2020), https://doi.org/10.1038/s41467-020-15578-1, 1720.
[71] B.H. Choi, M. Pagano, C. Huang, Dai W. Cdh1, A substrate-recruiting component of anaphase- promoting complex/cyclosome (APC/C) ubiquitin E3 ligase, specifically interacts with phosphatase and tensin homolog (PTEN) and promotes its removal from chromatin, J. Biol. Chem. 292 (48) (2017), https://doi.org/10.1074/jbc.AAC117.000864, 19609.
[72] R. Ali, M. Alabdullah, I. Miligy, et al., ATM regulated PTEN degradation is XIAP E3 Ubiquitin Ligase mediated in p85α deficient cancer cells and influence platinum sensitivity, Cells 8 (10) (2019), https://doi.org/10.3390/cells8101271, 1271. Published 2019 Oct 18.
[73] H. Maccario, N.M. Perera, A. Gray, C.P. Downes, N.R. Leslie, Ubiquitination of PTEN (phosphatase and tensin homolog) inhibits phosphatase activity and is enhanced by membrane targeting and hyperosmotic stress, J. Biol. Chem. 285 (17) (2010) 12620–12628, https://doi.org/10.1074/jbc.M109.072280.
[74] P. Tibarewal, G. Zilidis, L. Spinelli, et al., PTEN protein phosphatase activity correlates with control of gene expression and invasion, a tumor-suppressing phenotype, but not with AKT activity, Sci. Signal. 5 (213) (2012), https://doi.org/10.1126/scisignal.2002138, 18.
[75] X.C. Zhang, A. Piccini, M.P. Myers, L. Van Aelst, N.K. Tonks, Functional analysis of the protein phosphatase activity of PTEN, Biochem. J. 444 (3) (2012) 457–464, https://doi.org/10.1042/BJ20120098.

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ORIGINAL PAPER

Phospho PTEN mediated dephosphorylation of mitotic kinase PLK1 and Aurora Kinase A prevents aneuploidy and preserves genomic stability

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Abstract

PTEN, dual phosphatase tumor suppressor protein, is found to be frequently mutated in various cancers. Post-translational modification of PTEN is important for its sub-cellular localization and catalytic functions. But how these modifications affect cytological damage and aneuploidy is not studied in detail. We focus on the role of phosphatase activity along with C-terminal phosphorylation of PTEN in perspective of cytological damage like micronucleus, nuclear bud, and nuclear bridge formation. Our data suggest that wild-type PTEN, but not phospho-mutant PTEN significantly reduces cytological damage in PTEN null PC3 cells. In case of phosphatase-dead PTEN, cytological damage markers are increased during 24 h recovery after DNA damage. When we use phosphorylation and phosphatase-dead dual mutant PTEN, the extent of different cytological DNA damage parameters are similar to phosphatase-dead PTEN. We also find that both of those activities are essential for maintaining chromosome numbers. PTEN null cells exhibit significantly aberrant γ-tubulin pole formation during metaphase. Interestingly, we observed that p-PTEN localized to spindle poles along with PLK1 and Aurora Kinase A. Further depletion of phosphorylation and phosphatase activity of PTEN increases the expression of p-Aurora Kinase A (T288) and p-PLK1 (T210), compared to cells expressing wild-type PTEN. Again, wild-type PTEN but not phosphorylation-dead mutant is able to physically interact with PLK1 and Aurora Kinase A. Thus, our study suggests that the phosphorylation-dependent interaction of PTEN with PLK1 and Aurora Kinase A causes dephosphorylation of those mitotic kinases and by lowering their hyperphosphorylation status, PTEN prevents aberrant chromosome segregation in metaphase.

Keywords Aneuploidy · Spindle pole · Micronucleus · PTEN · Aurora Kinase · Polo-like kinase

Introduction

PTEN (Phosphatase and Tensin homolog deleted on chromosome 10) is frequently mutated in a variety of malignancies, including prostate cancer, breast cancer, endometrial cancer, and glioblastoma [1–3]. It is a dual protein and lipid phosphatase, whose classical function is to negatively regulate the oncogenic PI3K/Akt pathway [4, 5]. PTEN has recently been identified as a DNA repair protein. Bestowing several studies PTEN null cells shows genomic instability and abnormal chromosomal characteristic like chromosomal breakage, telomere fusion, nucleoplasmic bridge formation, etc. [6]. Significant pool of PTEN localizes to the nucleus/chromatin and plays vital functions in the maintenance of genomic stability and chromatin architecture [6]. PTEN inhibits genomic instability by a variety of functions, including regulation of spindle assembly checkpoint (SAC) [7, 8], controlling cell cycle checkpoints [9], homologous recombination (HR) repair [10], and replication fork stability [11]. According to the findings, PTEN stabilizes the MAD2 protein, a component of the MCC complex, and protects it from ubiquitin-mediated degradation [12]. This results in the prevention of unregulated chromatin segregation and aneuploidy during the metaphase of cell division [13–15].

Number of reports suggested that the catalytic functions of PTEN may be attributed to its post-translational modulations, including phosphorylation [16], ubiquitination, sumoylation [17], acetylation [18], and oxidation

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[19]. Several protein kinases, including PLK1 [20], CK2 [21], and GSK-3 [22] phosphorylate PTEN extensively in the C-terminal region (Ser 370, Ser 380, Thr 382, Thr 383, and Ser 385). Whereas PTEN stability is enhanced upon its phosphorylation, its phosphatase activity is diminished simultaneously [22]. It has been proposed that dephosphorylation or mutations at the STT cluster of PTEN advance its ubiquitination [23] while increasing its phosphatase activity [24, 25]. Thus, the PTEN C –terminal motif regulates its stability and catalytic activity, which may be related to its tumor suppressive functions through maintaining genomic stability. Recently several reports recommended that PTEN C-terminal phosphorylation has been linked with chromatin association and DNA repair [26, 27].

Polo-like kinase1 (PLK1), a mitotic kinase, regulates a variety of cell cycle events, including mitotic entry, bipolar spindle formation, and DNA repair [28–34]. PTEN is directly phosphorylated by PLK1 at Ser 380 and regulating its chromatin interaction [20]. On the other hand, Aurora kinases (AURKs), another mitotic kinase, are a group of preserved serine/threonine protein kinases that play a critical role in carcinogenesis when constitutively activated [35]. AURKA is involved in spindle assembly, mitotic entry, and centrosome activity [36]. Evidence suggests that the build-up of the CEP192 complex in pericentriolar material causes autophosphorylation of AURKA, which activates its kinase activity and leads to PLK1 phosphorylation. During microtubule nucleation, the CEP192-AURKA-PLK1 complex generates γ-tubulin ring complex attachment sites, whereas hyperactivity disrupts normal chromosomal segregation, resulting in aneuploidy [37]. Overexpression of AURKA has been reported in numerous cancers, including bladder cancer, esophageal squamous cell carcinoma, and breast cancer [38–40]. AURKA hyperactivation promotes centrosome amplification, which causes cytokinesis failure and subsequent multinucleation formation [35], which is one of the phenotypes of PTEN null cells [41]. Furthermore, AURKA overexpression in cells skips the spindle checkpoint in mitosis and causes cells to reach anaphase despite the creation of an aberrant spindle, resulting in chromosomal fragmentation or breakage, anaphase bridge, and lagging chromosomes [42].

PTEN phosphorylation and phosphatase activity have been studied individually in the context of genomic stability. However, to the best of our knowledge, there are no reports on the interplay of phosphorylation and phosphatase activity of PTEN together. Hence, in this study, we want to look at the relevance of PTEN's phosphorylation, as well as phosphatase activity, in preserving genomic integrity and subsequent consequence on cytological damage. In this study, in light of ectopic expression of (1) wild-type PTEN, (2) phospho-deficient PTEN mutant, (3) phosphatase-dead PTEN-mutant and (4) phospho-phosphatase dual dead PTEN mutant constructs in the PTEN-null glioma cell line U87MG and the prostate cancer cell line PC3, chromosomal instability was evaluated. Our findings imply that both PTEN phosphorylation and phosphatase activity are important in maintaining genomic stability.

Materials and methods

Cell culture, reagents, and antibodies

PC3 (Human Prostate Cancer Cell); U87MG (Human glioblastoma cell) and Wi-38 (normal lung fibroblast) were purchased from NCCS Pune India. PC3, derived from a prostatic adenocarcinoma grade 4, of a 62-year-old Caucasian male was cultivated in RPMI 1640 medium, U87MG, established from a malignant glioma of a female patient, and Wi-38, derived from a 3 month old female fetus were cultured in MEM, supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml). Cells were kept at 37 °C, 5% CO_2, and 95% relative humidity (RH). Dimethyl Sulphoxide (Sigma) was used to dissolve Nocodazole (Sigma), Etoposide (Sigma), and Cytochalasin B (Sigma) at concentrations of 1 mg/ml, 25 mM, and 5 mg/ml, respectively. Subsequent dilutions were made fresh in the medium. Anti-Aurora Kinase A (9980S), anti-PLK1 (4513S), anti-P-Aurora Kinase A (T288) (8025S), anti-P-PLK1 (T210) (3738S), and anti-P-PTEN antibodies were purchased from Cell Signalling Technology. Total PTEN antibody (32,199) was purchased from Abcam. Secondary antibodies like anti-rabbit IgG conjugated with Alexa Fluor 568 (1:150, Life Technologies, USA), anti-mouse IgG conjugated with Alexa Fluor 488 (1:150, Life Technologies, USA), and anti-rabbit IgG conjugated with horseradish peroxidase (HRP, 1:25,000, Abcam), were used.

Plasmids, site-directed mutagenesis, and transfection

Mammalian expression vectors for phospho-deficient pSG5L-HA-A4-PTEN (Ser380Ala, Thr382Ala, Thr383Ala, Ser385Ala), phosphatase-dead pSG5L-HA-C124S-PTEN (Cys124Ser) and phospho-deficient phosphatase-dead pSG5L-HA-A4PD-PTEN (Ser380Ala, Thr382Ala, Thr383Ala, Ser385Ala, Cys124Ser) encode hemagglutinin-tagged mutant proteins were created by site-directed mutagenesis kit (Stratagene) as per manufacturer's instruction and were confirmed by sequencing. The following primer was used for phosphatase-dead mutant PTEN:

Forward: 5′ GTTGCAGCAATTCACTCTAAAGCTGGAAAGGG 3′, Reverse: 5′CCCTTTCCAGCTTTAGAGTGAATTGCTGCAAC 3′

The following sequencing primer was used for sequencing the clone:

Sence- 5′GCTAGATCTCGAGCTCTTCAAGAGGATGGATT 3′, Antisence- 5′CGGGCCCGGGATCCACCGGATCTAG 3′

(HA-WT-PTEN) wild-type pSG5L-HA-Wt-PTEN encodes hemagglutinin-tagged Wt-PTEN protein. Lipofectamine 3000 (Invitrogen) was used for transient transfection. For transient transfection PTEN null PC3 and U87MG cells were cultured in an antibiotic-depleted medium. Transfections were done according to manufacturer instructions.

Cytokinesis-block micronucleus (CBMN) assay

PTEN null PC3 cells were plated on coverslips, transiently transfected, and then subjected to 100 µM etoposide for 4 h, followed by cytochalasin B (final concentration of 5 µg/ml). After 36 h, the coverslips were then incubated for 5 min at 37 °C in a hypotonic solution (75 mM KCl) before being fixed for 15 min at room temperature with paraformaldehyde (4%). The coverslips were put on microscopic slides with mounting medium containing 4 -6-diamidino-2-phenylindole (DAPI, Vecta Shield Laboratories, USA) and observed under a fluorescence microscope (Leica, Germany). The numbers of Micronuclei (MNi), Nucleoplasmic Bridge (NPBs), and Nuclear Buds (NBUDs) were computed in 1000 BNC (binucleated cells) for the respective experimental time points and documented using Fenech's [43] criteria.

Immuno-fluorescence

Cells cultured on glass coverslips were transiently transfected with PTEN clones and synchronized using a double thymidine block where cells were incubated for 18 h with thymidine (2 mM), then 9 h with fresh medium and again 18 h with thymidine (2 mM). After 8 h cells were fixed in a 4% paraformaldehyde solution and permeabilized on ice with 0.2% Triton X-100. The cells were then re-incubated in blocking solution (0.5% FBS in PBS), followed by overnight incubation with anti-α-tubulin and anti-γ-tubulin antibody or anti-γ-tubulin with either anti-PLK1 or anti-Aurora Kinase A antibody in wash buffer. The same technique was used to synchronize WI-38 cells, which were then treated with total PTEN or phospho-PTEN using γ-tubulin antibodies. The cells were then washed with 0.5% FBS and 0.05% Tween 20 in PBS before being probed with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 568 antibodies at room temperature for 1 h. Cells were mounted with DAPI before being observed under a fluorescence or confocal microscope (Leica, Germany).

Aneuploidy analyses

After transient transfection of different PTEN clones in both PC3 and U87MG cells, nocodazole (50 ng/ml) was added, and treated cells for 16 h. The cell pellet was then collected and incubated for 15 min in 75 mM KCl before being centrifuged. The pellet was then fixed for 15 min at 4 °C in an ice-cold fixative (methanol:acetic acid in a 3:1 ratio) before being centrifuged. The pellet was then resuspended in ice-cold fixative and dropped on a grease-free, cold glass slide, flame dried instantaneously, mounted with DAPI, and studied under a fluorescence microscope (Leica, Germany).

Western blotting

Different PTEN clones were transiently transfected into both PC3 and U87MG cells. Following transfection, the cells were treated for 16 h with nocodazole (50 ng/ml). Using RIPA lysis buffer(ab156034) and protease and phosphatase inhibitors (G Bioscience), whole cell lysates were produced. Protein concentrations in the supernatant were determined by Bradford assay, and an identical amount (90 µg) of protein-containing cell lysates were run on SDS-PAGE. The proteins were then transferred to PVDF (polyvinylidine difluoride) membrane (Merck). The membrane was incubated with appropriate primary antibodies, followed by appropriate secondary antibodies conjugated with horseradish peroxidase (HRP). The membrane was immunoblotted with anti-Aurora Kinase A, anti-PLK1, anti-p-Aurora Kinase A, anti-p-PLK 1, and anti-PTEN. As a loading control, an anti-GAPDH antibody was utilized.

Immunoprecipitation

After transient transfection with various PTEN clones, cell lysates were processed with 1X RIPA lysis buffer containing protease and phosphatase inhibitors. After clearing the lysate with protein A/G conjugated agarose bead, the lysates were incubated with anti-HA antibody overnight at 4 °C. (1:150). Then a protein A/G agarose bead was added and incubated at 4 °C for 2 h. The lysates were then centrifuged, and the beads were washed with RIPA buffer. Proteins were then eluted with 50 µl of 2X Lamellae dye, boiled for 10 min, and blotted with anti-PLK1, anti-AURKA, and anti-PTEN antibodies as previously described.

Statistical analysis

The data in the graphs were all represented as means and standard deviations (SD). GraphPad Prism 8.0 or Microsoft

Excel software was used to make the graphs. Microsoft Excel analytical tools were used to do statistical calculations. The statistical difference between groups was calculated using a student's *t*-test. $p < 0.05$ was determined to be statistically significant. R Studio was used to do the PCA analysis.

Results

PTEN phosphatase activity and phosphorylation results in suppressing cytological damage.

The Cytokinesis Block Micronucleus Assay is the basic technique to detect biomarkers of chromosomal instability such as micronuclei, nucleoplasmic bridges, and nuclear buds. Mis-segregated chromosomes or their pieces generate cytoplasmic DNA in the nucleus, resulting in micronuclei build-up during subsequent cell divisions. At first $PTEN^{-/-}$ PC3 cells were transfected with Wt-PTEN, A4-PTEN, C124S-PTEN, and A4-C124S-PTEN (Fig. 1A) separately, and subsequently, they were treated for 4 h with etoposide followed by CBMN assay. Micronuclei (MNi), Nucleoplasmic Bridges (NPBs), and Nuclear Buds (NBUDs) were counted at 0 h and 24 h recovery time points (Table 1). After 24 h of recovery from etoposide treatment, we found MNi formation is significantly increased (Fig. 1BIV) in both C124S-PTEN (phosphatase-dead) and A4-C124S-PTEN (phosphatase and phosphorylation-dead) transfected cells (49 ± 3.20 and 66 ± 2.88) respectively compared to Wt-PTEN (25 ± 2.06) transfected cells. Nuclear bud development is similarly increased in phosphatase-dead and double mutant transfected cells versus Wt-PTEN (C124S-PTEN-27 ± 3.30 and A4-C124S-PTEN 47 ± 2.5 vs 9 ± 1.26 at 24 h) (Fig. 1BIII). NPBs (Fig. 1BII), which are indications of amplified DNA exclusion and telomere end fusion or DNA mis-repair, were also higher in C124S-PTEN (14 ± 2.38) and A4-C124S-PTEN (20 ± 2.94) transfected cells than in Wt-PTEN (3 ± 0.5) transfected cells. Interestingly, phosphorylation-dead PTEN (A4-PTEN), which has increased phosphatase activity, eliminates MNi more efficiently than other PTEN mutant transfected cells but less efficiently than Wt-PTEN transfected cells (Table 1). This study implies that classical phosphatase activity is critical for excluding accumulated cytological damage.

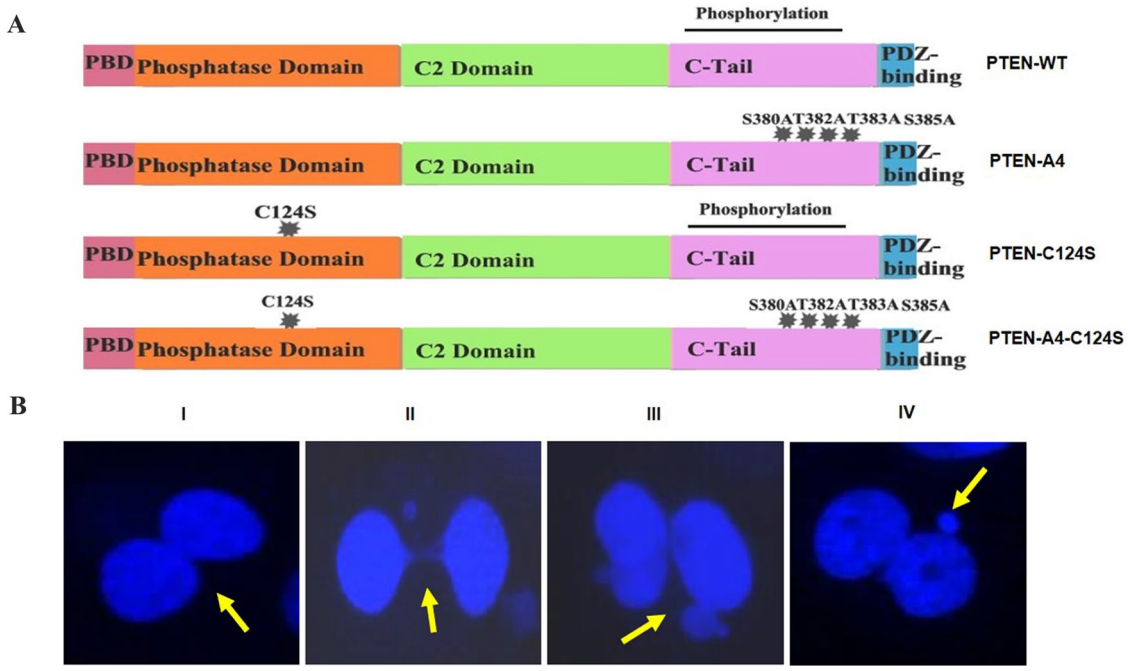

Fig. 1 Occurrence of cytological damage in different mutants of PTEN A. Map of different mutants of PTEN (Wt-PTEN, A4-PTEN, C124S-PTEN, and A4-C124S-PTEN) B. Microscopic images of cells incubated in the presence of etoposide (100 µM for 4 h) and allowed to recover for 24 h. Cells were stained with DAPI. Arrowheads indicate I. binucleated cell II. Binucleated cell with Mni and NPB III. Binucleated cell with NBud IV. Binucleated cell with Mni

Table 1 The results of cytokinesis-block micronucleus assay in different clones of PTEN after 4 h exposure of 100μ M etoposide

Mutants	MNi per 1000 BN cells		NBud per 1000 BN cells		NPB per 1000 BN cells	
	0 h	24 h	0 h	24 h	0 h	24 h
UT	77 ± 2.98	65 ± 3.79	28 ± 1.82	37 ± 1.5	17 ± 2.21	20 ± 2.1
PTEN-WT	49 ± 1.89[a]	25 ± 2.06[b]	14 ± 1.73[a]	9 ± 1.26[b]	6 ± 0.58[a]	3 ± 0.5[b]
PTEN-A4	48 ± 1.15	36 ± 2.16	36 ± 0.82	20 ± 1.82	10 ± 1.29	7 ± 0.96
PTEN-C124S	23 ± 2.36[c]	49 ± 3.20[d]	15 ± 3.09[c]	27 ± 3.30[d]	5 ± 0.5[c]	14 ± 2.38[d]
PTEN-A4PD	56 ± 3.16[c]	66 ± 2.88[d]	32 ± 2.58[c]	47 ± 2.5[d]	15 ± 2.75[c]	20 ± 2.94[d]

Values shown as for means ± SEM of three independent experiments

MNi Micronuclei; *NBud* Nuclear Buds; *NPBs* Nucleoplasmic Bridges

[a] $p < 0.05$ compared with UT 0 h (Student's *t*-test)

[b] $p < 0.05$ compared with UT 24 h (Student's *t*-test)

[c] $p < 0.05$ compared with PTEN 0 h (Student's *t*-test)

[d] $p < 0.05$ compared with PTEN 24 h (Student's *t*-test)

Phosphatase and phosphorylation activities of PTEN prevent multiple γ-tubulin pole formation in metaphase and suppress spontaneous aneuploidy

The most important stage in chromosomal segregation during metaphase is the formation of a γ-tubulin pole at the centrosome. Multiple pole development during metaphase is harmful to cell destiny because they result in chromosome missegregation and aneuploidy. Aneuploidy occurs spontaneously in PTEN-deficient cells, indicating whole-chromosome instability. Microscopic pictures exhibit the variance in the number of pole formations following various PTEN mutant transfection in PTEN$^{-/-}$ U87MG and PC3 cell lines (Fig. 2A). The absence of PTEN phosphatase and phosphorylation activity resulted in a 1.3-fold reduction in normal bipolar cell percentage in PTEN clones against Wt-PTEN (Fig. 2B). Percentage of multiple poles in untransfected, C124S-PTEN, and A4-C124S-PTEN cells is 10–12-fold higher, and 8–10-fold rise in A4-PTEN transfected cells in comparison to Wt-PTEN (Fig. 2C) transfected U87MG cells. Compared to Wt-PTEN transfected PC3 cells, PTEN phosphorylation- and phosphatase-dead clone, phosphorylation-dead clone, and phosphatase-dead clone including untransfected cells, had a 6–11-fold intensification in the percentage of multipolar cells formation (Fig. 2F). Next, we investigated the ploidy status of several mutant PTEN clones after transient transfection in PTEN$^{-/-}$ PC3 as well as U87MG cell lines. After 16 h of treatment with a microtubule depolymerizing reagent, nocodazole, the aneuploidy status of each mutant was investigated. In this study, we detected a 1.6-fold rise in aneuploidy percentage in A4-PTEN, and an approximately twofold increase in both C124S-PTEN and A4-C124S-PTEN transfected U87MG cells and untransfected had a 1.9-times increase in aneuploidy percentage compared with Wt-PTEN-transfected cells (Fig. 2D). This pattern holds true for the other PTEN-negative PC3 cell line. We found a 1.9, 2.6, and 2.7-fold surge in aneuploidy in A4-PTEN, C124S-PTEN, and A4-C124S-PTEN transfected PC3 cells, when compared to Wt-PTEN transfected cells, respectively, and a 2.7-fold increase in untransfected cells when compared with Wt-PTEN transfected cells (Fig. 2G). This finding indicates that the phosphatase activity of PTEN and its phosphorylation is required for proper chromosomal segregation.

Phospho-PTEN co-localized with γ- tubulin at the spindle pole during metaphase.

Previous research has suggested that PTEN controls chromosomal segregation [15]. Owing to the fact that PTEN deletion causes aneuploidy, we want to investigate whether PTEN is localized to the spindle pole. According to our immunofluorescence study, PTEN, or in particular the phosphorylated version of PTEN (Ser 380, Thr 382,383), is localized to the spindle pole and associated with γ-tubulin, as demonstrated in PTEN-positive lung fibroblast Wi-38 cell (Fig. 3A). PLK1 and AURKA, on the other hand, co-localize with γ-tubulin to the centrosomes of the PTEN-negative cell line PC3. (Fig. 3B).

Loss of PTEN phosphatase and phosphorylation activities lead to differential expression of Aurora Kinase A and PLK1

Several investigations have shown that during the early stages of mitosis, AURKA autophosphorylation is the key regulatory mechanism in centrosome formation [44]. Overactivation of these mitotic kinases results in unregulated chromosomal segregation and mitotic pole disintegration [45]. To test this idea, we ectopically expressed various

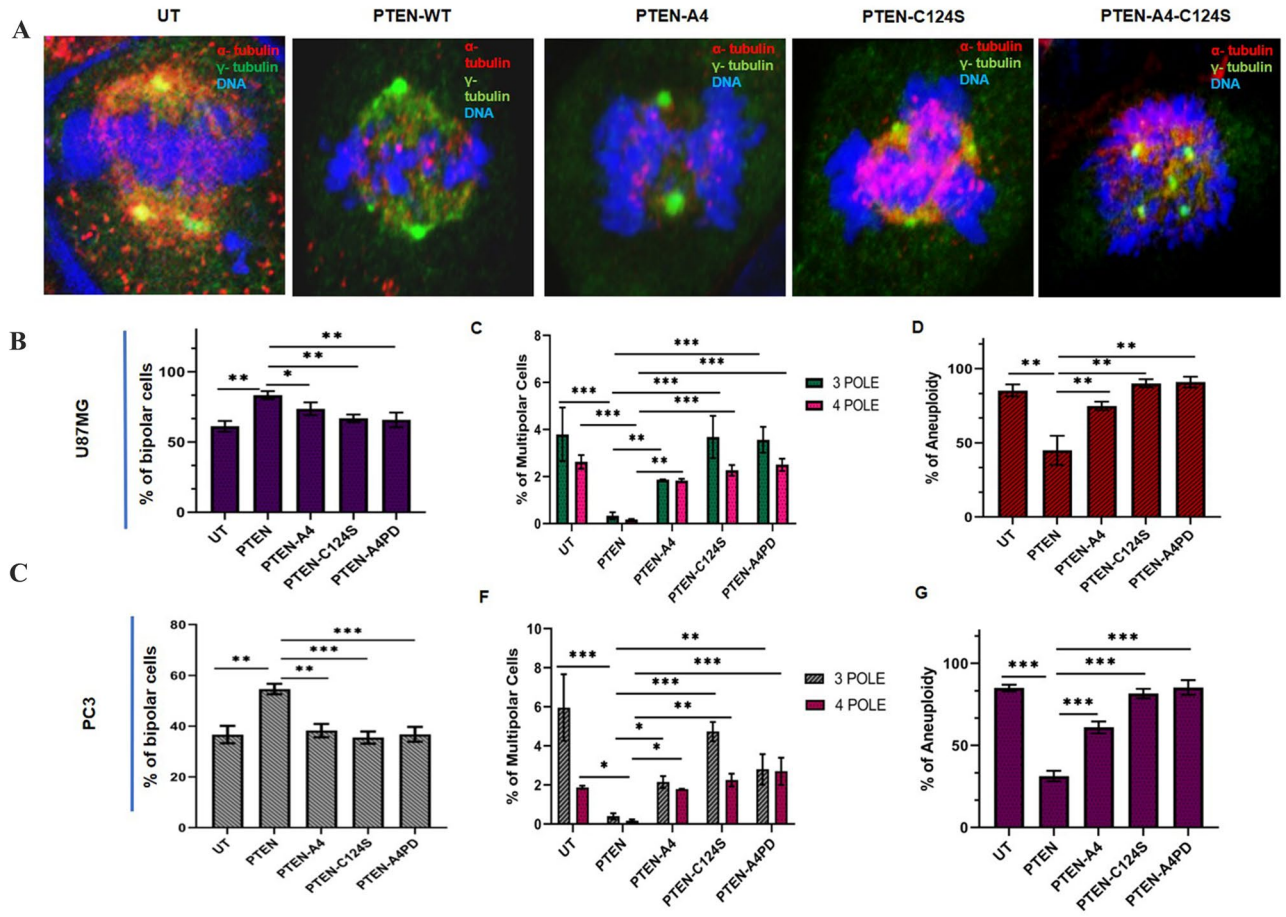

Fig. 2 Multiple pole formation causes chromosome missegregation. **A** Microscopic images of the formation of multiple poles during metaphase in different PTEN-transfected cells Images are labeled with α-tubulin (red), γ-tubulin (green), and DNA (blue). Graphical representation of decrease in the percentage of bipolar and increase in the percentage of multipolar cells in U87MG (**B, C**) and in PC3 cells (**E, F**) transfected with different PTEN clones compared to Wt-PTEN. The increase in aneuploidy in various mutant PTEN and the decrease in aneuploidy in Wt-PTEN transfected U87MG (**D**) and PC3 cells (**G**) were quantified. The values represent the mean SEM of three independent experiments. $*p<0.05$; $**p<0.01$; $***p<0.001$

clones of PTEN in PTEN-deficient PC3 and U87MG cells separately. After 24 h, the cells were treated for 16 h with nocodazole, and western blots were used to evaluate the levels of PLK1 and Aurora Kinase A expression. Both phospho-Aurora Kinase A (T210) and phospho-PLK1 (T288) expression were significantly amplified in untransfected PC3 and U87MG cell lines as compared to Wt-PTEN transfected cells. These mitotic kinases were also observed to be overexpressed in C124S-PTEN and A4-C124S-PTEN transfected cells in PC3 (Fig. 4A) and U87MG cells when compared to Wt-PTEN transfected cells (Fig. 4B). Interestingly, phosphorylation-dead PTEN-transfected cells have higher levels of phospho-PLK1 (T210) and phospho-Aurora Kinase A (T288) though it possesses increased phosphatase activity. Thus, it appears that alternative mechanisms exist in cells that play a role in the inability of A4-PTEN to dephosphorylate certain proteins in vivo.

Wt-PTEN but not A4-PTEN or A4-C124S PTEN physically interact with PLK1/Aurora Kinase A

We next used immunoprecipitation study to see if PTEN and its mutants physically interact with PLK1 and Aurora Kinase A. PTEN clones were transfected into PTEN-null PC3 cells for 24 h before being treated with nocodazole. Anti-HA antibody was used to immunoprecipitate whole cell lysates, which were then blotted with PLK1 and Aurora Kinase A. We observed that phosphorylation-dead mutant PTEN (A4-PTEN) (lane 3) and double mutant PTEN (A4-C124S-PTEN) (lane 5) did not physically interact with PLK1 as efficiently as Wt-PTEN (lane 2). However, phosphatase-dead PTEN (lane 4) interacted

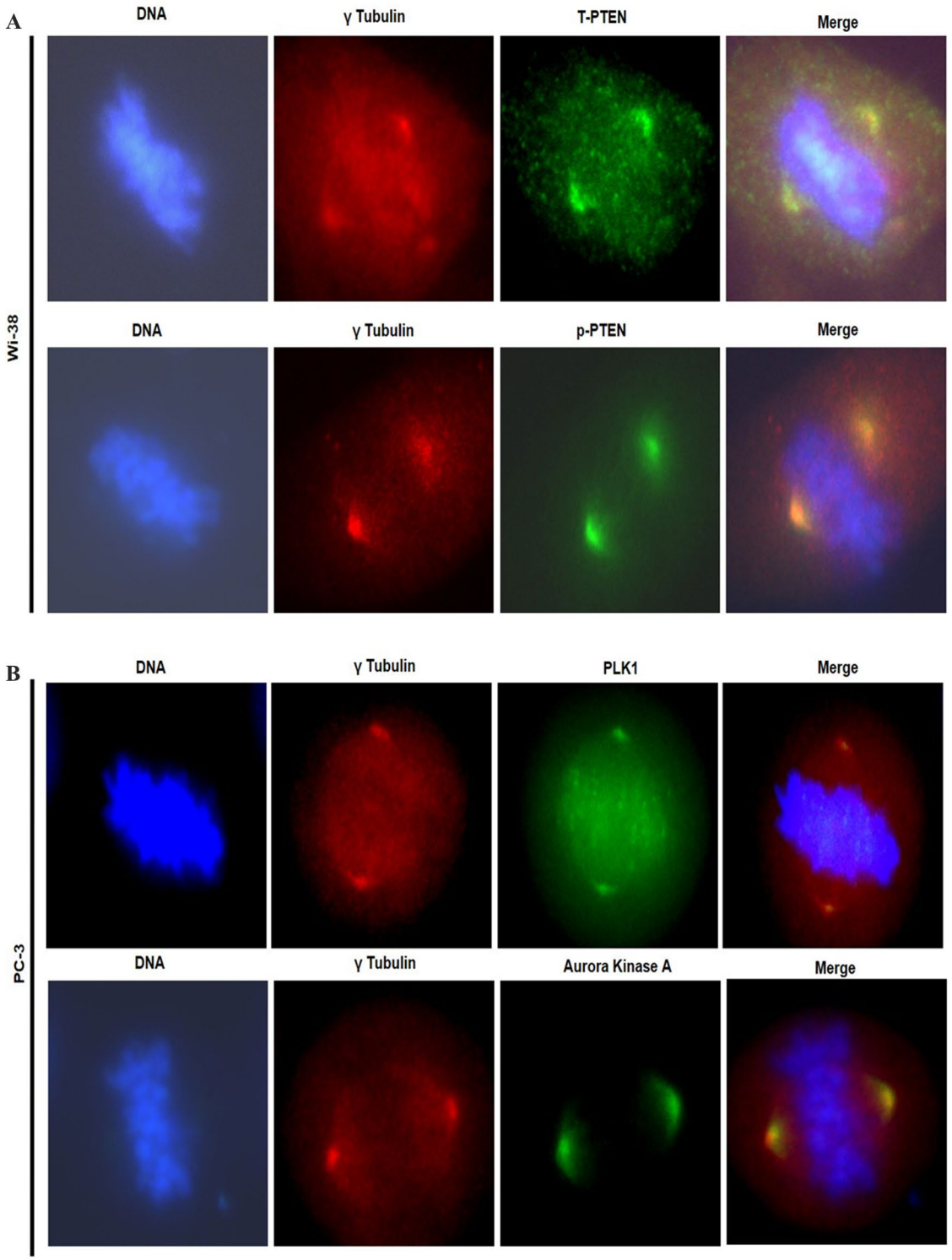

Fig. 3 PTEN localizes at the spindle pole. **A** Metaphase Wi-38 cells are immunolabeled with γ-tubulin antibody (red) and either total PTEN antibody (green) or phospho-Ser 380, Thr 382, and 383 PTEN antibody (green). **B** Synchronized PTEN-null PC3 cells are immunolabeled with γ-tubulin (red) and either PLK1 or Aurora Kinase A (green) antibodies

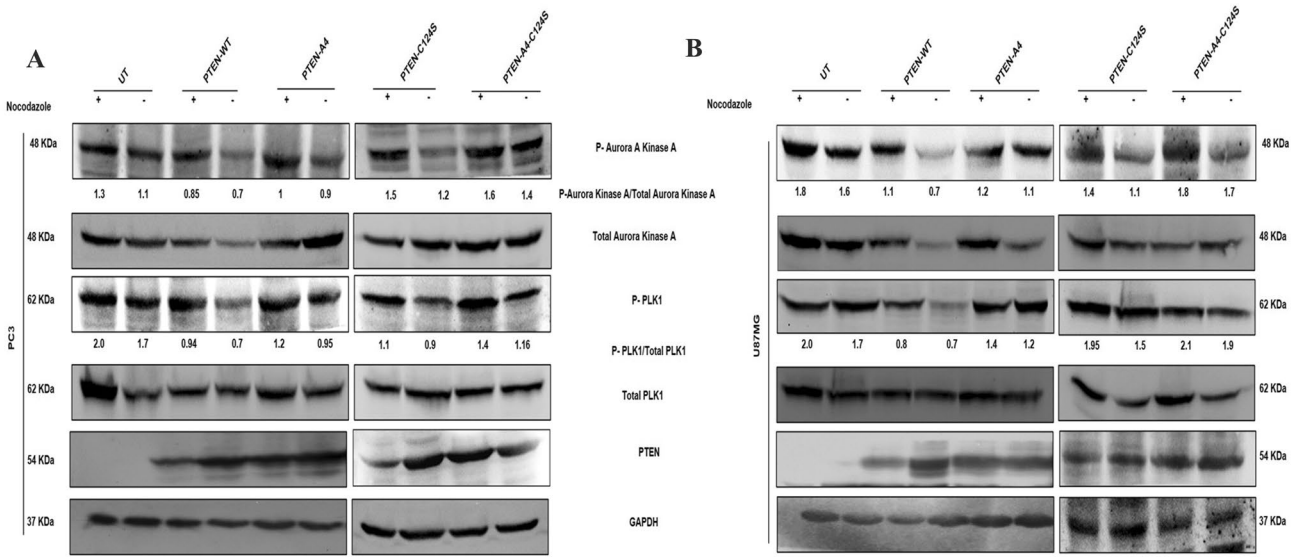

Fig. 4 Differential expression of mitotic kinases in different PTEN mutants A and B Phosphorylation of AURKA and PLK1 was reduced in Wt-PTEN but increased in UT, C124S-PTEN, and A4-C124S-PTEN, PTEN clones after transfection in PTEN-/- cell lines (PC3 and U87MG) before and after nocodazole treatment for 16 h. GAPDH acts as a loading control. Values were calculated by image J software, which indicates the ratio of p-PLK1/ total PLK1 and p-AURKA/ total AURKA antibody. Untransfected control is normalized to 1

with PLK1 efficiently. Next, we found that hosphor-dead PTEN (lane 3) and double mutant PTEN (lane 5) were also unable to associate with Aurora Kinase A, although wild-type PTEN (lane 2) and phosphatase-dead PTEN (lane 4) did. Thus, the C-terminal phosphorylation of PTEN may be critical for its physical interaction with PLK1 and Aurora Kinase A. Even though A4-PTEN has increased phosphatase activity, it is unable to engage efficiently with PLK1 and Aurora Kinase A and so cannot dephosphorylate both, as demonstrated by our western blot assay. (Fig. 5).

Discussion

Maintenance of genomic stability is crucial to prevent neoplastic characteristic of cells. PTEN is one of the important tumor suppressor proteins that not only regulates traditional PI3K/AKT pathway but also plays a critical role in the maintenance of genomic stability by controlling cell cycle regulation, heterochromatin structure maintenance [46, 47], HR repair [46], accurate chromosomal segregation [12, 48]and progression of DNA replication [11, 49]. The importance of phosphatase activity and phosphorylation of PTEN have been investigated in various literature, however, addressing the integrated activity of PTEN phosphatase and phosphorylation activity have not yet been examined in connection to chromosomal integrity [50, 51]. In this study, we used PTEN null PC3 and U87MG cells as a model system as no functional PTEN expressed in these cell lines [52, 53]. The PTEN status of both of these cells is negative. When different mutants of PTEN were transfected, we found PTEN phosphorylation and phosphatase function are both required to reduce damage induced Mni accumulation indicating compromised DNA repair with non-functional cell cycle checkpoint functionary. When phosphorylation- and phosphatase-impaired double mutant (A4-C124S-PTEN) and phosphatase-dead (C124S-PTEN) were compared to Wt-PTEN, Nbud, NPBs also increased. It appears that overexpressed p-Akt in C124S-PTEN transfected cells drives cell division with unrepaired damaged DNA, overcoming the checkpoint barrier, and inducing the build-up of Mni, Nbud, and NPB during the 24 h recovery period. Sporadic phospho-deficient PTEN may also contribute to the Mni build-up in the absence of DNA repair system. As a result, when we utilized both phosphatase-dead and phosphorylation-dead PTEN (A4-C124S-PTEN), excessive damage build-up was detected due to the dual influence of elevated p-AKT levels and reduced DNA repair capabilities. As previously reported, we also observed that PTEN null cells show abnormal chromosomal segregation leading to aneuploidy [12]. This is the consequence of compromised MCC/ SAC functions. PTEN has been shown to physically interact with the Mad1, Mad2, and Cdc20 proteins, all of which are required for MCC [54, 55], and establishes docking sites for dlg1 and Eg-5, both of which are required for normal bipolar spindle formation. Going upstream to this pathway we found that multiple γ-tubulin pole formation occurs in PTEN null

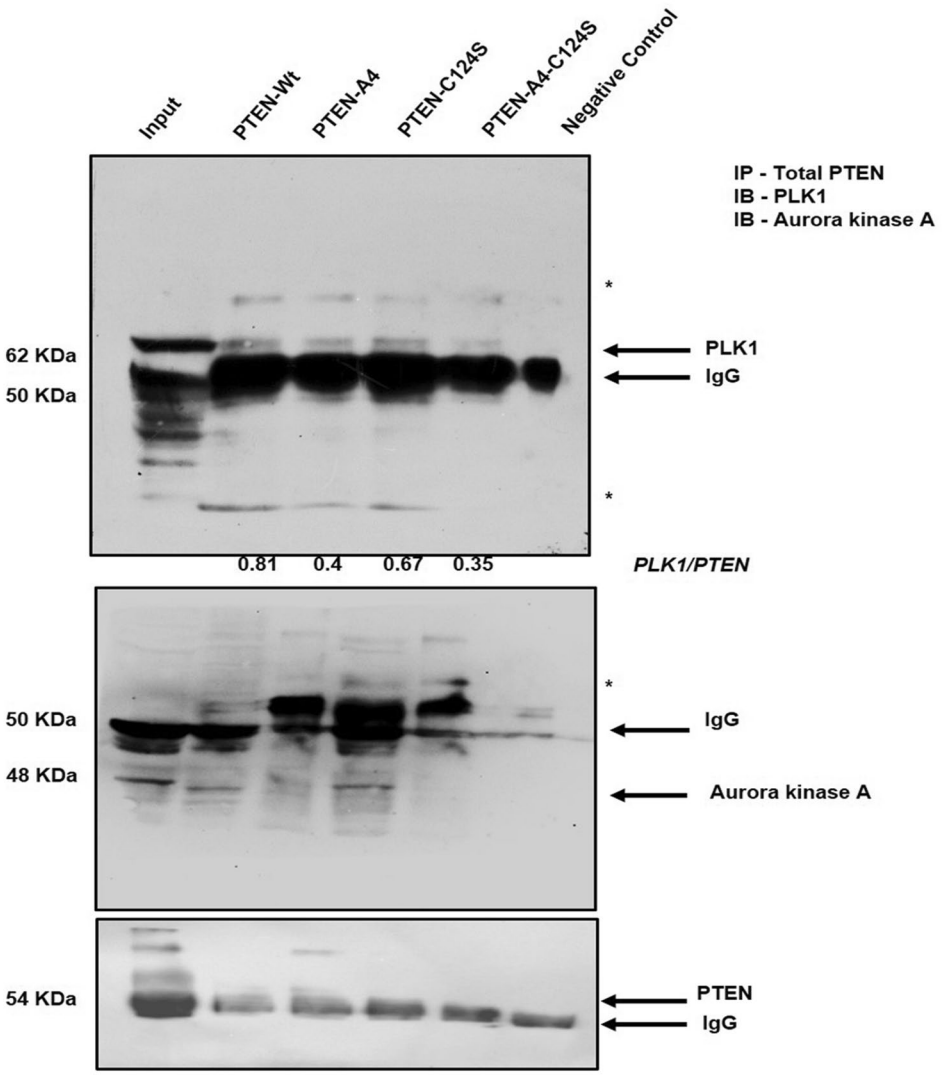

Fig. 5 PTEN null PC3 cells were transfected with the indicated PTEN clone, and after nocodazole treatment, whole cell lysate was prepared and immune precipitated with anti-HA antibody followed by immunoblotting with PLK1 and Aurora Kinase A antibody showed that PTEN-C124S and PTEN-A4-C124S were unable to efficiently interact with PLK1 and Aurora Kinase A and dephosphorylate them

cells. Interestingly we observed that increased multiple pole formation also occurs in PTEN phosphatase-dead and phosphorylation-dead transfected cells when compared to Wt-PTEN transfected cells. A recently published study revealed that PTEN lacking phosphorylation domain (A4-PTEN) is unable to localize at metaphase chromosomes [56]. However, the results of our study strongly suggest that p-PTEN localized to the spindle pole with γ-tubulin. Studies suggest that PTEN-deficient cells have increased γ-tubulin levels, which may lead to centrosome amplification [57]. Another cause of centrosome amplification is the hyperactivity of mitotic kinases like PLK1 and Aurora Kinase A. Nuclear PTEN interacts with APC/C, promotes its interaction with CDH1, and so enhances the tumor suppressive activity of the APC-CDH1 complex by degrading Cyclin A2, Aurora A, PLK1 [58] and regulates proper mitotic exit [9]. Thus, PTEN regulates Aurora A, PLK1 expression indirectly. Accordingly, we found that PTEN null cells show increased expression of those mitotic kinases and their phosphorylated form. PTEN phosphatase and phosphorylation-dead mutant transfected cells show the expression of hyperphosphorylated mitotic kinases compared to Wt-PTEN. Expression of Wt-PTEN suppresses hosphor-PLK1 (T210) and hosphor-Aurora Kinase A (T288) expression, and this is independent of nocodazole treatment. In the case of C124S-PTEN and A4-C124S-PTEN, the expression of both phospho-PLK1 and phospho-Aurora Kinase A proteins is equal before and after nocodazole treatment. A4-PTEN, despite having higher phosphatase activity, showed a negligible decrease in phospho-PLK1 and phospho-Aurora Kinase A expression. To address this question, we carried out an immunoprecipitation assay, where we found that phospho-dead PTEN is unable to physically interact with Aurora Kinase A and diminished association with PLK1 compared to WT-PTEN. Thus, compromised association with these proteins with phospho-mutant PTEN renders it in hyperphosphorylated

form, thus promoting centrosome amplification hence causing aneuploidy. This is supported by principal component analysis (PCA), which explained 87.0% of the total variation. The aberrations (3- and 4-pole, Mni-24 h, Nbud-24 h, and NPB-24 h) and aneuploidy are positively related to A4-C124S-PTEN and negatively related to wild-type-PTEN. A4-C124S-PTEN, on the other hand, is negatively related to two poles whereas wild-type PTEN is positively related to two poles (S1). Taken together our data are in a model where we suggest that phosphorylation-mediated interaction of PTEN with PLK1 and Aurora Kinase A causes dephosphorylation of those proteins and hence maintained those mitotic kinases in hyperphosphorylated form. Thus, phospho PTEN preserves genomic stability. We are the first ones to indicate that PTEN phosphatase activity in conjunction with phosphorylation is crucial in preserving cell genomic integrity.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflict of interest All the authors declare there is no conflict of interest.

References

1. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science. 1997;275(5308):1943–7. https://doi.org/10.1126/science.275.5308.1943. (**PMID: 9072974**).
2. Eng C. PTEN: one gene, many syndromes. Hum Mutat. 2003;22(3):183–98. https://doi.org/10.1002/humu.10257. (**PMID: 12938083**).
3. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet. 1997;15(4):356–62. https://doi.org/10.1038/ng0497-356. (**PMID: 9090379**).
4. Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP, Tonks NK. The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc Natl Acad Sci USA. 1998;95(23):13513–8. https://doi.org/10.1073/pnas.95.23.13513.PMID:9811831;PMCID:PMC24850.
5. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell. 1998;95(1):29–39. https://doi.org/10.1016/s0092-8674(00)81780-8. (**PMID: 9778245**).
6. Shen WH, Balajee AS, Wang J, Wu H, Eng C, Pandolfi PP, Yin Y. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell. 2007;128(1):157–70. https://doi.org/10.1016/j.cell.2006.11.042. (**PMID: 17218262**).
7. Bassi C, Ho J, Srikumar T, Dowling RJ, Gorrini C, Miller SJ, Mak TW, Neel BG, Raught B, Stambolic V. Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress. Science. 2013;341(6144):395–9. https://doi.org/10.1126/science.1236188.Erratum.In:Science.2013Sep6;341(6150):1064.PMID:23888040;PMCID:PMC5087104.
8. Hou SQ, Ouyang M, Brandmaier A, Hao H, Shen WH. PTEN in the maintenance of genome integrity: From DNA replication to chromosome segregation. BioEssays. 2017;39(10):1700082. https://doi.org/10.1002/bies.201700082.
9. Brandmaier A, Hou SQ, Shen WH. Cell cycle control by PTEN. J Mol Biol. 2017;429(15):2265–77. https://doi.org/10.1016/j.jmb.2017.06.004.
10. Mansour WY, Tennstedt P, Volquardsen J, Oing C, Kluth M, Hube-Magg C, Borgmann K, Simon R, Petersen C, Dikomey E, Rothkamm K. Loss of PTEN-assisted G2/M checkpoint impedes homologous recombination repair and enhances radio-curability and PARP inhibitor treatment response in prostate cancer. Sci Rep. 2018;8(1):3947. https://doi.org/10.1038/s41598-018-22289-7.PMID:29500400;PMCID:PMC5834544.
11. He J, Kang X, Yin Y, Chao KS, Shen WH. PTEN regulates DNA replication progression and stalled fork recovery. Nat Commun. 2015;9(6):7620. https://doi.org/10.1038/ncomms8620.PMID:26158445;PMCID:PMC4499867.
12. Sun Z, Lu J, Wu M, Li M, Bai L, Shi Z, Hao L, Wu Y. Deficiency of PTEN leads to aberrant chromosome segregation through downregulation of MAD2. Mol Med Rep. 2019;20(5):4235–43. https://doi.org/10.3892/mmr.2019.10668.
13. Yuen KW, Montpetit B, Hieter P. The kinetochore and cancer: what's the connection? Curr Opin Cell Biol. 2005;17(6):576–82. https://doi.org/10.1016/j.ceb.2005.09.012. (**Epub 2005 Oct 17 PMID: 16233975**).
14. Puc J, Keniry M, Li HS, Pandita TK, Choudhury AD, Memeo L, Mansukhani M, Murty VV, Gaciong Z, Meek SE, Piwnica-Worms H, Hibshoosh H, Parsons R. Lack of PTEN sequesters CHK1 and initiates genetic instability. Cancer Cell. 2005;7(2):193–204. https://doi.org/10.1016/j.ccr.2005.01.009. (**PMID: 15710331**).
15. Sun Z, Huang C, He J, Lamb KL, Kang X, Gu T, Shen WH, Yin Y. PTEN C-terminal deletion causes genomic instability and tumor development. Cell Rep. 2014;6(5):844–54. https://doi.org/10.1016/j.celrep.2014.01.030.
16. Vazquez F, Grossman SR, Takahashi Y, Rokas MV, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. J Biol Chem. 2001;276(52):48627–30. https://doi.org/10.1074/jbc.C100556200. (**Epub 2001 Nov 13 PMID: 11707428**).
17. Lang V, Aillet F, Da Silva-Ferrada E, Xolalpa W, Zabaleta L, Rivas C, Rodriguez MS. Analysis of PTEN ubiquitylation and SUMOylation using molecular traps. Methods. 2015;77–78:112–8. https://doi.org/10.1016/j.ymeth.2014.09.001. (**Epub 2014 Sep 16 PMID: 25224693**).

18. Meng Z, Jia LF, Gan YH. PTEN activation through K163 acetylation by inhibiting HDAC6 contributes to tumour inhibition. Oncogene. 2016;35(18):2333–44. https://doi.org/10.1038/onc.2015.293. (**Epub 2015 Aug 17 PMID: 26279303**).
19. Zhang Y, Park J, Han SJ, Yang SY, Yoon HJ, Park I, Woo HA, Lee SR. Redox regulation of tumor suppressor PTEN in cell signaling. Redox Biol. 2020;34:101553. https://doi.org/10.1016/j.redox.2020.101553.
20. Choi BH, Pagano M, Dai W. Plk1 protein phosphorylates phosphatase and tensin homolog (PTEN) and regulates its mitotic activity during the cell cycle. J Biol Chem. 2014;289(20):14066–74. https://doi.org/10.1074/jbc.M114.558155.
21. Al-Khouri AM, Ma Y, Togo SH, Williams S, Mustelin T. Cooperative phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) by casein kinases and glycogen synthase kinase 3beta. J Biol Chem. 2005;280(42):35195–202. https://doi.org/10.1074/jbc.M503045200. (**Epub 2005 Aug 17 PMID: 16107342**).
22. Vazquez F, Ramaswamy S, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail regulates protein stability and function. Mol Cell Biol. 2000;20(14):5010–8. https://doi.org/10.1128/mcb.20.14.5010-5018.2000.PMID:10866658;PMCID:PMC85951.
23. Torres J, Pulido R. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus: implications for PTEN stability to proteasome-mediated degradation. J Biol Chem. 2001;276(2):993–8. https://doi.org/10.1074/jbc.M009134200. (**PMID: 11035045**).
24. Ginn-Pease ME, Eng C. Increased nuclear phosphatase and tensin homologue deleted on chromosome 10 is associated with G0–G1 in MCF-7 cells. Cancer Res. 2003;63(2):282–6 (**PMID: 12543774**).
25. Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J, Gavrilova N, Mueller B, Liu X, Wu H. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci USA. 1999;96(11):6199–204. https://doi.org/10.1073/pnas.96.11.6199.PMID:10339565;PMCID:PMC26859.
26. Ma J, Benitez JA, Li J, Miki S, Ponte de Albuquerque C, Galatro T, Orellana L, Zanca C, Reed R, Boyer A, Koga T, Varki NM, Fenton TR, Nagahashi Marie SK, Lindahl E, Gahman TC, Shiau AK, Zhou H, DeGroot J, Sulman EP, Cavenee WK, Kolodner RD, Chen CC, Furnari FB. Inhibition of nuclear PTEN tyrosine phosphorylation enhances glioma radiation sensitivity through attenuated DNA repair. Cancer Cell. 2019;35(3):504–18.e7. doi: https://doi.org/10.1016/j.ccell.2019.01.020. Epub 2019 Feb 28. Erratum in: Cancer Cell. 2019 May 13;35(5):816. Erratum in: Cancer Cell. 2019 Dec 9;36(6):690–91. PMID: 30827889; PMCID: PMC6424615.
27. Misra S, Chowdhury SG, Ghosh G, Mukherjee A, Karmakar P. Both phosphorylation and phosphatase activity of PTEN are required to prevent replication fork progression during stress by inducing heterochromatin. Mutation Res/Fundamental Mol Mech Mutagen. 2022;825:111800. https://doi.org/10.1016/j.mrfmmm.2022.111800.
28. Barr FA, Silljé HH, Nigg EA. Polo-like kinases and the orchestration of cell division. Nat Rev Mol Cell Biol. 2004;5(6):429–40. https://doi.org/10.1038/nrm1401. (**PMID: 15173822**).
29. Strebhardt K. Multifaceted polo-like kinases: drug targets and antitargets for cancer therapy. Nat Rev Drug Discov. 2010;9(8):643–60. https://doi.org/10.1038/nrd3184. (**PMID: 20671765**).
30. Liu X, Erikson RL. Polo-like kinase (Plk)1 depletion induces apoptosis in cancer cells. Proc Natl Acad Sci USA. 2003;100(10):5789–94. https://doi.org/10.1073/pnas.1031523100.
31. Smits VA, Klompmaker R, Arnaud L, Rijksen G, Nigg EA, Medema RH. Polo-like kinase-1 is a target of the DNA damage checkpoint. Nat Cell Biol. 2000;2(9):672–6. https://doi.org/10.1038/35023629. (**PMID: 10980711**).
32. Liu XS, Li H, Song B, Liu X. Polo-like kinase 1 phosphorylation of G2 and S-phase-expressed 1 protein is essential for p53 inactivation during G2 checkpoint recovery. EMBO Rep. 2010;11(8):626–32. https://doi.org/10.1038/embor.2010.90.
33. Bassermann F, Frescas D, Guardavaccaro D, Busino L, Peschiaroli A, Pagano M. The Cdc14B-Cdh1-Plk1 axis controls the G2 DNA-damage-response checkpoint. Cell. 2008;134(2):256–67. https://doi.org/10.1016/j.cell.2008.05.043.PMID:18662541;PMCID:PMC2591934.
34. van Vugt MA, Brás A, Medema RH. Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells. Mol Cell. 2004;15(5):799–811. https://doi.org/10.1016/j.molcel.2004.07.015. (**PMID: 15350223**).
35. Meraldi P, Honda R, Nigg EA. Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53-/- cells. EMBO J. 2002;21(4):483–92. https://doi.org/10.1093/emboj/21.4.483.PMID:11847097;PMCID:PMC125866.
36. Fu J, Bian M, Jiang Q, Zhang C. Roles of Aurora kinases in mitosis and tumorigenesis. Mol Cancer Res. 2007;5(1):1–10. https://doi.org/10.1158/1541-7786.MCR-06-0208. (**PMID: 17259342**).
37. Joukov V, De Nicolo A. Aurora-PLK1 cascades as key signaling modules in the regulation of mitosis. Sci Signal. 2018;11(543):4195. https://doi.org/10.1126/scisignal.aar4195. (**PMID: 30108183**).
38. Sen S, Zhou H, Zhang RD, Yoon DS, Vakar-Lopez F, Ito S, Jiang F, Johnston D, Grossman HB, Ruifrok AC, Katz RL, Brinkley W, Czerniak B. Amplification/overexpression of a mitotic kinase gene in human bladder cancer. J Natl Cancer Inst. 2002;94(17):1320–9. https://doi.org/10.1093/jnci/94.17.1320. (**PMID: 12208897**).
39. Staff S, Isola J, Jumppanen M, Tanner M. Aurora-A gene is frequently amplified in basal-like breast cancer. Oncol Rep. 2010;23(2):307–12 (**PMID: 20043089**).
40. Yang SB, Zhou XB, Zhu HX, Quan LP, Bai JF, He J, Gao YN, Cheng SJ, Xu NZ. Amplification and overexpression of Aurora-A in esophageal squamous cell carcinoma. Oncol Rep. 2007;17(5):1083–8 (**PMID: 17390048**).
41. Mukherjee A, Misra S, Howlett NG, Karmakar P. Multinucleation regulated by the Akt/PTEN signaling pathway is a survival strategy for HepG2 cells. Mutat Res. 2013;755(2):135–40. https://doi.org/10.1016/j.mrgentox.2013.06.009. (**Epub 2013 Jun 21 PMID: 23796964**).
42. Anand S, Penrhyn-Lowe S, Venkitaraman AR. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. Cancer Cell. 2003;3(1):51–62. https://doi.org/10.1016/s1535-6108(02)00235-0. (**PMID: 12559175**).
43. Fenech M. The in vitro micronucleus technique. Mutat Res. 2000;455(1–2):81–95. https://doi.org/10.1016/s0027-5107(00)00065-8. (**PMID: 11113469**).
44. Zorba A, Buosi V, Kutter S, Kern N, Pontiggia F, Cho YJ, Kern D. Molecular mechanism of Aurora A kinase autophosphorylation and its allosteric activation by TPX2. Elife. 2014;3:e02667. https://doi.org/10.7554/eLife.02667.
45. Lens SM, Voest EE, Medema RH. Shared and separate functions of polo-like kinases and aurora kinases in cancer. Nat Rev Cancer. 2010;10(12):825–41. https://doi.org/10.1038/nrc2964. (**Epub 2010 Nov 24 PMID: 21102634**).
46. Mansour WY, Tennstedt P, Volquardsen J, Oing C, Kluth M, Hube-Magg C, Borgmann K, Simon R, Petersen C, Dikomey E, Rothkamm K. Loss of PTEN-assisted G2/M checkpoint impedes homologous recombination repair and enhances radio-curability and PARP inhibitor treatment response in prostate cancer. Sci Rep. 2018;8(1):1–2.

47. Gong L, Govan JM, Evans EB, Dai H, Wang E, Lee SW, Lin HK, Lazar AJ, Mills GB, Lin SY. Nuclear PTEN tumor-suppressor functions through maintaining heterochromatin structure. Cell Cycle. 2015;14(14):2323–32.
48. He J, Zhang Z, Ouyang M, Yang F, Hao H, Lamb KL, Yang J, Yin Y, Shen WH. PTEN regulates EG5 to control spindle architecture and chromosome congression during mitosis. Nat Commun. 2016;5(7):12355. https://doi.org/10.1038/ncomms12355.PMID:27492783;PMCID:PMC4980451.
49. Feng J, Liang J, Li J, Li Y, Liang H, Zhao X, McNutt MA, Yin Y. PTEN controls the DNA replication process through MCM2 in response to replicative stress. Cell Rep. 2015;13(7):1295–303. https://doi.org/10.1016/j.celrep.2015.10.016.
50. Misra S, Mukherjee A, Karmakar P. Phosphorylation of PTEN at STT motif is associated with DNA damage response. Mutat Res. 2014;770:112–9.
51. Steelman LS, Navolanic PM, Sokolosky ML, Taylor JR, Lehmann BD, Chappell WH, Abrams SL, Wong EW, Stadelman KM, Terrian DM, Leslie NR, Martelli AM, Stivala F, Libra M, Franklin RA, McCubrey JA. Suppression of PTEN function increases breast cancer chemotherapeutic drug resistance while conferring sensitivity to mTOR inhibitors. Oncogene. 2008;27(29):4086–95. https://doi.org/10.1038/onc.2008.49.
52. Schmitz M, Grignard G, Margue C, Dippel W, Capesius C, Mossong J, Nathan M, Giacchi S, Scheiden R, Kieffer N. Complete loss of PTEN expression as a possible early prognostic marker for prostate cancer metastasis. Int J Cancer. 2007;120:1284–92. https://doi.org/10.1002/ijc.22359.
53. Da-M Li, Sun H. PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. PNAS. 1998;95(26):15406–11. https://doi.org/10.1073/pnas.95.26.15406.
54. Liu Y, Du X, Zhang S, Liu Y, Zhang Q, Yin Q, McNutt MA, Yin Y. PTEN regulates spindle assembly checkpoint timing through MAD1 in interphase. Oncotarget. 2017;8(58):98040–50. https://doi.org/10.18632/oncotarget.20532.PMID:29228672;PMCID:PMC5716712.
55. Choi BH, Xie S, Dai W. PTEN is a negative regulator of mitotic checkpoint complex during the cell cycle. Exp Hematol Oncol. 2017;29(6):19. https://doi.org/10.1186/s40164-017-0079-0.PMID:28670501;PMCID:PMC5492438.
56. van Ree JH, Nam HJ, Jeganathan KB, Kanakkanthara A, van Deursen JM. Pten regulates spindle pole movement through Dlg1-mediated recruitment of Eg5 to centrosomes. Nat Cell Biol. 2016;18(7):814–21. https://doi.org/10.1038/ncb3369.
57. Leonard MK, Hill NT, Bubulya PA, Kadakia MP. The PTEN-Akt pathway impacts the integrity and composition of mitotic centrosomes. Cell Cycle. 2013;12(9):1406–15. https://doi.org/10.4161/cc.24516.
58. Song MS, Carracedo A, Salmena L, Song SJ, Egia A, Malumbres M, Pandolfi pp. Nuclear PTEN regulates the APC-CDH1 tumor-suppressive complex in a phosphatase-independent manner. Cell. 2011;144(2):187–99. https://doi.org/10.1016/j.cell.2010.12.020.