ROLE OF PTEN'S POST-TRANSLATIONAL MODIFICATIONS IN AUTOPHAGY

Thesis submitted to

Jadavpur University

for the degree of

Doctor of Philosophy (Science)

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "Role of PTEN's post-translational modifications in autophagy" submitted by Mr.Debojyoti De who got his name registered on 21/04/2021 for the award of Ph.D. (Science) degree of JadavpurUniversity, is absolutely based upon his own work under the supervision of Prof. ParimalKarmakar and that neither this thesis nor any part of it has been submitted for either any degreeor any other academic award anywhere before.

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Declaration

I do hereby declare that the work embodied in this thesis entitled "Role of PTEN's post-translational modifications in autophagy" submitted for the award of Doctorate 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.

Date 03/05/2024

Debojyoti De
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Preface

The importance of PTEN lies in the fact that it serves as a guardian of the genome and preserves cellular health. Autophagy is a fundamental cellular phenomenon which also safeguards cells against any undesirable outcome. As we know that post-translational modifications fine-tune several characteristics of a protein, we based our work on finding out the impact of such modifications of PTEN in autophagy. Our work will not only advance the knowledge of these three elements that we based our research on, but also the holistic understanding of cell biology. The entire work has been structured into three chapters. The first chapter comprises of the foundational literature that is required for the understanding of the following results. The discourse on the PTEN domains, PTEN regulation, autophagy regulation is especially important regarding this. The second chapter contains all the results that we recorded, depicting the relationship between sumoylation as well as phosphorylation of PTEN and autophagy. In the third chapter, we talked about the significance of our results and analysed all implications stemming out from each observation. All the materials and methods that we applied are mentioned in a separate section, with a separate segment at the end for the overall conclusion as well.

Acknowledgment

First and foremost, I would want to sincerely thank Prof. Parimal Karmakar for believing in me and giving me the opportunity to work on this project. His insightful criticism and persistent support allowed me to stay motivated and create my finest work. In addition, I want to thank him for his guidance and direction, which made it easier for me to get a lot of the resources I required to complete my project.

I would also like to extend my thanks to our esteemed professors of Department of Life Science & Biotechnology, Jadavpur University, as their encouragement have motivated me to strive for excellence.

I want to express my heartfelt gratitude to my wonderful labmates and colleagues. Your camaraderie and collaborative spirit have made our work environment truly special.

I would also extend my heartfelt appreciation to each member of our dedicated administrative and technical staff for their assistance.

I am grateful to Prof. Prosenjit Sen and Prof. Deepak Kumar Sinha (IACS, Kolkata) as well as Dr. Moumita Dutta (NICED, Kolkata) for allowing me to access their instrumentation facility.

Last but not least I would like to acknowledge DBT, India for awarding me the fellowship to carry out this work as well as DST, India and DSTBT, West Bengal for their financial assistance towards this project.

Index

Abbreviations

AKT: Ak strain transforming

Ambra1: Activating molecule in Beclin 1-regulated autophagy

AMPK: AMP-activated protein kinase

ASD: Autism spectrum disorder

ATF6: Activating Transcription Factor 6

Atg: Autophagy-related genes

ATP: Adenosine triphosphate

Bcl-2: B-cell leukemia/lymphoma 2 protein

Beclin 1: Bcl-2 interacting protein

Bif-1: Bax-interacting factor 1

BiP: Binding immunoglobulin protein

Bmi-1: B cell-specific Moloney murine leukemia virus integration site 1

BSA: Bovine serum albumin

CaMKKβ: Ca2+/calmodulin-dependent protein kinase kinase Beta

cAMP: Cyclic adenosine monophosphate

CBF1: C-repeat binding factor 1

CDK: Cyclin-dependent kinase

ceRNA: Competitive endogenous RNA

CK2: Casein kinase 2

CMA: Chaperone-Mediated Autophagy

DAPI: 4',6-diamidino-2-phenylindole

DAPK1: Death associated protein kinase 1

DFCP1: Double FYVE containing protein 1

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

EBSS: Earle's Balanced Salt Solution

ECL: Enhanced chemiluminescence

EDTA: Ethylenediaminetetraacetic acid

EGR1: Early growth response 1

eIF2α: Eukaryotic initiation factor 2α

EMT: Epithelial–mesenchymal transition

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinase

EVI1: Ecotropic viral integration site 1

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

GABARAP: Gamma-aminobutyric acid receptor-associated protein

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GRP78: Glucose-regulated protein 78

GSK3: Glycogen synthase kinase 3

HA: Hemagglutinin

Hac1: Homologous to ATF/CREB 1

HRP: Horseradish peroxidase

hVPS34: Human Vacuolar protein sorting 34

ID1: Inhibitor of Differentiation 1

IGF: Insulin-like growth factor

IRE1: Inositol-requiring enzyme type 1

IRS: Insulin receptor substrate

JNK: Jun N-terminal kinase

LC3: Light chain 3

LKB1: Liver Kinase B1

MAP: Microtubule-associated protein

MDC: Monodansylcadaverine

MEK: Mitogen-activated protein kinase kinase

MEM: Minimal Essential Medium

miRNA: MicroRNA

mRNA: Messenger ribonucleic acid

Msn: Multicopy suppressor of SNF1 mutation proteins

mTOR: Mammalian target of Rapamycin

NF-κB: Nuclear factor kappa B

NHERF1: Na+/H+ exchanger regulatory factor 1

NOTCH 1: Neurogenic locus notch homolog protein 1

NP-40: Nonidet P-40

PAGE: Polyacrylamide gel electrophoresis

PAS: Pre-autophagosomal structure

PBD: PIP2-binding domain

PBS: Phosphate buffered saline

PDK1: 3-Phosphoinositide-dependent kinase 1

PDZ-BD: PDZ-binding domain

PE: Phosphatidylethanolamine

PERK: Protein kinase RNA-like ER kinase

pH: Potential of Hydrogen

PHTS: PTEN Hamartoma Tumor Syndrome

PI3K: Phosphoinositide-3-kinase

PI3KC3: Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3

PIP3/PIP2: Phosphatidylinositol (3, 4, 5)-trisphosphate/ Phosphatidylinositol (4, 5)-bisphosphate

PKA: Protein kinase A

PKCθ: Protein Kinase C Theta

POPC/POPS: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/serine

PP2A: Protein phosphatase 2A

PPARγ: Peroxisome proliferator-activated receptor gamma

PtdIns3K: Phosphatidylinositol 3-kinase

PTEN: Phosphatase and tensin homolog deleted on chromosome 10

PTENP1: PTEN Pseudogene 1

PTM: Post-translational modifications

PVDF: Polyvinylidene fluoride

Raf: Rapidly accelerated fibrosarcoma

Rag: Recombination-activating genes

Rheb: Ras homolog enriched in brain

RIPA: Radioimmunoprecipitation assay Buffer

RNA: Ribonucleic acid

ROS: Reactive Oxygen Species

RPMI-1640: Roswell park memorial institute-1640

RT: Room Temperature

RTK: Receptor tyrosine kinases

SALL4: Sal-like protein 4

SD: Standard Deviation

SDS: Sodium Dodecyl Sulphate

siRNA: Small interfering RNA

SNAIL: Zinc finger protein SNAI1

SQSTM1: Sequestosome 1

SUMO: Small Ubiquitin-related Modifier

Tap42: Two A phosphatase Associated Protein

TBST: Tris Buffered Saline with 0.1% Tween 20

TEM: Transmission electron microscopy

TEMED: N,N,N'N'-Tetramethylethylenediamine

TORC1: Target of Rapamycin Complex 1

TRIS: Tris(hydroxymethyl)aminomethane

TSC: Tuberous sclerosis complex

ULK1: Unc-51 like autophagy activating kinase 1

UPR: Unfolded protein response

UTR: Untranslated region

WT: Wildtype

Symbols

% percentage

°C degree centigrade

± Plus-minus

h hours

min minutes

L litre

ml millilitre

µg microgram

mm millimetre

µm micrometre

nm nanometre

M molar

mM millimoles

N Number of observations

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Abstract

Autophagy is a cellular phenomenon that simply eliminates 'unwanted' compounds. But beyond this apparent 'destruction', autophagy effectively plays the role of a recycling system. PTEN is a protein, primarily known for its tumour-suppressive actions but it is also involved in varied domains of cell metabolism. Like every other protein, post-translational modifications (PTM) modulate the various functions of PTEN. PTM are chemical modifications that occur on a protein after it has been synthesized and subsequently regulate the activity, localization, and stability of proteins. PTEN can be localized to the nucleus, cytoplasm or cell membrane which accordingly affects its functions and interactions. The domains that constitute PTEN are N-terminal PBD, phosphatase domain, C2 domain, C-terminal tail, and the PDZ-BD. The N-terminal PBD is the attachment site for PTEN's primary substrate PIP2 whereas PTEN's enzymatic nature is instilled in it by the phosphatase domain. The C2 domain assists in the association of this phosphatase domain with the plasma membrane where PTEN can express its phosphatase nature. The C-terminal tail is mainly involved in maintaining stability and function and the PDZ-BD is associated with additional signalling. PTEN exists in two conformations namely open and closed which serve as the gateway for the enzyme's active site. Numerous factors including PTM can modulate this alternating conformation shift. The PI3K/AKT intracellular signaling pathway plays a crucial role in regulating cellular events like autophagy. At the plasma membrane, AKT attaches itself to PIP3 and gets activated. PI3K assists in this conversion of PIP2 to PIP3 while PTEN dephosphorylates PIP3 back to PIP2. Hence, deleted or dysfunctional PTEN results in a dysregulated PI3K/AKT pathway. Two most important PTM that

modulate PTEN function are phosphorylation and sumoylation. PTEN is phosphorylated at several locations, especially within the C-terminal tail, whereas the possible sumoylation sites are all located in the C2 domain.

We wanted to carry out this study on PTEN as it is frequently mutated or deleted in various types of cancer. Also, defective PTEN causes syndrome like PHTS which give rise to a propensity of developing cancers throughout life. Moreover, the paramount importance of a phenomenon like autophagy lies in the exhaustive role it displays in both physiology and pathophysiology. Taking both of these into account, we wanted to examine the effects of PTEN's phosphorylation and sumoylation on autophagy. We set off with finding out the alteration in autophagy patterns with respect to these PTM and the reasons behind it. We tried to further examine these alterations with respect to different causative processes of autophagy namely nutrient stress and ER stress. We finally tested our hypotheses on multiple cell lines, both PTEN null and PTEN positive cells, to reach a befitting consensus.

We found out that transfection of WT PTEN recovered autophagy in PTEN-null PC3 and U87MG cells. Sumoylation of PTEN was found to have boosted autophagy as observed from the reduced expression of autophagy related proteins in sumoylation deficient PTEN mutant transfected cells. Phosphorylation of PTEN curtailed autophagy as observed from the expression of autophagy in dephosphorylation-mimicking and phosphorylation-mimicking PTEN mutants transfected cells. This modulation by sumoylation and phosphorylation was also observed in PTEN positive normal (WI38) and cancer (A549, Hela) cells. The overall reason behind this modulation was that sumoylation of PTEN assisted whereas phosphorylation hindered plasma membrane localisation of PTEN. The

observed effects of alteration in autophagy were similar when surfacing from both nutrient deprivation as well as ER stress, induced by EBSS and Tunicamycin treatment respectively.

WT PTEN transfection had triggered autophagy, and as the starvation period increased, there was a consistent rise in the number of active cells exhibiting autophagy. Because phosphatase deficient PTEN mutant couldn't fully recover autophagy, we could infer that it was catalytically inactive and PTEN's lipid phosphatase activity was deemed essential for the induction of autophagy. The C2 domain of PTEN is mostly responsible for its interaction with the plasma membrane as well as for the correct orientation of its catalytic phosphatase domain. This is crucial from a functional standpoint because PTEN's enzymatic efficiency rely both on its penetration and orientation within the membrane. The location of cellular substrates inside the cell is determined by their sumoylation. PTEN was in fact directed to the cytoplasm by decreased sumoylation, as shown in the sumoylation deficient PTEN mutant transfected cells. PTEN's net positive charge on the C2 domain had supposed to have increased from sumoylation and it was subsequently drawn towards the negatively charged cell membrane, hence facilitating its association. As a result, sumoylation deficient mutant transfected cells showed prolonged AKT and mTOR phosphorylation both of which are deemed essential for subduing autophagy. On the other hand, the phosphorylation-mimicking and dephosphorylation-mimicking PTEN mutant transfected cells showed decreased and increased plasma membrane affinity respectively. Needless to say, the autophagy levels in the cells got changed accordingly. The C-terminal phosphorylation behaved as a molecular clamp on the C2 domain and

consequently determined the aforementioned alteration in open and closed conformation of PTEN.

The parameters that we checked in our experiments for successful autophagy occurrence are namely MDC dye staining that preferentially accumulates in the autophagic vesicles; immunolabeling of LC3B which determines the formation of autophagosomes; western blot of cargo receptor protein p62 which binds to LC3B and provides perception about the autophagic flux; immunoblot of other autophagic marker proteins Beclin1, ATG5, ATG7 which are distinctly regulated during the whole process, and ultimately the ultrastructure of autophagic vesicles inside the cells using TEM. PTEN's sumoylation and phosphorylation status after autophagy was found out using a combination of both immunoprecipitation and western blot. Finally, PTEN's subcellular localisation was ascertained by immunolabeling followed by subsequent inspection under confocal microscopy. PTEN's physiological functions have been repeatedly linked to its defective membrane recruitment. Here, we were able to show how autophagy and PTEN's post-translational modifications were related with respect to cell membrane association. In a nutshell, we had successfully rescued autophagy (stemming from both nutrient deprivation as well as ER stress) in PTEN null cells by transfecting WT PTEN. We had found out that the lipid phosphatase activity of PTEN was responsible for inducing autophagy via PI3K/Akt/mTOR axis. Most importantly we had observed (in both PTEN null and PTEN positive cells) that sumoylation of PTEN was aiding in autophagy while phosphorylation was curbing it. This was occurring due to the modulation of PTEN's cell membrane localisation by the PTM where PTEN's enzymatic activity controls autophagy.

Chapter1

Introduction

1.1 Overview

Overall, our study is based on three broad topics namely PTEN, autophagy and post-translational modifications.

PTEN is a protein that is essential for controlling a variety of cellular processes including autophagy [1][2]. The most important cellular functions like apoptosis, angiogenesis, and cell size can be influenced by PTEN [1][3][4]. Additionally, it can also modulate the actin cytoskeleton and cell adhesion dynamics, which are essential for cell migration and invasion [5][6]. But it's most well-known for its function as a tumour suppressor gene, implying that it stops malignant tumours from growing and spreading [1][3][4][5][6]. PTEN protects the genome by preventing cells from proliferating uncontrolled or avoiding planned cell death [1][3][4][5][6]. So, when PTEN is lost or mutated, unchecked cell proliferation and survival take place, which eventually increases the propensity to form malignant tumours [1][3][4][5][6]. Additionally, its depletion or inactivation is linked to resistance to specific cancer therapies [7]. Furthermore, PTEN's significance goes beyond studies on cancer. According to recent research, it may also be involved in neurological diseases and disorders such as ASD, intellectual difficulties, and developmental delays have all been linked to PTEN abnormalities [8][9]. This demonstrates the complexity of PTEN function and the extensive effects that its faulty action may have on human health. Therefore, it may be possible to develop innovative treatment therapies by comprehending the mechanisms underlying PTEN inactivation and investigating methods to restore its activity.

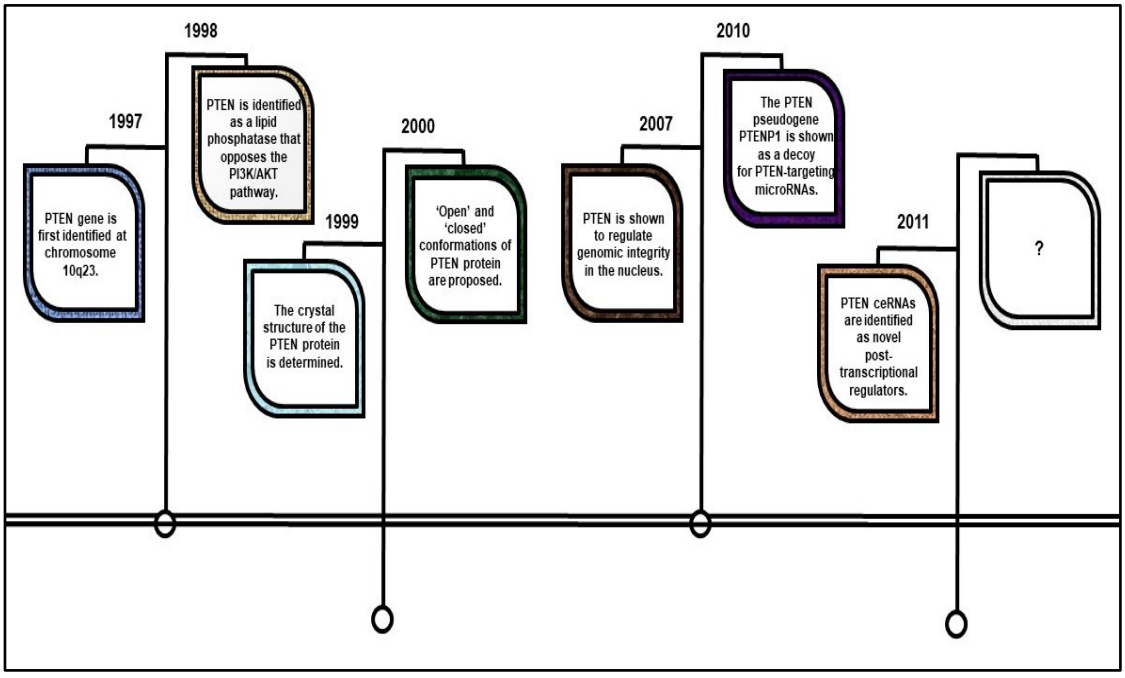


Figure 1.1

Timeline of key events in PTEN research.

Adapted from Song, M. S., Salmena, L., &Pandolfi, P. P. (2012). The functions and regulation of the PTEN tumour suppressor. Nature reviews. Molecular cell biology, 13(5), 283–296. https://doi.org/10.1038/nrm3330

Cells recycle their components through a highly dynamic and regulated process called autophagy. It functions as a cellular quality control mechanism, destroying and recycling intracellular pathogens, misfolded proteins, and damaged organelles in a selective manner, in order to preserve cellular homeostasis [10][11]. The phrase autophagy somewhat implies that cells "eat themselves" during this process [12]. The targeted cargo is engulfed by a double membrane structure known as autophagosome [12][13]. The lysosome, a cellular organelle packed with potent enzymes, and the autophagosome combine to produce an autolysosome [12]. These enzymes then break down the cargo inside the autolysosome, releasing the end products back into the cytoplasm for further use

[13][14]. Autophagy can be broadly classified into three categories: macroautophagy, microautophagy and CMA [15][16][17][18]. The kind of autophagy that has been researched and understood the most is macroautophagy. It entails the widespread breakdown of cytoplasmic elements such as aggregates of proteins, long-lived proteins, and organelles. On the other hand, microautophagy entails the direct invasion of lysosomal membranes to absorb cytoplasmic particles. CMA is a relatively selective type in contrast to the other two forms [15][16][17][18]. Being a highly regulated process, autophagy's dysregulation can cause a plethora of diseases such as metabolic disorders, neurological diseases and even cancers [19]. In the context of tumorigenesis, autophagy can have both favourable and deleterious effects [20]. In one way autophagy can function as a tumour suppressor by breaking down damaged cell parts and stopping the spread of dangerous mutations [20]. On the other hand, autophagy has the potential to accelerate the growth of tumours by giving cancer cells sustenance and energy during nutrient shortage or hypoxia [20]. Autophagy seems promising from a therapeutic perspective, for instance, it has been demonstrated that increasing autophagy can aid in the removal of harmful aggregates of proteins that are typical of neurodegenerative diseases like Alzheimer's and Parkinson's disease [19][21]. In a similar vein, blocking autophagy has been investigated to curate novel anticancer treatments [19]. Hence, the development of innovative therapies for a variety of illnesses could benefit greatly from in-depth study into the mechanics and regulation of autophagy.

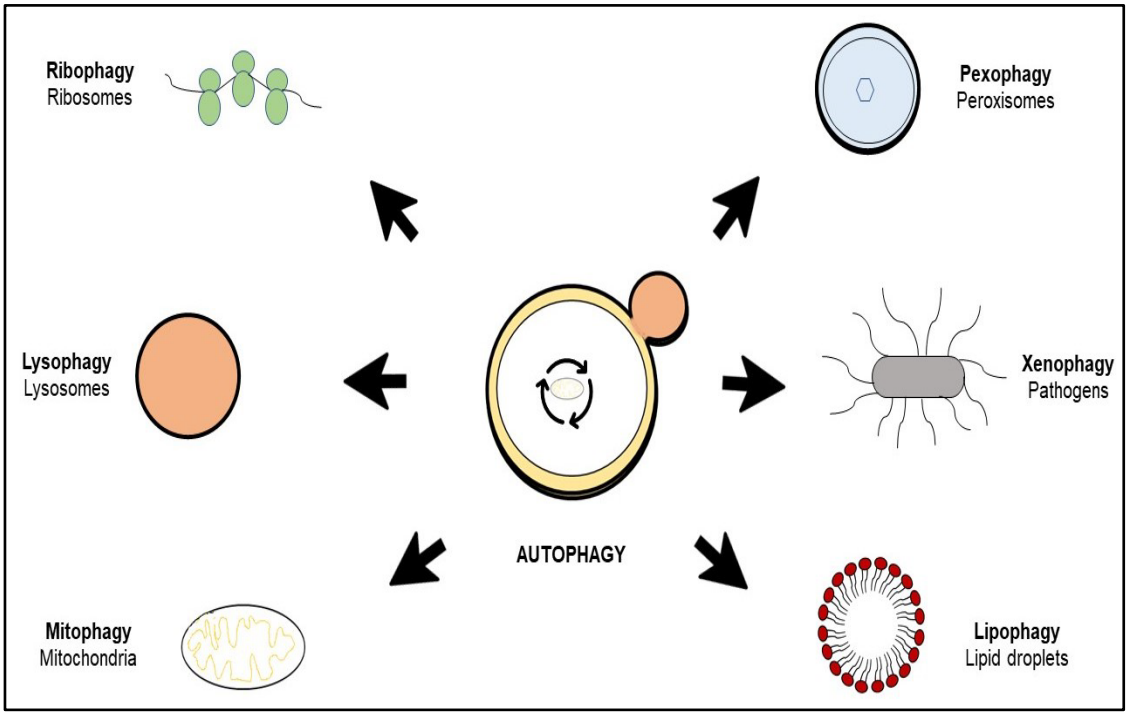


Figure 1.2

Both non-selective or selective types of autophagy can target a broad and heterogeneous range of cytoplasmic entities for lysosomal destruction, whether they are intracellular or extracellular.

Adapted from Galluzzi, L., Baehrecke, E. H., Ballabio, A., Boya, P., Bravo-San Pedro, J. M., Cecconi, F., Choi, A. M., Chu, C. T., Codogno, P., Colombo, M. I., Cuervo, A. M., Debnath, J., Deretic, V., Dikic, I., Eskelinen, E. L., Fimia, G. M., Fulda, S., Gewirtz, D. A., Green, D. R., Hansen, M., … Kroemer, G. (2017). Molecular definitions of autophagy and related processes. The EMBO journal, 36(13), 1811–1836. https://doi.org/10.15252/embj.201796697

Proteins undergo chemical changes known as post-translational modifications (PTM) following synthesis [22][23]. The regulation of protein structure, function, localization, and stability is greatly aided by these alterations [22][23]. They adjust protein activity in response to different biological signals and add to the

proteome's enormous diversity [23]. PTM come in several forms, such as acetylation, glycosylation, sumoylation, methylation and phosphorylation [24][25]. They provide precise control of cellular activities by adding to the total complexity of the proteome [23]. Additionally, PTM have the ability to act combinatorially, which increases the specificity and functional variety of protein regulation [26]. PTMs' dynamic and reversible properties enable cells to carry out intricate biological activities with efficiency and to react swiftly to external stimuli [22][23][24][25][26][27]. To decipher the intricacy of cellular processes and create treatment approaches that specifically target these alterations, it is essential to comprehend the role of PTM and their functional implications.

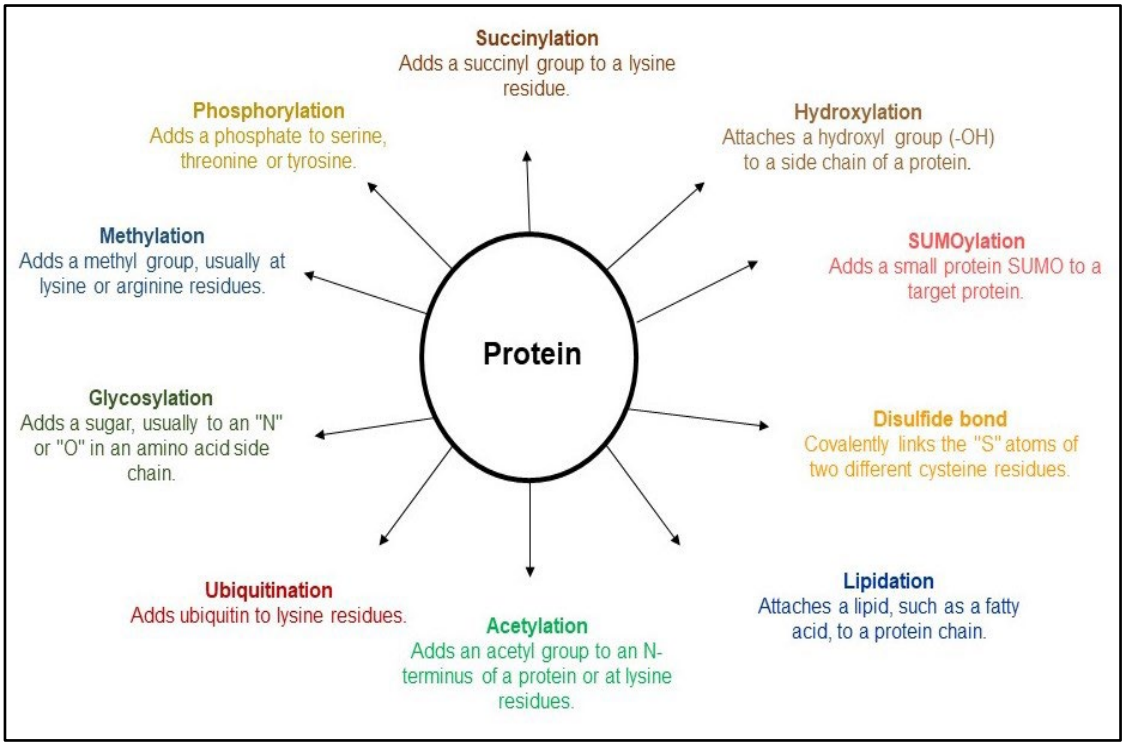


Figure 1.3

Post-translational modifications at a glance.

Adapted from https://www.creative-proteomics.com/pronalyse/protein-modifications-analysis.html

Literature Review

1.2 PTEN domains

The PTEN protein is made up of several domains, each of which performs a distinct function [1][4]. One of the PTEN domains that has been explored the most is the phosphatase domain [1][4]. The phosphatase domain of PTEN inhibits AKT-mediated cellular signaling and PI3K activity by dephosphorylating PIP3, which eventually limits biological phenomenon like cell proliferation [28][29]. In addition to its catalytic roles, this domain also aids in interaction with membranes [28][29]. The C2 domain of PTEN is another essential domain that has been demonstrated to play a role in controlling PTEN's subcellular localization [1][4]. PTEN is targeted to plasma membrane by the C2 domain and this affinity towards membrane association is greater when compared to that of phosphatase domain [28][29]. The location is essential to PTEN's correct operation, as for example, its absence from the plasma membrane would make it more difficult to regulate PI3K/AKT signaling and encourage unchecked cell proliferation [28][29]. Also, by keeping the catalytic domain of PTEN stable, the C2 domain helps to maintain the protein’s effective activation [28][29]. Another essential part of PTEN is its C-terminal tail, which also interacts with different molecules to regulate and contribute to PTEN's overall activity [1][4]. The PTEN's C-terminal tail plays an important role in regulating the protein's subcellular distribution. The C-terminal tail contains distinct motifs that can lead PTEN to the plasma membrane where it subsequently exerts enzymatic activity [28][29]. Furthermore, the PTEN C-terminal tail serves as a location for PTMs such as phosphorylation and ubiquitination, which further modulate PTEN's stability and degradation [28][29]. In addition, the C-terminal tail of PTEN provides binding sites for a variety of

interacting proteins, including the PDZ domain-containing proteins [28][29]. This brings us to the last domain known as PDZ-BD, which helps to engage in interactions with proteins that have PDZ domains [1][4][28][29]. PDZ proteins exhibit dynamic function as scaffolding molecules to arrange and control different signaling pathways [28][29].

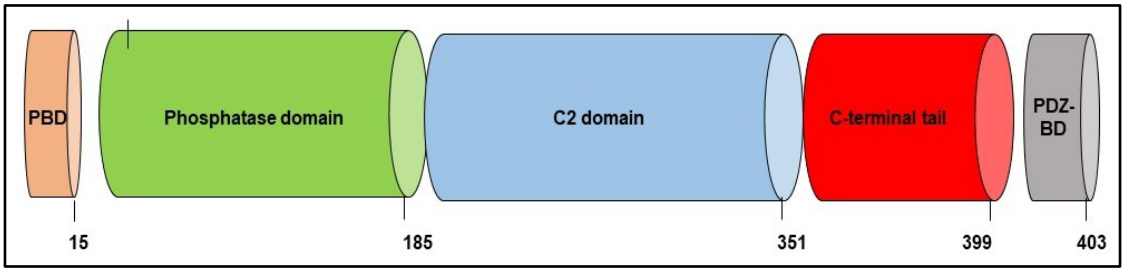


Figure 1.4

The domain structure of PTEN. PTEN is comprised of 403 amino acids and can be divided into five domains.

Adapted from Song, M. S., Salmena, L., &Pandolfi, P. P. (2012). The functions and regulation of the PTEN tumour suppressor. Nature reviews. Molecular cell biology, 13(5), 283–296. https://doi.org/10.1038/nrm3330

1.3 Subcellular Distribution of PTEN

Understanding the subcellular distribution of proteins is critical in cellular biology for unravelling the mysteries of diverse cellular processes. Numerous factors, including lipid binding and protein-protein interactions, affect PTEN localization [29][30]. For example, the interaction between PTEN and NHERF1 enhances PTEN translocation to the plasma membrane, so improving its ability to regulate cell growth and survival [31]. On the other hand, PTEN's tumour-suppressive effects can be diminished by selective ubiquitylation depending on specific subcellular localization [32]. PTEN’s location at the plasma membrane allows it

to directly block PI3K signaling, preventing Akt from being hyperactivated and promoting greater motility and invasive behaviour [29][30]. PTEN expression has also been observed in cellular sites like mitochondria [29][30]. PTEN localization in mitochondria is crucial for preserving its integrity and functionality [33]. PTEN controls the generation of reactive oxygen species in the mitochondria, which are important for a number of biological functions, including cell cycle progression and death [34]. The fact that it is found in mitochondria indicates a further function of PTEN in preserving cellular homeostasis and delaying the development of pathological diseases [33][34]. Another important organelle where PTEN has been seen is the ER [35]. Under stressful ER conditions, PTEN has been shown to affect the UPR. eIF2α is dephosphorylated by PTEN, which leads to the reduction of protein synthesis, hence reducing ER stress and re-establishing cellular homeostasis [36][37]. PTEN is also found in the nucleus, where it directly or indirectly dictates a plethora of cellular activity. PTEN interacts with other proteins in the nucleus and performs phosphatase-independent activities [38][39]. It regulates the expression of several target genes involved in DNA repair and cell cycle control by acting as a transcriptional regulator [40][41]. Furthermore, it has been demonstrated that PTEN interacts with chromatin remodeling complexes to affect transcription factors' ability to access DNA [42]. PTEN's range of nuclear actions broadens its role as a dynamic protein that controls gene expression and preserves genomic stability [38][39][40][41][42]. It has recently been found that PTEN is capable of being secreted from cells, which allows it to continue playing its vital function outside of the cell [28][29][30]. PTEN isoforms are exceptional and potent tools in the fight against cancer because, once produced, it can use its tumour-inhibiting abilities on nearby cells

or distant locations [43]. Secreted PTEN also operates by dephosphorylating PIP3 and inhibiting the PI3K/AKT pathway [43]. Furthermore, secreted PTEN has distinct characteristics that set it apart from its intracellular equivalent. Most importantly, released PTEN is not restricted to the tumour site, rather it can travel throughout the bloodstream, reaching distant metastases and successfully stopping their growth [43]. Thus, the identification of secreted PTEN presents encouraging opportunities for therapeutic intervention [28][29][30]. To take advantage of PTEN's ability to prevent tumour growth, researchers are presently exploring a number of delivery systems for the protein. Such methods entail delivering genes encoding secreted PTEN directly to tumour locations using polymer-lipid nanoparticles or expression of modified viruses [44][45]. As an alternative, PTEN can be delivered to cancer cells specifically via exosomes, which are tiny, membrane-bound vesicles released by cells [46]. There is hope for precise, tailored treatments with little side effects thanks to these creative approaches, like our research on exosomes which suggest the possibility of delivering PTEN from PTEN positive cells to PTEN null cells via exosomes and consequently alleviating DNA damage induced senescence [46].

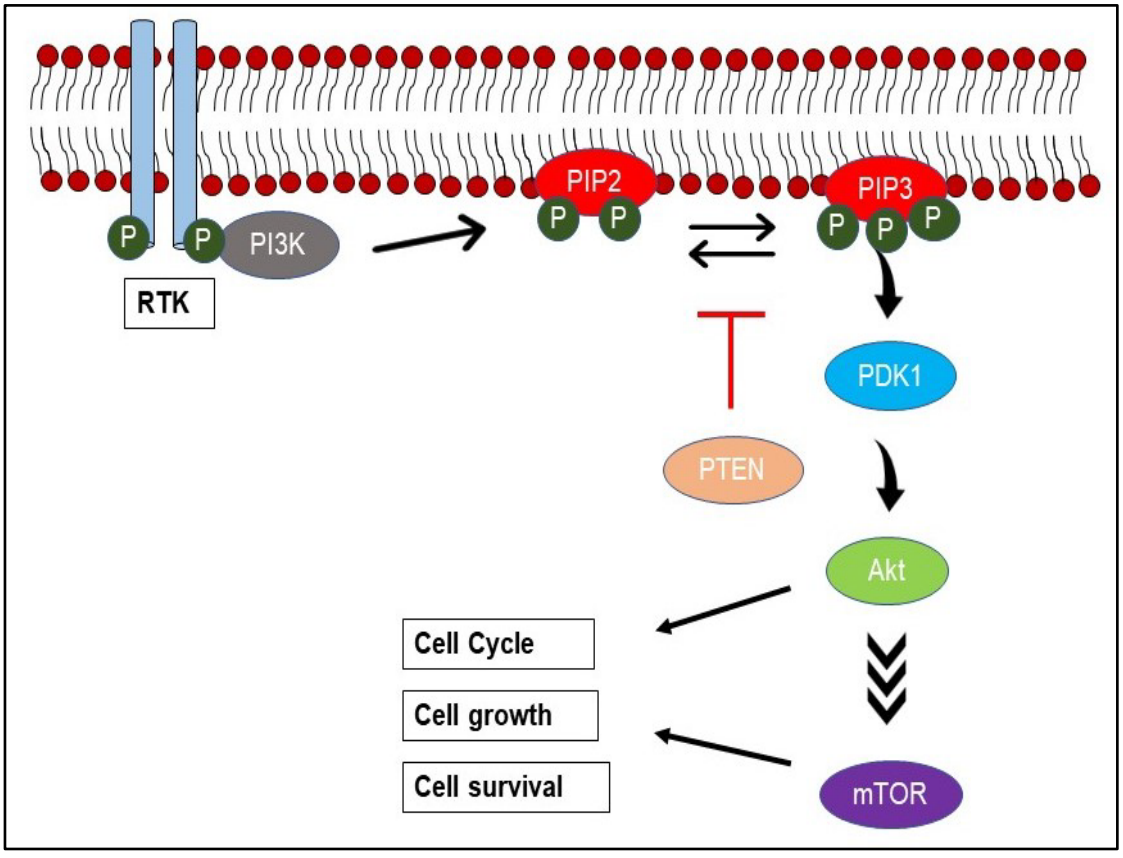


Figure 1.5

The PTEN/PI3K/Akt/mTOR pathway.

Adapted from Phin, S., Moore, M. W., & Cotter, P. D. (2013). Genomic Rearrangements of PTEN in Prostate Cancer. Frontiers in oncology, 3, 240. https://doi.org/10.3389/fonc.2013.00240

1.4 PTEN Regulation

PTEN regulation begins at the gene expression level where it is closely regulated to provide precise PTEN levels [47][48]. The abnormal methylation of the PTEN promoter results in epigenetic silencing and subsequently tumourigenesis [49]. Additionally, the epigenetic silencing is carried out by histone deacetylases and chromatin remodeling enzymes, recruited by transcription factors like SALL4 [50][51]. The transcription factor SNAIL and ID1 have been shown to negatively

regulate PTEN transcription by competing with p53, another transcriptional activator of PTEN [52][53]. NOTCH1 controls PTEN transcription through MYC and CBF1, in a positive and negative manner, respectively [54][55]. PTEN transcription is additionally repressed by three other transcription factors, EVI1, BMI1 and cJUN, which are dysregulated in a variety of malignancies [56][57][58]. PTEN transcription also appears to be downregulated by NF-κB signaling either directly or indirectly, and upregulated by transcription factors EGR1 and PPARγ [59][60][61]. These transcription factors highlight the significance of PTEN in preserving genomic integrity under influence of varied physiological cues, such as oxidative stress and DNA damage [47][48][51].

miRNAs play a function in PTEN regulation as well. It has been shown that many miRNAs downregulate PTEN expression, which in turn promotes cancer or metabolic diseases [62][63]. These comprise, but are not limited to, the miR-17~92 cluster in autoimmunity and lymphoproliferative disorders, miR-19 in colorectal cancer and osteosarcoma, and miR-21 in oral squamous cell carcinoma, breast cancer, and recurrent spontaneous abortions [62][63][64][65].

The identification of a novel mechanism of gene regulation resulted from the revelation that PTENP1 regulates PTEN expression through an unexpected coding independent function [66][67][68]. Significant sequence homology exists between the PTENP1 transcript and PTEN mRNA, especially in the coding region and the first third of its 3′ UTR, which are both enriched for known miRNA target regions [66][67][68]. As a consequence, miRNAs that aim at PTEN, mistakenly target PTENP1 instead [66][67][68]. The undesired effect of miRNAs on PTEN expression and function is prevented because of this [66][67][68]. The finding that

lower PTENP1 expression coincides with disease progression lends credence to the significance of this mechanism [66][67][68].

According to a paradigm called the ceRNA binding hypothesis, both non-coding and protein-coding genes have a unique mRNA-dependent non-coding role that allows them to behave as a decoy to prevent the action of specific miRNAs on other RNAs [68][69]. This vast regulatory network has also been found to regulate PTEN expression by sequestering PTEN-targeting miRNAs in cancers [68][70]. For example, the tumour suppressive function of PTEN ceRNAs was shown in an oncogenic BRAF-induced mouse model of melanoma [68][70]. Concurrently, a novel computational method has identified over 7000 potential ceRNAs that could potentially mediate thousands of pairwise gene interactions in tumours [70].

The previously mentioned post-translational modifications also regulate both the stability and function of PTEN [71][72][73]. We explored two of them in our research, phosphorylation as well as sumoylation. PTEN's C-terminal tail contains particular residues where phosphorylation events primarily occur [74]. These residues are regulated by several kinases, such as CK2, GSK3 [75]. PTEN can be phosphorylated at a number of locations, including tyrosine, serine, and threonine residues [71][73][75]. PTEN experiences structural changes upon phosphorylation, which influences its stability, intracellular location, and enzymatic activity [71][72][73][74][75]. PTEN's interaction with other proteins and the following signaling cascades are also modulated by its phosphorylation [75]. One of the most important effects of PTEN’s phosphorylation is on the PI3K/Akt signaling pathway. When PTEN is phosphorylated, its phosphatase activity is inhibited, which consequently activates Akt and triggers further

signaling events, like enhanced cell survival and proliferation [74][75]. Furthermore, additional proteins involved in cellular processes such as cell migration and nuclear export can interact with phosphorylated PTEN [71][73][74][75]. PTEN's phosphorylation-induced inactivation promotes cancer by enabling unchecked cellular proliferation as mentioned above [75]. In addition, PTEN phosphorylation anomalies may give resistance to targeted cancer treatments that obstruct the PI3K/Akt pathway [74][75]. Furthermore, abnormal neural connections and anomalies in brain are caused by disrupted PTEN phosphorylation, which impairs PTEN's normal physiological roles in neuronal development [74][76].

Sumoylation is the reversible covalent binding of SUMO proteins to particular target proteins which generally contain lysine residues [77]. PTEN sumoylation is achieved by conjugating either SUMO1 or SUMO2/3, mainly at residues 254, 266, and 289 [73][75][78]. According to experimental results, sumoylation increases PTEN phosphatase activity, stabilizes PTEN, and inhibits ubiquitin-mediated degradation of PTEN [73][75][78]. Furthermore, it has been demonstrated that sumoylation controls PTEN subcellular localization, including facilitating its translocation to the nucleus, where it can influence a range of transcriptional processes [73][75]. All these results suggest that PTEN sumoylation is essential for preserving PTEN-mediated cellular homeostasis. A number of malignancies and even EMT have been linked to aberrant sumoylation patterns [79]. PTEN function may be compromised by these dysregulated sumoylation events, which can result in increased DNA damage, cell proliferation, and altered subcellular localization-all characteristics that mark the advancement of cancer [73][75][78].

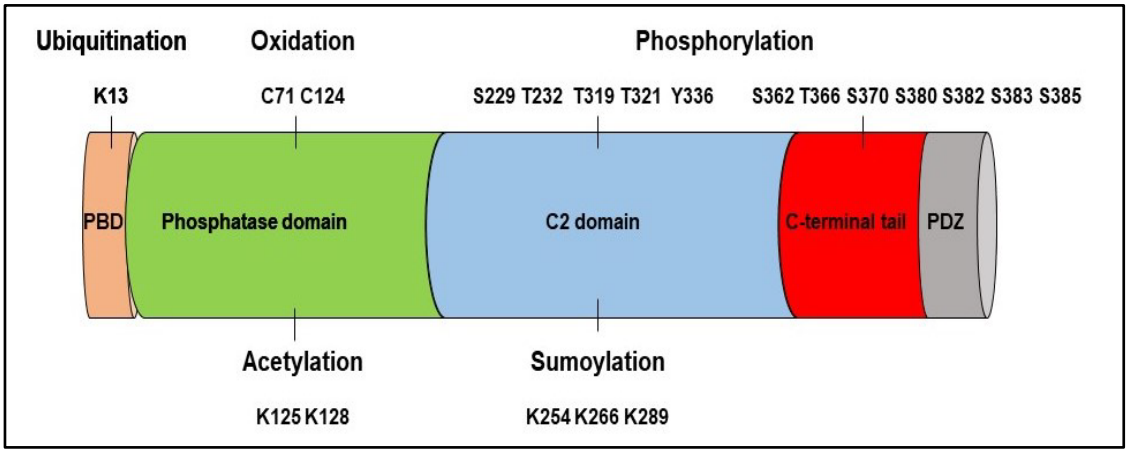


Figure 1.6

Major sites for post-translational modifications of PTEN.

Adapted from https://www.cytoskeleton.com/april-newsletter-posttranslational-regulation-of-phosphatase-and-tensin-homolog-pten

1.5 PTEN and Autophagy

The capacity of PTEN to control the PI3K/AKT/mTOR signaling pathway is fundamental to its role of controlling the cellular processes including autophagy [80][81][82][83]. Overall, activation of PI3K leads to the catalysis of PIP2 lipids to PIP3 [80][81][82][83]. Akt binds to PIP3 at the cell membrane, allowing PDK1 to access and subsequently phosphorylate it, activating it as a result [81][82][83]. AKT further phosphorylates and activates a number of downstream targets, including mTOR which in turn exhibits active role in curtailing autophagy [80][81][82][83][84][85]. PTEN inhibits the PI3K/AKT pathway by dephosphorylating PIP3 to PIP2 and as a result preventing PIP3 membrane recruitment and activation of AKT [81][83]. This impairs downstream signaling via the mTOR proteins and consequently induces autophagy [84][85].

1.6 Autophagy Machinery

The process of autophagy can be broken down into mechanistically separate processes, such as induction, vesicle production, autophagosome-lysosome fusion, cargo detection and selection, and the resultant breakdown that is followed by release of the degradation products back into the cytosol [86]. These stages involve several sets of Atg proteins, which make up the basic autophagic machinery [87]. The double membrane structure better known as the isolation membrane or phagophore sequesters the designated cargo. The two primary stages of phagophore production are initiation and elongation [86]. The first step in this process is the activation of Atg1 kinase complex or ULK1 complex in case of yeast or mammalian cells respectively [88]. Nutrient sensors control this complex, which transduces signals to synchronize the onset of autophagy [88]. The phagophore assembly site, which is frequently located in certain subcellular areas like the ER or Mitochondria, is where the Atg1/ULK1 complex attracts more proteins during activation [89][90]. After the aforementioned initiation, vesicles from different cellular compartments are recruited and integrated, causing the phagophore to lengthen [89][90]. PI3KC3 complex catalyzes the synthesis of PI3P, which is essential for phagophore elongation as it attracts particular effector proteins [91]. The phagophore's elongation and subsequent maturation depends on such effectors like DFCP1 [86][90]. Beclin-1, Vps34, BIF-1, Ambra1 and ATG14L are additional components that are either part of or help to form this complex [86][88][89][90][91]. Phagophore development can occur via a variety of processes, resulting in distinct forms of phagophores. One type is the Atg9 reservoir-derived phagophore, in which the transmembrane protein Atg9 functions as a mobile source of membranes to generate phagophores [87][90][92]. To

supply lipids for phagophore production, Atg9 traffics between many compartments, including the endosomes, Golgi complex and even membranes associated with the mitochondria [88][93]. Another kind is the phagophore formed from omegasomes, which develops from the membrane extensions of ER [94]. Rich in PI3P, omegasomes attract the previously mentioned effector DFCP1 which can additionally serve as their marker [92][94]. Lipids from the ER are supplied to the accumulating phagophore, facilitating its growth [95]. Apart from ER, mitochondria also aid in the creation of phagophores by supplying lipids like cardiolipin, through membranes generated from the mitochondria [96]. Next, the conjugation between Atg5 and Atg12, which is essential for autophagosome formation, is at the centre of the autophagic pathway [97][98][99]. The development of Atg8/LC3-positive autophagosome membranes, which are essential for carrying out the sequestration and destruction of cargo, is mostly mediated by Atg5 and Atg12 [97][98][99]. During the initial phase of the Atg5-Atg12 conjugation process, Atg7 (resembling E1 enzyme) forms a thioester bond with the C-terminus of Atg12 to activate Atg12 [88]. After this activation, a transthiolation process transfers the activated Atg12 to Atg10 (resembling E2 enzyme) [100]. The conjugation between Atg5 and Atg12 is the next phase in this complex dance [97][98][99]. This mechanism is also mediated by Atg10, which catalyzes the formation of an isopeptide bond between Atg5 and Atg12 [88]. Further promoting autophagosome formation, the ensuing Atg5-Atg12 conjugation serves as a platform for the recruitment and assembly of other Atg proteins [86]. For example, Atg16L (resembling E3 enzyme) produces a large molecular weight complex that localizes to the site of autophagosome formation when it binds to the Atg12 moiety of the conjugate [88]. Proper autophagosomal

membrane expansion and assembly depend on the interaction between Atg16L and the Atg5-Atg12 conjugation [88][89]. The next important player in this signaling cascade is LC3B [95][98]. Atg4, a cysteine protease, cleaves LC3B proteolytically to produce LC3B-I when autophagy is induced. Similarly, like the aforementioned ubiquitin-like system, the exposed glycine from cleaving gets bound to ATG7 (resembling E1 enzyme) [88][95]. Then, following its transfer to Atg3 (resembling E2 enzyme), LC3B-I is fully activated and subsequently converted to processed LC3B-II by conjugating PE to the carboxyl glycine [88][95]. So, LC3B-II is basically assembled and incorporated into the isolation membrane, aided by Atg5–Atg12 [95]. Both the outer and inner surfaces of autophagosome comprise of LC3B-II [95]. This molecule is not only associated with the hemifusion of the phagophore membrane but also the 'hand-picking' of the materials to be recycled [88][95]. Another ATG8 ortholog GABARAP undergoes similar activation and is located at autophagosomes along with LC3 [88][95]. To guarantee that LC3B-PE conjugation plays a precise spatiotemporal role in autophagosome formation, its dynamics are strictly regulated. Unwanted and excessive LC3B-PE conjugation is prevented by the same Atg4, which acts now as a deconjugating enzyme [88][90]. As a result, LC3B-PE conjugation gets released from the autophagosome membrane, thus preventing unchecked autophagy induction and preserving the delicate balance between autophagy and cell survival [88][90]. Because autophagy seems to consume cytosol without discrimination, it has generally been thought of as a random process [95]. On the contrary, there is mounting evidence that the expanding phagophore membrane can interact with organelles and protein aggregates in a targeted manner. As previously mentioned, LC3B-II, for selection of the cargo, correspond to adaptor

molecules on them [89][90][92][98][99]. In this context, the most well-characterized molecule is p62/SQSTM1, a multifunctional adaptor molecule that facilitates the turnover of aggregates of poly-ubiquitinated proteins [89][90][92][95][98][99]. Lysosome is a single membrane bound organelle which merges with the autophagosome to build another specific compartment called autolysosome [98][99]. This is the penultimate stage before the final degradative process, after fusing the growing ends of the phagophore membrane has been properly executed [86][98][99]. But before the ultimate degradative action of the acid proteases of the lysosome, the pH of the compartment gets already reduced by the combination of the endosomes and the autophagosomes [95]. Cathepsin proteases such as B and D (lysosomal proteases) are additionally necessary for autophagosome turnover and autolysosome maturation within the lysosome [95].

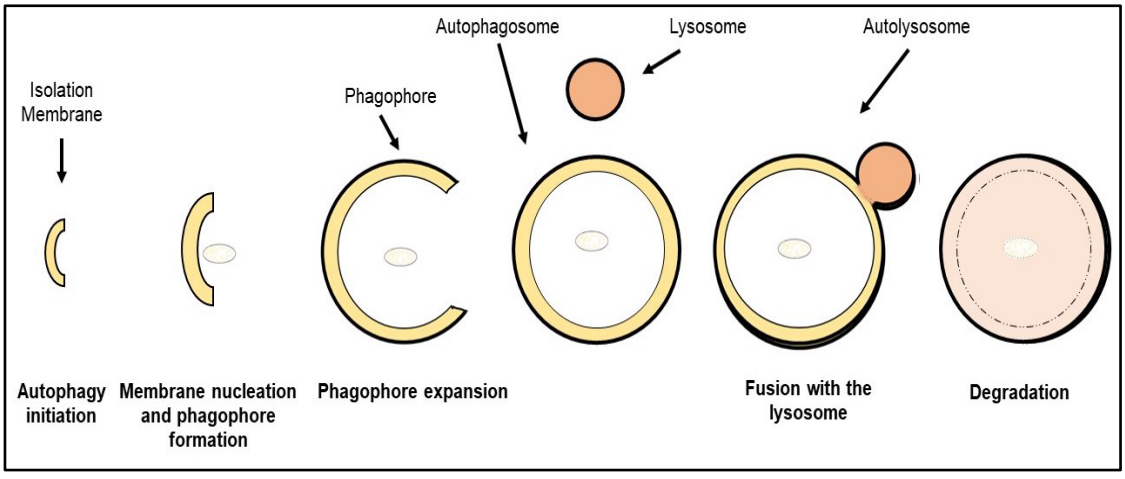


Figure 1.7

The major steps of macroautophagy are Initiation, Elongation, Autophagosome formation, Fusion and Degradation.

Adapted from Hansen, M., Rubinsztein, D. C., & Walker, D. W. (2018). Autophagy as a promoter of longevity: insights from model organisms. Nature

reviews. Molecular cell biology, 19(9), 579–593. https://doi.org/10.1038/s41580-018-0033-y

1.7 Autophagy Regulation

A significant increase in autophagosome production occurs during nutrient restriction [101]. The Ras-cAMP-PKA and TOR signaling pathways perceive nutrient condition, and control autophagy [101]. Rapamycin has the ability to inhibit TORC1 [102]. Even in the presence of nutrients, rapamycin-induced inactivation of TORC1 promotes autophagy, indicating that TOR inhibits autophagy [102]. It is hypothesized that mTORC1 directly detects nutrient signals and is subsequently phosphorylated in response [85]. As a reaction to amino acid feedback, Rag proteins induce TORC1 by translocating it to its activator Rheb [103][104][105][106]. Additionally, amino acids use class III PtdIns3K (hVps34) to activate mTOR [105][107][108]. Amino acid stimulation of hVps34 results in autophagy inhibition and mTOR activation [105][107][108]. In addition to controlling the Atg1/ULK complex, TORC1 inhibits autophagy in yeast by phosphorylating Tap42, which triggers the catalytic subunits of PP2A, a negative autophagy regulator [109][110][111]. From yeast to mammals, the Ras/cAMP dependent PKA signaling pathway is crucial for glucose sensing [111]. The regulatory component Bcy1 is present in yeast PKA [110][111][112]. When there is an ample availability of nutrients, Ras1 as well as Ras2 promote adenylyl cyclase's production of cAMP [111]. Increased cAMP binds to Bcy1 and causes PKA to be inhibited [111]. In yeast, constitutive activation of the Ras/PKA route inhibits autophagy produced by TOR inhibition, indicating that the Ras-PKA system and the TOR pathway both downregulate autophagy [110]. Ras/PKA may

impede autophagy via regulating Atg1, which has been found to be a PKA phosphorylation substrate [111]. While Atg1 is dephosphorylated and localized to the PAS during starvation, PKA phosphorylation causes Atg1 to be mostly cytosolic and dissociated from the PAS in the presence of nutrient availability [101]. Nutrient sensing also involves protein kinase Sch9, the closest yeast homolog to both the TOR target S6K and the mammalian Akt [111]. Autophagy is induced by simultaneous inactivation of PKA and Sch9 which can be further enhanced by inactivating TORC1 [112]. This indicates that autophagy in yeast is negatively controlled by a minimum of three concurrent pathways: TORC1, Ras/PKA, and Sch9. Ras/PKA and Sch9 may regulate autophagy at the transcriptional level but not TOR [112]. This can be inferred from the observation that transcription factors Rim15 kinase as well as Msn2/4 are needed for PKA and Sch9 activity but not TOR [112].

Even in the presence of adequate nutrients, autophagy is triggered when growth factors are removed from the extracellular milieu [113]. The routes by which nutrients and hormones control autophagy in higher eukaryotes differ, but all ultimately intersect at mTOR [101]. mTOR is regulated by insulin as well as IGFs via class I PtdIns3K [114][115]. Insulin binding causes IRS1 and IRS2 to be recruited and phosphorylated [101][114][115]. This process produces a docking scaffold that facilitates the binding of class I PtdIns3K subunits and other adaptor proteins [101][114][115]. Akt and its activator PDK1 are recruited to the membrane more frequently when PIP3 is generated by the class I PtdIns3K [80][81]. This causes PDK1 to phosphorylate and activate Akt [80][81]. Akt activation further upregulates the tumour suppressor TSC2 protein's phosphorylation [80][81]. As a consequence, TSC1 TSC2 interaction gets blocked

leading to the lack of TSC1/2 complex [80][81]. The successful formation of this complex negatively regulates mTOR [80][81][116]. The lack of hormones causes mTOR to become inactive, which relieves the autophagy-inhibiting action [114][115]. In addition to TOR, Ras signaling is involved in growth factor-mediated autophagy control [117]. Ras transmits signals from growth factor RTK to effectors such as class I PtdIns3K as well as Raf-1/MAP kinases [117]. One important Ras effector Raf-1 upregulates autophagy by sensing amino acids deficiency [118]. In this case, the Raf-1 kinase is the target of amino acids, which suppress its activity and consequently downregulates the activity of the downstream MEK1/2 as well as ERK1/2 kinases and subsequently autophagy [118]. This inhibition is reversed and ERK1/2 as well as autophagy are induced by amino acid deficiency [118]. This implies that the Ras-Raf-1-ERK1/2 and the Ras-PtdIns3K cascade act against each other while influencing autophagy, in a situation of amino acids deprivation vs growth factors shortage [101]. AMPK detects a decreased level of cellular energy (ATP) in mammalian cells [119]. A lower ATP/AMP ratio activates AMPK via the upstream LKB1 kinase [119]. The previously mentioned TSC1/2 complex is also phosphorylated and activated by active AMPK, which further inhibits mTOR action by means of Rheb [116][119]. The resulting autophagy leads to increased ATP generation via nutrition recycling [119]. Furthermore, in response to stress caused by nutrient deprivation and growth factor withdrawal, cell cycle arrest can take place via CDK inhibitor p27kip1 through LKB1-AMPK pathway [120]. This process is necessary to stop cells from going through apoptosis and instead trigger autophagy for survival [120].

Autophagy is also strongly induced by numerous stress factors that can stem from both inside and outside of cells [101]. The expression of aggregate-prone proteins, glucose deprivation (which reduces glycosylation and energy for chaperone activity), hypoxia and oxidative stress (which reduces disulfide bond formation), and Ca^{2+} efflux from the ER are few examples of ER stress stimuli that cause the ER to accumulate more unfolded proteins than it can process [101][121]. ER stress causes autophagy which depending upon the particular stress conditions and the species, connect to several signaling pathways [101][121]. Tunicamycin hampers N-glycosylation and can thus act as an ER stressor [122]. The consequent cell survival is achieved by compensatory clearance of the misfolded proteins within the ER as well as the enlarged and disorderly ER that arises from UPR [101][121]. In yeast, the UPR signaling pathway is mediated by Ire1 and its substrate Hac1 [123]. The Ire1-Hac1 pathway appears to be dispensable for the transcriptional upregulation of ATG8, despite being necessary for the induction of autophagy by ER stress [124]. Grp78/BiP, the UPR regulator, positively regulates the formation of the phagophore which becomes evident when its siRNA inhibition stops the formation of autophagosome [125]. Compared to yeast, mammalian UPR signaling is more intricate and involves three different downstream pathways: IRE1, ATF6, and PERK [121][126]. These elements trigger the transcription of several target genes after receiving an indication of the presence of misfolded proteins in the ER [121][126]. JNK is one of IRE1's downstream targets which is necessary for the tunicamycin-influenced lipid conjugation of LC3 [121][126]. PERK phosphorylates eIF2α which leads to the conversion of LC3-I to LC3-II, in response to ER stressors like mutant polyQ72 proteins [127]. ATF6 can also sense misfolded proteins and gets activated. In

order to induce autophagy, it binds to the promoter of DAPK1 which consequently gets activated and phosphorylates Beclin1 [128]. ER stress also causes the release of luminal Ca^{2+} into the cytosol in addition to UPR signaling [129]. An increase in intracellular Ca^{2+} levels stimulate CaMKKβ, which in turn activates AMPK to switch on autophagy [129]. PKCθ is also phosphorylated in response to elevated Ca^{2+} levels, leading to LC3 conversion and autophagy in reaction to ER stressors like tunicamycin [130]. While the aforementioned research indicates that autophagy triggered by ER stress aids in mammalian cell survival, other findings imply that autophagic cell death could result from ER stressors [131]. When renal tubules of mice are injected with tunicamycin, DAPK catalyzes both autophagy and apoptosis, resulting in cell death [132]. Activated DAPK presumably initiates autophagy by phosphorylating Beclin1 and encouraging Beclin1 separation from Bcl-2 [133]. One recurring topic is this particular possibility that autophagy has parallel roles in deciding cell destiny, depending on particular cell types and stimuli [131].

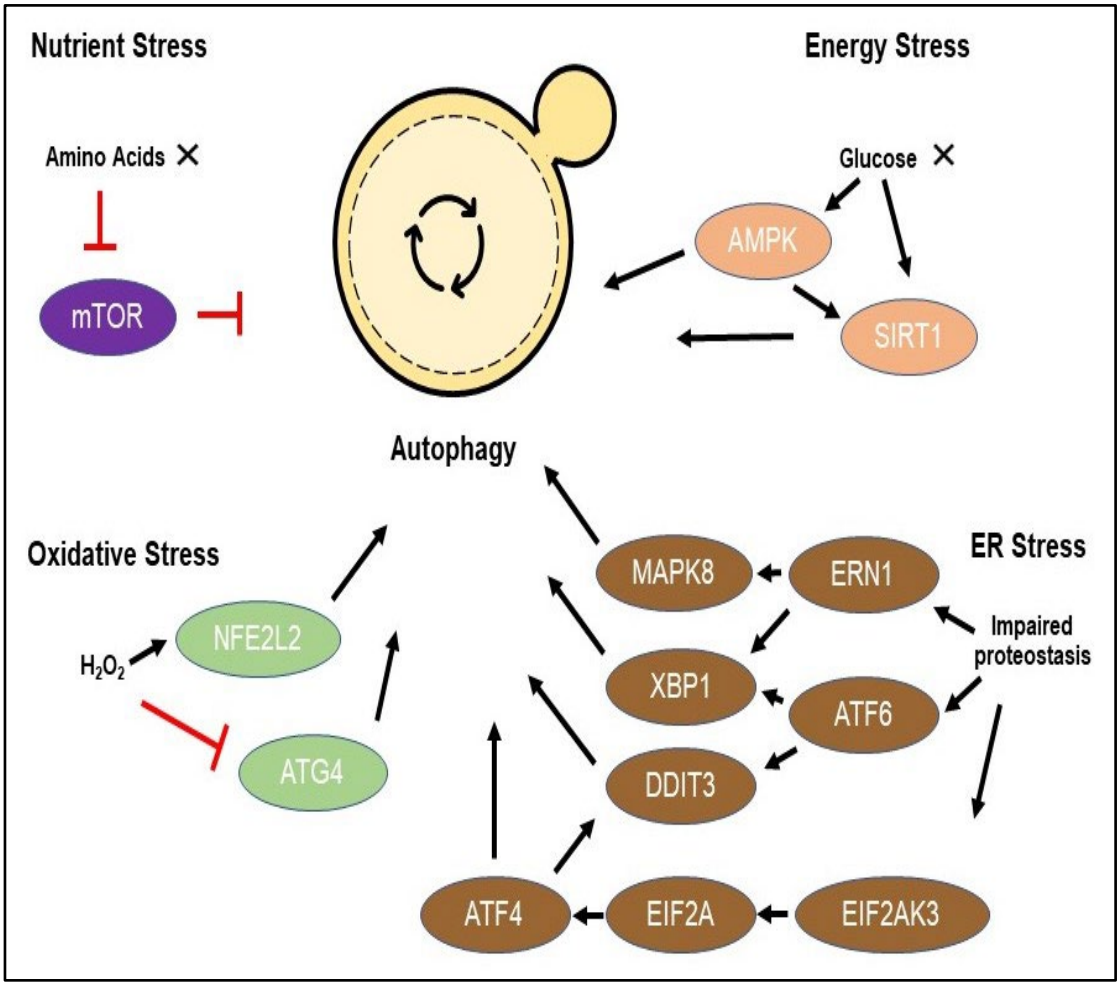


Figure 1.8

Autophagy regulation under different kinds of stress.

Adapted from Lei, Y., Huang, Y., Wen, X., Yin, Z., Zhang, Z., &Klionsky, D. J. (2022). How Cells Deal with the Fluctuating Environment: Autophagy Regulation under Stress in Yeast and Mammalian Systems. Antioxidants (Basel, Switzerland), 11(2), 304. https://doi.org/10.3390/antiox11020304

Rationale

Different malignancies can form and progress as a result of compromised or transformed PTEN [134][135][136][137]. A variety of cancers, including those of the prostate, uterus, brain, have been linked to PTEN's dysregulation [134][135][136][137]. When PTEN function is lost as a result of genetic or epigenetic changes, PI3K signaling is hyperactivated, which promotes cell survival, proliferation, and resistance to apoptosis [134][135][136][137]. Furthermore, poor clinical outcomes and resistance to traditional cancer therapy are frequently correlated with PTEN changes [134][135][136][137]. The importance of PTEN mutations in cancer goes beyond prognosis and diagnostics towards treatment approaches. Targeting the PI3K pathway has grown in popularity as a treatment strategy since PTEN loss frequently causes this pathway to become activated [134][135][137]. Numerous PI3K inhibitors, including copanlisib and idelalisib, have demonstrated encouraging efficacy in preclinical and clinical investigations [138]. Moreover, determining PTEN mutations in cancer patients can aid in predicting therapeutic outcomes and customizing treatment approaches [134][135][136][137]. A rare genetic disorder known as PHTS is characterized by the development of different benign growths, or hamartomas, and a higher risk of malignancy [139][140]. PHTS encompasses abnormalities including Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome [139][140]. Germline mutations in PTEN are frequently the cause of these illnesses, which can damage several organs and cause a range of symptoms, such as skin deformities and cognitive deficits [139][140]. PTEN is also linked to a number of neurological illnesses in addition to its roles in cancer and developmental problems [141][142]. PTEN mutations have been discovered in

patients with macrocephaly, a disorder marked by an increased head circumference, and ASD [141][142]. ASD and other neurodevelopmental disorders may arise as a result of PTEN loss or malfunction, which alters the complex signaling pathways in the brain and affects neuronal growth and connections [141[142]. The general health and well-being of those who suffer from these disorders may be enhanced by novel approaches to the treatment and prevention of diseases linked to PTEN deficiency. Also, diabetes and cardiovascular disorders are among the plethora of illnesses linked to PTM dysregulation [143].

Disease	PTEN defects	Cancer susceptibility
Cowden's syndrome	Splice variants Deletion: coding sequence promoter Nonsense mutation Missense mutation C124: no phosphatase activity, G129: no lipid phosphatase activity, K289: no nuclear translocation.	Breast Thyroid Endometrium.
Bannayan-Riley-Ruvalcaba syndrome	Deletion Nonsense mutation Missense mutation	Breast Thyroid Endometrium Colorectal
Proteus/Proteus-like syndrome	Missense mutation	Cystadenoma of the ovary Testicular tumours CNS tumours Parotid monomorphic adenoma.

Figure 1.9

PHTS and associated cancer susceptibility.

Adapted from Rodriguez, S., & Huynh-Do, U. (2012). The Role of PTEN in Tumor Angiogenesis. Journal of oncology, 2012, 141236. https://doi.org/10.1155/2012/141236

As autophagy can either stimulate or inhibit tumour growth depending on the situation, the intricate interaction between autophagy and cancer is still being studied. One way autophagy affects the progression of cancer is by functioning as a mechanism for tumour suppression [144][145][146][147][149]. Under normal circumstances, autophagy guarantees the removal of damaged organelle, thus inhibiting the build-up of harmful substances [146][148][151][153]. By doing so, the growth of cancerous cells is inhibited and the likelihood of cancer developing is decreased [146][148][151][153]. Furthermore, autophagy eliminates dangerous reactive oxygen species, which have been shown to induce DNA damage and genomic instability, further resulting in the creation of cancer cells [144][145][147][149][150]. However, autophagy can act in two ways in tumours that have already advanced, it can either encourage or impede the growth of the malignancy [148][151]. Autophagy has the ability to stop the build-up of damaged proteins and organelles during the early stages of carcinogenesis [147][148][151][152]. This is necessary because it stops the formation of tumours, as previously mentioned. On the other hand, under nutritional scarcity, autophagy can supply cancer cells with vital nutrients and energy, allowing them to withstand adverse circumstances and carry-on proliferating [148][151]. This shows that autophagy may give cancer cells a survival edge and increase their resistance to therapeutic interventions [144][148][151]. Comprehending the correlation between autophagy and cancer holds noteworthy consequences for the advancement of innovative cancer treatments. Creating tailored therapies for different kinds of cancer may depend on controlling autophagic responses. For example, inhibitors of autophagy may be used in conjunction with current medicines to increase their effectiveness in tumours where autophagy supports

cancer cell survival [147][148][149][151][152]. On the other hand, treatments that promote or activate autophagy may be helpful in stopping the spread of cancer in tumours where autophagy inhibits growth [147][148][149][151][152]. The pathophysiology of various diseases, including metabolic syndromes and viral infections, is also linked to dysregulation of autophagy [154][155][156]. Alpha-synuclein and other aggregated proteins build up in Parkinson's disease due to defective autophagy, preventing them from being cleared and causes neurodegeneration [154]. Moreover, a number of metabolic disorders such as type 2 diabetes and obesity have also been linked to abnormalities in autophagy [155]. These metabolic illnesses are partly caused by impaired autophagy in metabolic tissues, including the liver and pancreas [154][155].

Autophagy is an essential component in preserving metabolic homeostasis because it controls insulin signaling, lipid droplet disintegration, and cellular energy metabolism [154][155]. To enhance diagnosis, mitigate and treat these crippling illnesses, more study is necessary to determine the precise mechanisms driving autophagy dysregulation and to devise practical methods of modulating this process in disease situations.

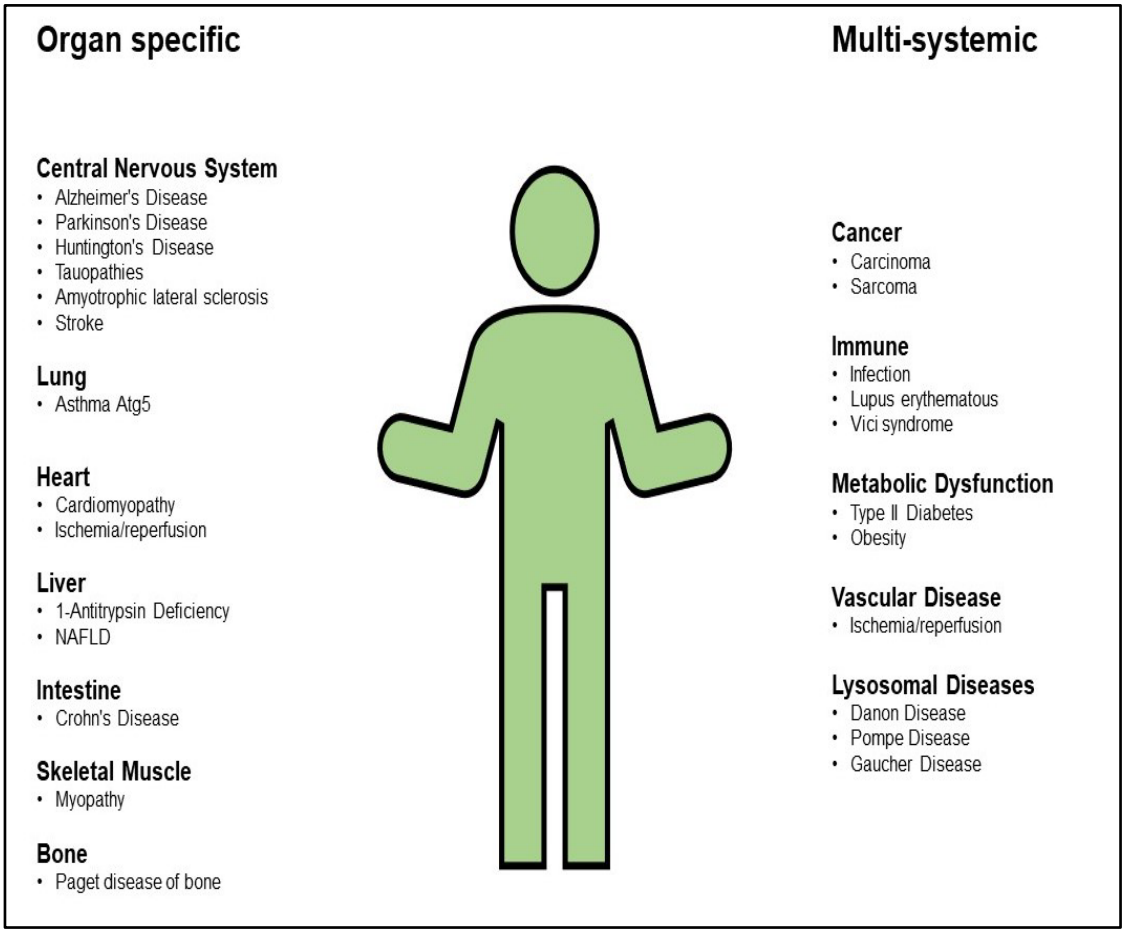


Figure 1.10

Autophagy-dependent human diseases.

Adapted from Schneider, J. L., &Cuervo, A. M. (2014). Autophagy and human disease: emerging themes. Current opinion in genetics & development, 26, 16–23. https://doi.org/10.1016/j.gde.2014.04.003

Aims and Objectives

Our study concerned the impact of PTEN's phosphorylation and sumoylation on autophagy. We aimed to find out the change in autophagy that these PTM caused and subsequently the reasons behind such change. Additionally, we wanted to examine any differences in expression of autophagy according to its causal processes – nutrient stress and endoplasmic reticulum stress. Also, to avoid any results arising from cell-type-specific effects, we wanted to have our hypotheses tested on multiple cell lines.

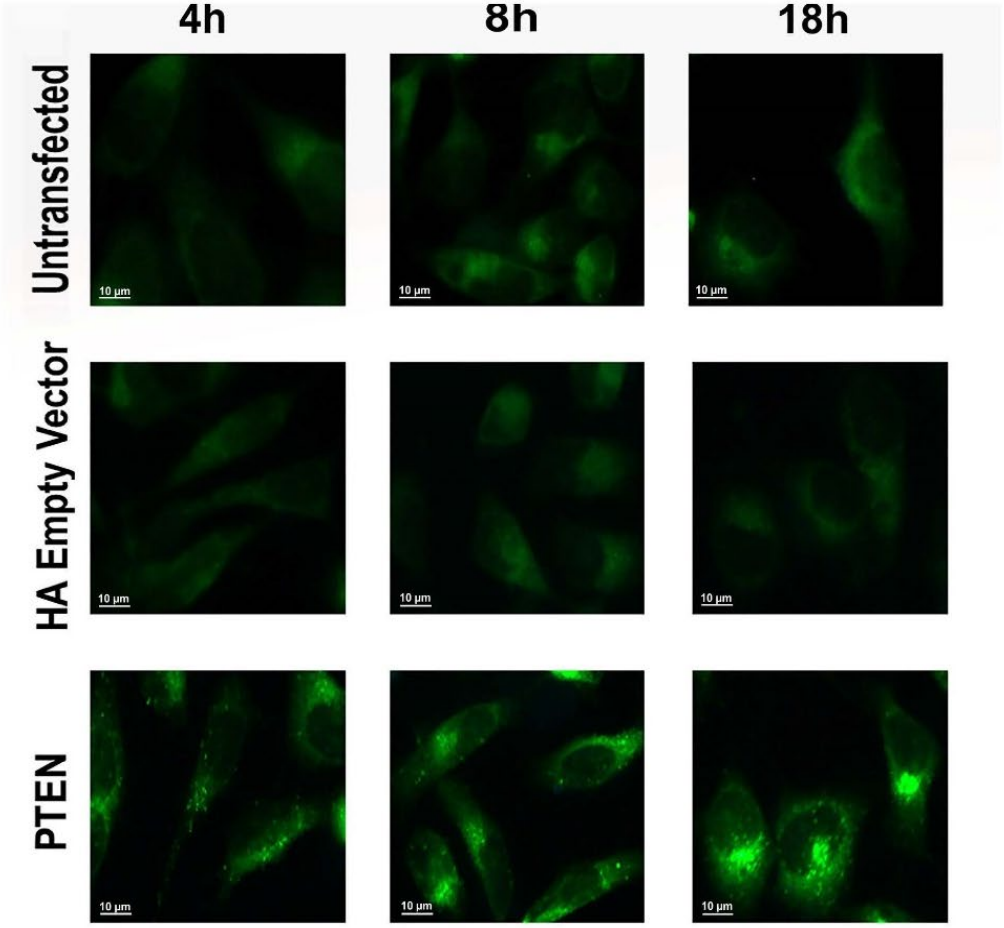
Chapter 2

Results

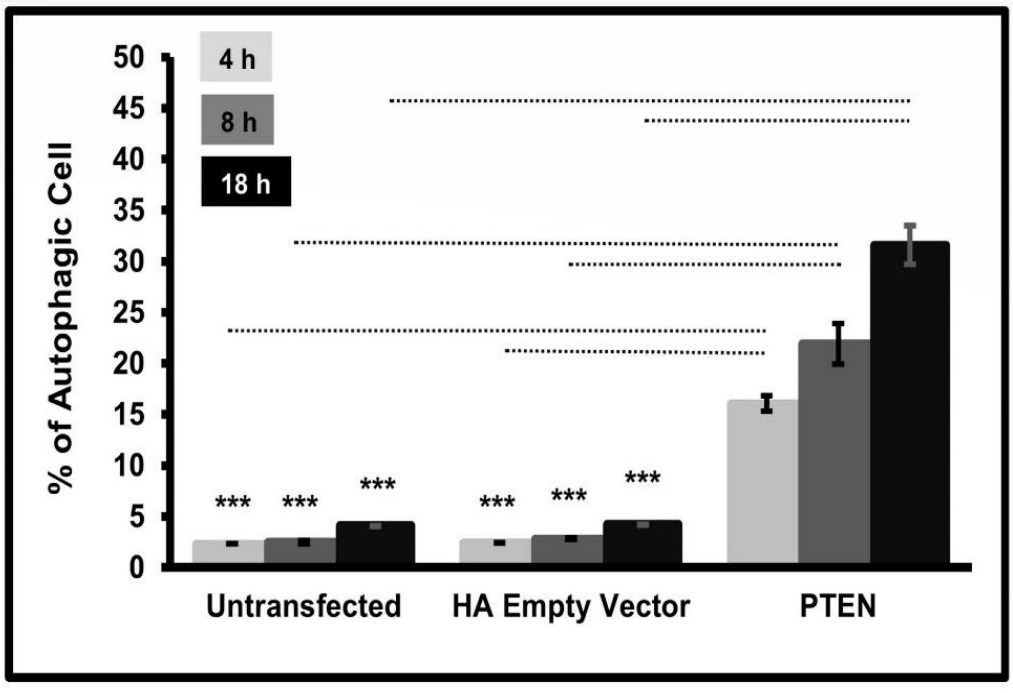
2.1 PTEN-null PC3 cells fails to exhibit autophagy caused by EBSS starvation, which is reversed by PTEN transfection.

We commenced our series of experiments with simply finding out whether transfection of PTEN into PTEN-null PC3 cells can fulfil our objective, that is to induce autophagy. We also had to check whether the induced autophagy was increasing with time of starvation (as long as the cells didn't die), because all our later experiments were designed to undergo starvation for a longer period of time. In untransfected PC3 cells, PC3 cells transfected with HA empty vector, and PC3 cells transfected with PTEN, autophagy by starvation was examined. Cells were starved by incubating them in EBSS for 4 hours, 8 hours, 18 hours (EBSS was used as it causes amino acid starvation). Autophagy was found to be limited in untransfected and empty vector transfected cells (lacking PTEN). However, autophagy was restored upon transfection of WT PTEN. First, all of these results were seen utilising a fluorescence microscope to visualize autophagic vacuoles as distinct puncta by MDC labelling (Figure 1A). After counting the percentage of cells with these puncta, it was discovered that 2.29 ± 0.13 %, 2.45 ± 0.16 % and 4.08 ± 0.15 % corresponded to 4 hours, 8 hours and 18 hours respectively for untransfected PC3 cells; 2.43 ± 0.13 %, 2.81 ± 0.06 % and 4.21 ± 0.16 % corresponded to 4 hours, 8 hours and 18 hours respectively for HA empty vector transfected PC3 cells and 16.06 ± 0.76 %, 21.90 ± 1.99 % and 31.57 ± 1.90 % corresponded to 4 hours, 8 hours and 18 hours respectively for PTEN transfected PC3 cells (Figure 1B). Therefore, compared to PTEN transfected PC3 cells, significantly lesser number of autophagic cells were observed in untransfected or HA empty vector transfected PC3 cells. This result was consistent across all examined time periods, where the number of autophagic cells showed an upward trend with starvation duration.

A


4h
8h
18h
Untransfected
HA Empty Vector
PTEN
10 µm

B


% of Autophagic Cell
50
45
40
35
30
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15
10
5
0
4 h
8 h
18 h
***
Untransfected
HA Empty Vector
PTEN

Figure 2.1

Detection of autophagy as a result of EBSS starvation in PC3 cells by MDC staining.

(A) (B) Autophagic (LC3) puncta were expressed in PC3 cells and quantified using MDC labelling. (A) Using a fluorescent microscope (100X magnification), images were taken. Scale bar: 10 μm. (B Percentage of cells that exhibit puncta at 4, 8, and 18 hours of starvation. Over 500 cells were counted. The statistical differences between the recorded values at each time point of untransfected and HA empty vector transfected cells and corresponding time point of PTEN transfected cells were computed using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

As previously mentioned, the expression of endogenous LC3B, a critical component of autophagosomes formed during autophagy, was next examined using indirect immunolabelling. The autophagic cells exhibited a high fluorescence that was directly related to the effective creation of autophagosomes, indicating similar outcomes of EBSS-induced autophagy (Figure 2A). The results were further consolidated by carrying out the immunoblotting of transfected cell extracts, which contained specific marker proteins associated to autophagy. Beclin1/GAPDH, ATG5/GAPDH, and ATG7/GAPDH band intensity ratios were computed. The ratios of all these marker proteins got elevated which again proved the existence of autophagy as a consequence of transfection of PTEN into PTEN-null PC3 cells. The increase of autophagic flux with increase in time interval of starvation was further proven by these blots (Figure 2B).

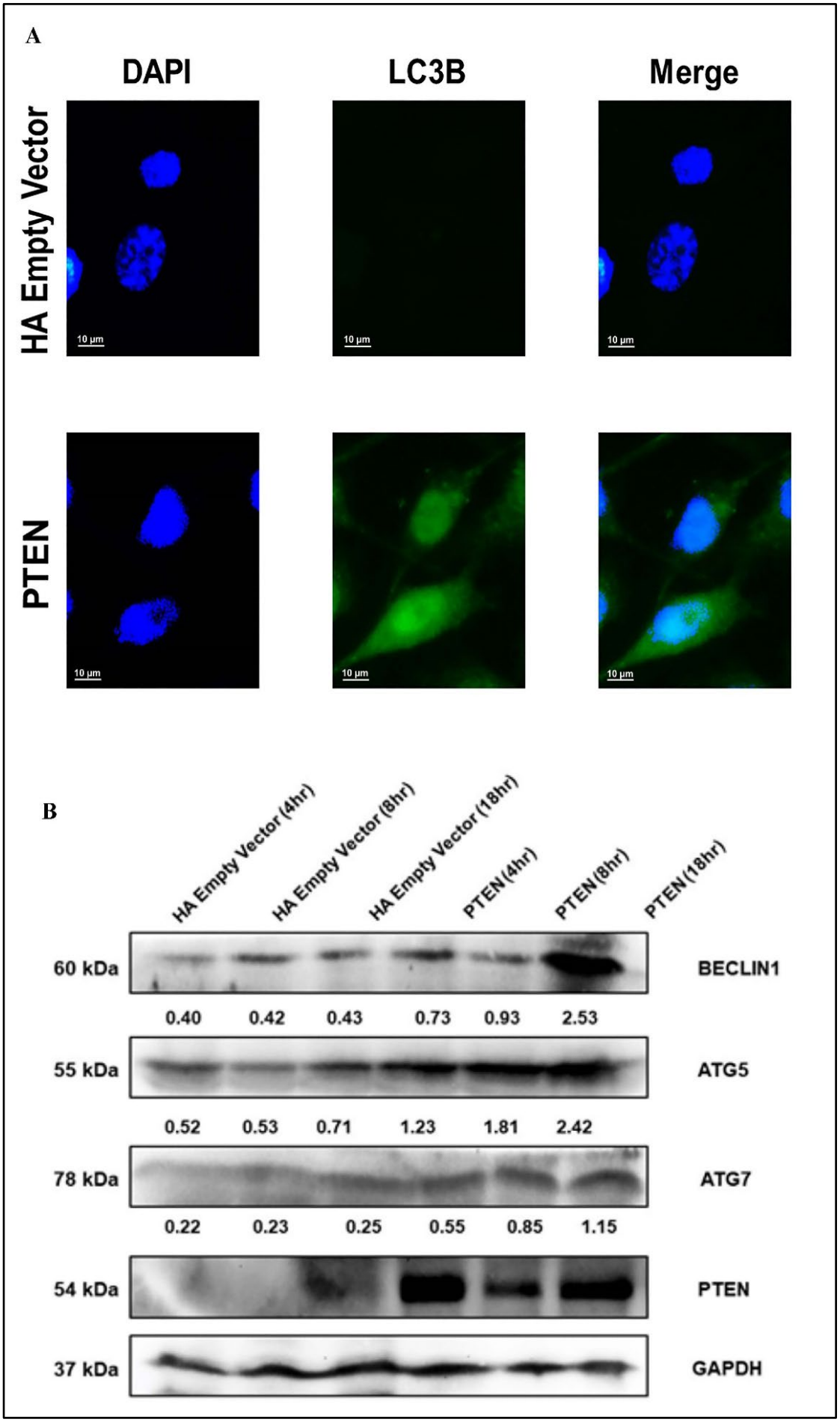

A
DAPI
LC3B
Merge
HA Empty Vector
PTEN
10 µm
B
HA Empty Vector (4hr)
HA Empty Vector (8hr)
HA Empty Vector (18hr)
PTEN (4hr)
PTEN (8hr)
PTEN (18hr)
60 kDa
BECLIN1
0.40 0.42 0.43 0.73 0.93 2.53
55 kDa
ATG5
0.52 0.53 0.71 1.23 1.81 2.42
78 kDa
ATG7
0.22 0.23 0.25 0.55 0.85 1.15
54 kDa
PTEN
37 kDa
GAPDH

Figure 2.2

Autophagy after EBSS starvation in PC3 cells was observed using Indirect Immunolabeling and Western Blot.

(A) Indirect immunolabelling generated Anti-LC3B expression in PC3 cells. The WT PTEN gene construct and HA empty vector transfected cells were starved for 18 hours. They were then examined under a fluorescent microscope (100X magnification) after being immunolabelled with Anti-LC3B and DAPI mounting medium. Scale bar: 10 μm. (B) Total cellular extract from PC3 was collected to estimate the expression of autophagy marker proteins Beclin1, ATG5, and ATG7 using Western Blot. As a loading control GAPDH was utilised. ImageJ software was used to calculate the ratios of band intensities between each marker protein and GAPDH, which are represented by the specified values.

2.2 The induction of autophagy is attributed to PTEN's lipid phosphatase activity. PTEN's phosphorylation prevents it from being able to induce autophagy, but PTEN's sumoylation promotes autophagy induction.

Using Site-directed mutagenesis kit, we created distinct PTEN mutant clones from WT PTEN in accordance with the instructions provided. Following the transformation, a single colony was isolated, sequenced, and verified using a sequencing primer. We were aware of the fact that PTEN's phosphatase nature renders its control over several physiological phenomenon. Also, as previously mentioned, the fine tuning by a protein's PTM alters its function and localization. So, in our experiments, we used PTEN mutants that were either phosphatase deficient (the fundamental nature of PTEN) and mutants bearing alterations in PTEN's phosphorylation as well as sumoylation capability. As it will be seen in

the following discourse, autophagy is affected by both these two types of mutations.

Utilizing the previously indicated techniques once more, we observed that transfection of a phosphatase inactive mutant form of PTEN (C124S) did not result in autophagy levels comparable to transfection of full-length WT PTEN. MDC labelling of autophagic vacuoles in PC3 cells transfected with PTEN (C124S) following EBSS-mediated starvation have 6.94 ± 0.78 % puncta as compared to 30.49 ± 1.81 % cells transfected with WT PTEN (Figure 3A, 3B).

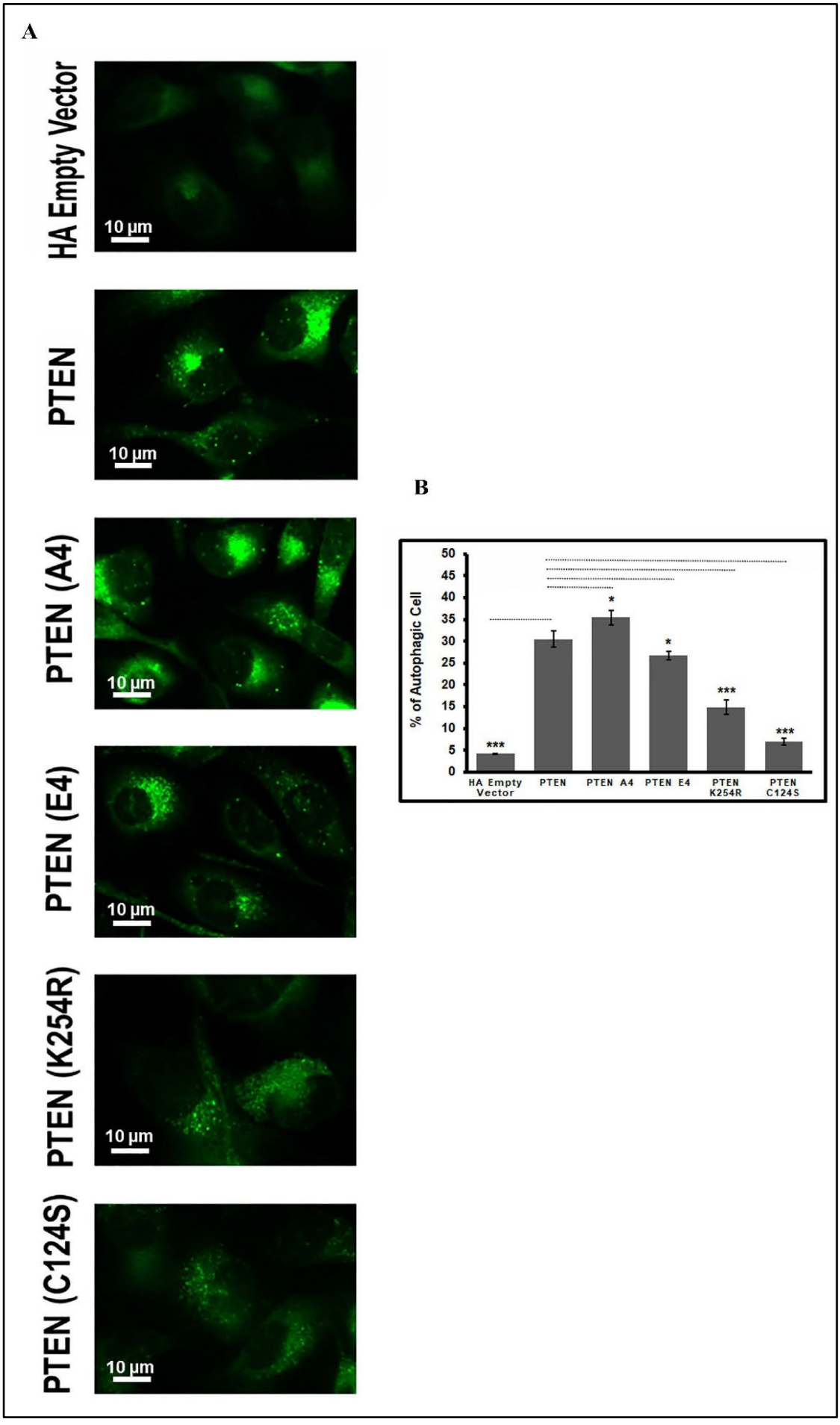

A
HA Empty Vector
PTEN
PTEN (A4)
PTEN (E4)
PTEN (K254R)
PTEN (C124S)
10 µm
B
% of Autophagic Cell
50
45
40
35
30
25
20
15
10
5
0
***
*
*
***
***
HA Empty Vector
PTEN
PTEN A4
PTEN E4
PTEN K254R
PTEN C124S

Figure 2.3

The autophagic (LC3) puncta expression of PC3 cells as a result of EBSS starvation, after transfection with PTEN mutants.

(A) (B) Autophagic (LC3) puncta were expressed in PC3 cells and quantified using MDC labelling. (A) Using a fluorescent microscope (100X magnification), images were taken. Scale bar: 10 μm. (B Percentage of cells that exhibit puncta at 18 hours of starvation with the respective PTEN gene constructs. Over 500 cells were counted. The statistical differences between the values recorded for each mutant and WT PTEN were computed using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

The LC3B study from immunolabelling experiments produced identical results (Figure 4). This showed lack of autophagosome production in mutated PTEN (C124S) transfected PC3 cells.

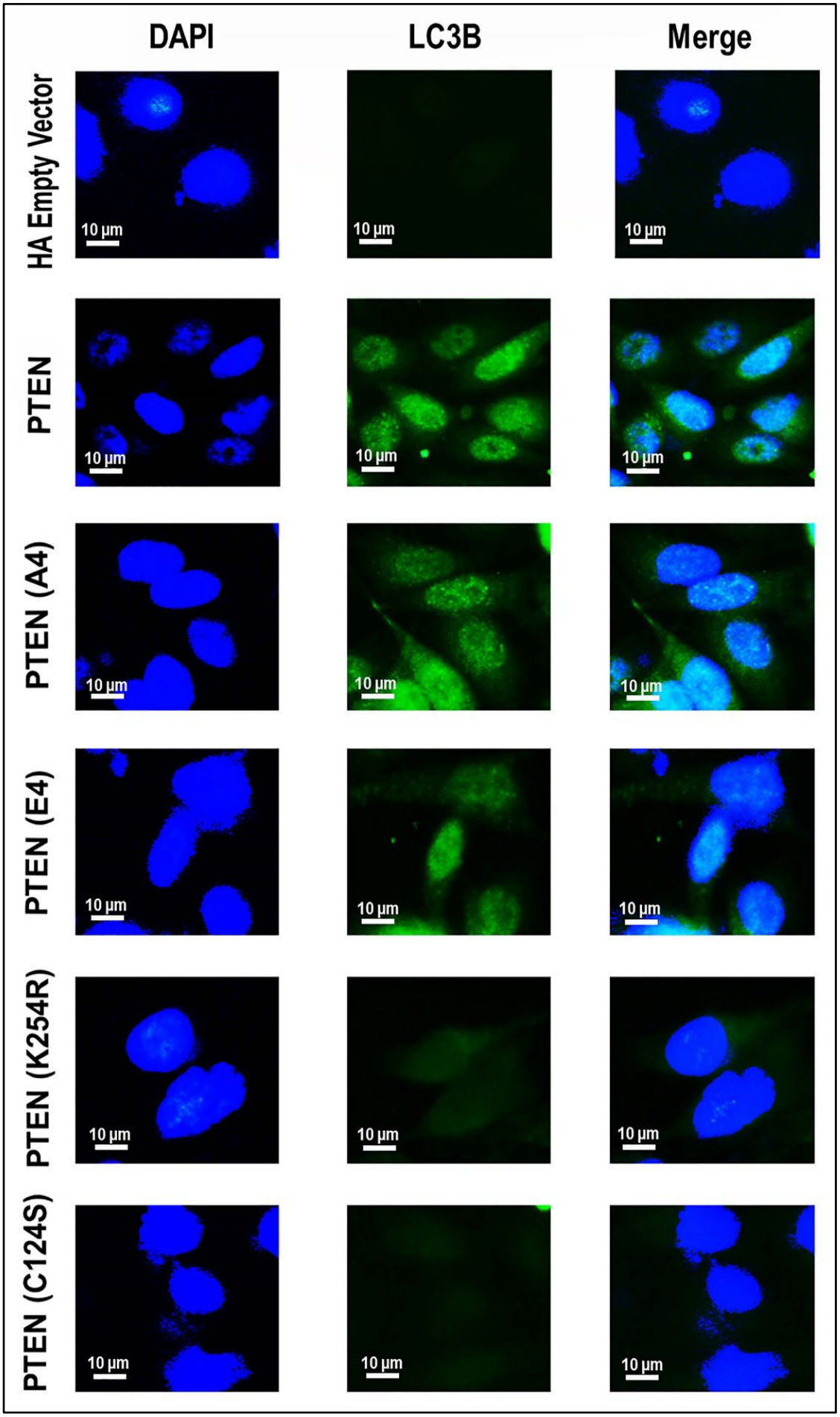

DAPI
LC3B
Merge
HA Empty Vector
PTEN
PTEN (A4)
PTEN (E4)
PTEN (K254R)
PTEN (C124S)
10 µm

Figure 2.4

PC3 cells' generation of anti-LC3B immunofluorescence following transfection with PTEN mutants and EBSS-mediated starvation.

All the respective gene constructs were used to transfect PC3 cells, and they were starved for 18 hours. They were then examined under a fluorescent microscope (100X magnification) after being immunolabelled with Anti-LC3B and DAPI mounting medium. Scale bar: 10 μm.

Following EBSS starvation, immunoblotting experiments in WT PTEN transfected PC3 cells revealed that the ratios of band intensities of Beclin1/GAPDH; ATG5/GAPDH; ATG7/GAPDH; p62/GAPDH and phospho-mTOR/mTOR were 0.76 ± 0.010; 0.96 ± 0.022; 1.14 ± 0.027; 0.89 ± 0.031 and 0.46 ± 0.019 respectively. Similarly, for PTEN (C124S) transfected cells, they were 0.19 ± 0.046; 0.43 ± 0.019; 0.72 ± 0.031; 1.36 ± 0.052 and 0.95 ± 0.020 respectively (Figure 5A, 5B, 6A, 6B). The expression of all the autophagic marker proteins got reduced in PTEN (C124S) transfected PC3 cells. On the contrary the phosphorylation of mTOR (negative regulator of autophagy) increased.

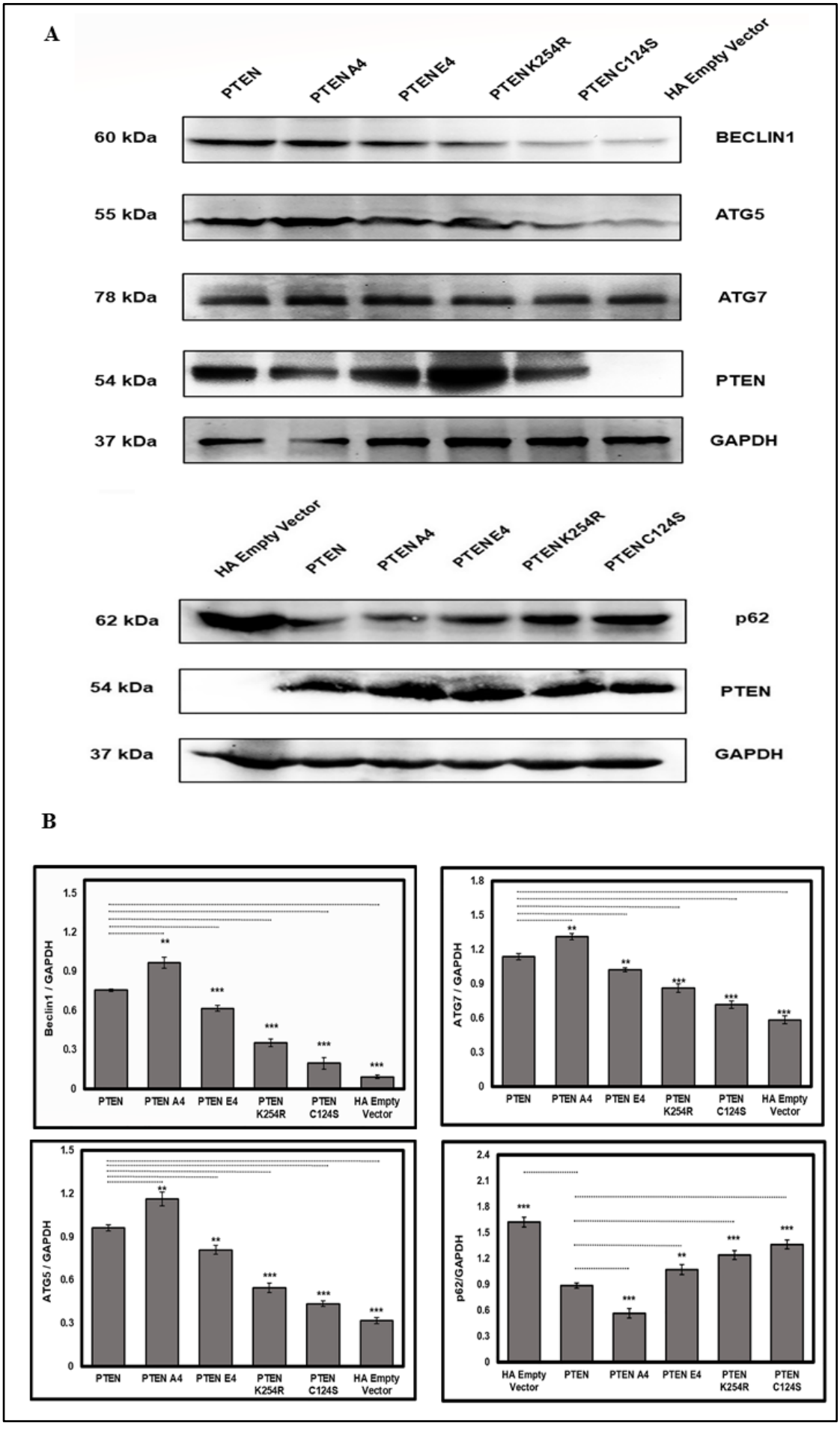

A
PTEN
PTEN A4
PTEN E4
PTEN K254R
PTEN C124S
HA Empty Vector
60 kDa
BECLIN1
55 kDa
ATG5
78 kDa
ATG7
54 kDa
PTEN
37 kDa
GAPDH
HA Empty Vector
PTEN
PTEN A4
PTEN E4
PTEN K254R
PTEN C124S
62 kDa
p62
54 kDa
PTEN
37 kDa
GAPDH
B
Beclin1 / GAPDH
ATG7 / GAPDH
ATG5 / GAPDH
p62/GAPDH
PTEN
PTEN A4
PTEN E4
PTEN K254R
PTEN C124S
HA Empty Vector

Figure 2.5

The quantitative assessment of autophagic marker proteins from PC3 cells transfected with PTEN mutants and treated with EBSS (18 h).

(A) Autophagy marker proteins Beclin1, ATG5, ATG7, and p62 were detected by Western Blot analysis of total cellular extracts from PC3 cells. As a loading control, GAPDH was utilized. (B) The ratios of band intensities between respective marker proteins and GAPDH were computed using ImageJ software, and were graphically represented. The statistical differences between the recorded values of each mutant and PTEN were determined using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

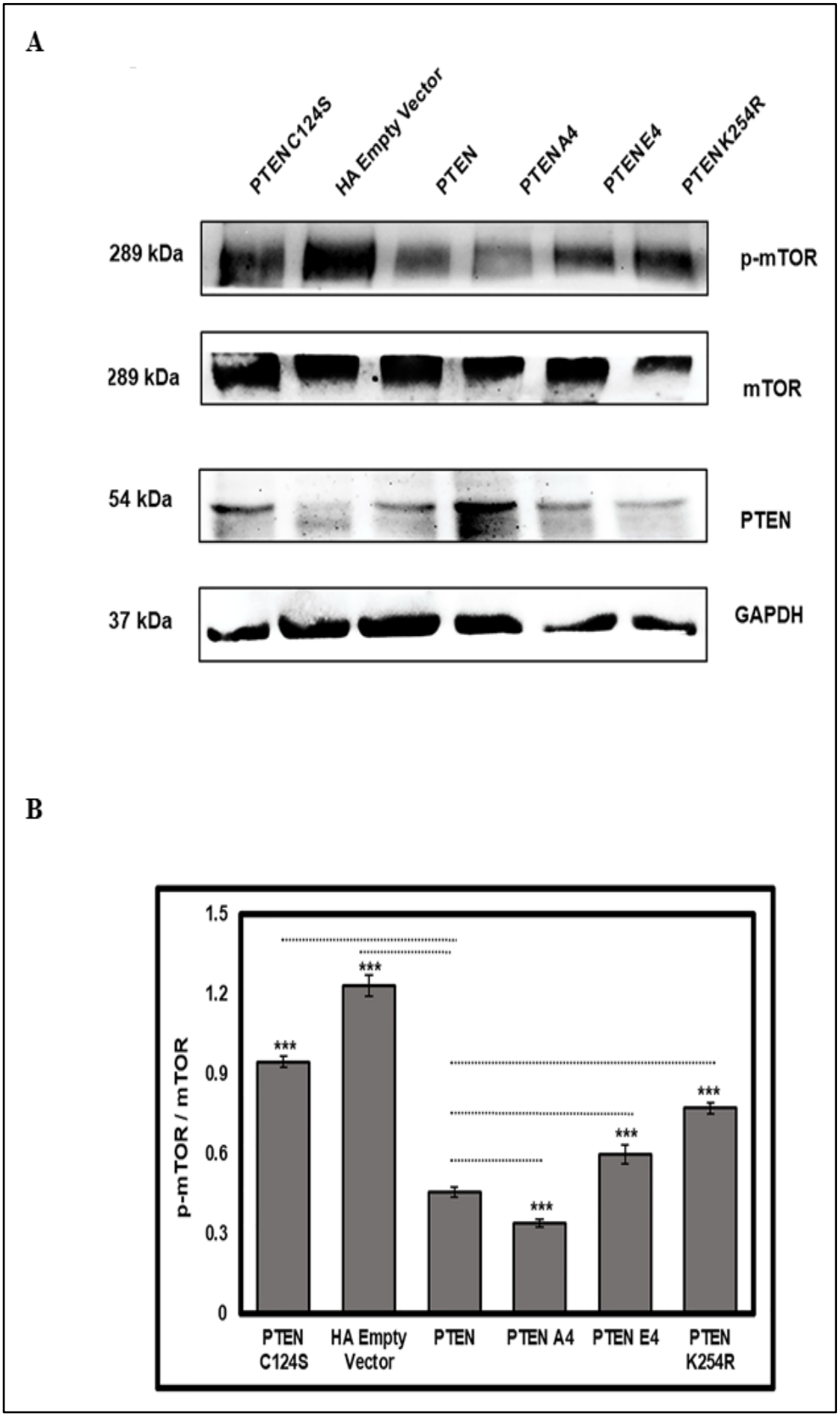

A
PTEN C124S
HA Empty Vector
PTEN
PTEN A4
PTEN E4
PTEN K254R
289 kDa
p-mTOR
289 kDa
mTOR
54 kDa
PTEN
37 kDa
GAPDH
B
p-mTOR / mTOR
1.5
1.2
0.9
0.6
0.3
0
***
PTEN C124S
HA Empty Vector
PTEN
PTEN A4
PTEN E4
PTEN K254R

Figure 2.6

The quantitative assessment of p-mTOR and mTOR from PC3 cells transfected with PTEN mutants and treated with EBSS (18 h).

(A) Autophagy marker protein p-mTOR was detected by Western Blot analysis of total cellular extracts from PC3 cells. As a loading control, GAPDH was utilized. (B) The ratios of band intensities between p-mTOR and mTOR were computed using ImageJ software, and were graphically represented. The statistical differences between the recorded values of each mutant and PTEN were determined using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

Finally, after 18 hours of EBSS-induced autophagy, we captured images of autophagic vacuoles using TEM (Figure 7). These further consolidated our findings as the error-free image of a subcellular components' ultrastructure is always deemed reliable. As mentioned in the following figure legend, we maintained utmost care in detecting autophagic vacuoles amongst other subcellular structures. WT PTEN-transfected cells showed numerous autophagic vacuoles, indicating the presence of autophagy. In contrast, autophagic vacuoles were hardly noticeable in the transfected mutant PTEN (C124S) cells.

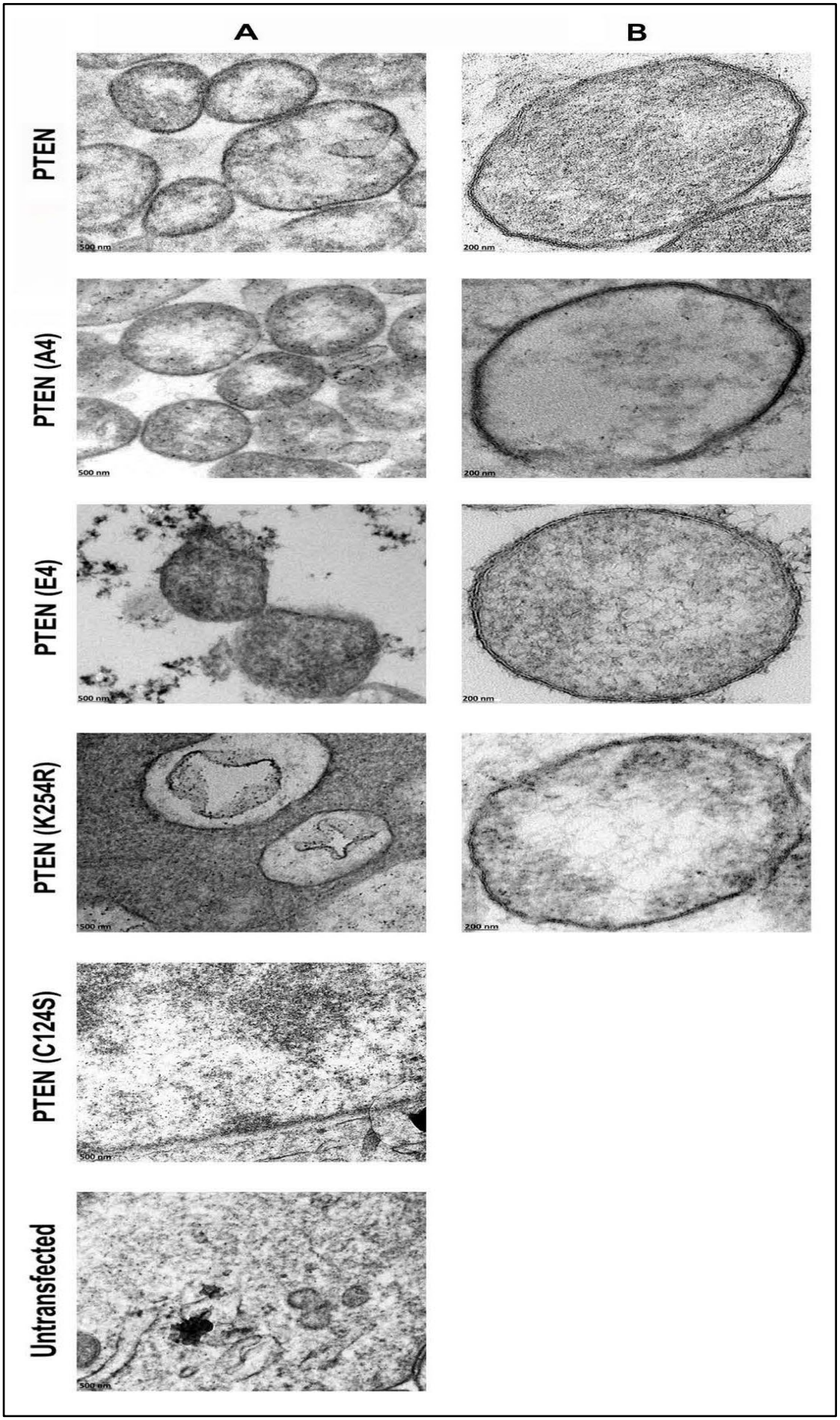

A
B
PTEN
PTEN (A4)
PTEN (E4)
PTEN (K254R)
PTEN (C124S)
Untransfected
500 nm
200 nm

Figure 2.7

Following transfection with PTEN mutants and subsequent EBSS starvation in PC3 cells, imaging of autophagic vacuoles using TEM.

PC3 cells were starved for 18 hours after being transfected with PTEN gene constructs. Sections were examined under TEM after cells were methodically fixed. (A) TEM image of autophagic vacuoles. Scale bar: 500 nm. (B) Each field's enlarged autophagic vacuole as specimen. Scale bar: 200 nm. The vacuoles were examined for the presence of cristae along with double membranes since autophagic vacuoles and mitochondria are sometimes mistaken for each other.

Now for our next series of experiments concerning PTEN's sumoylation and phosphorylation, we not only included another PTEN-null cell line U87MG but also applied another causal agent of autophagy: tunicamycin-mediated ER stress. This was done in order to have additional evidence to support our results and to avoid any cell-specific erroneous results. Cells transfected with sumoylation-deficient mutant form of PTEN (K254R) and WT PTEN transfected cells were compared to inspect the differences in expression of PTEN-mediated autophagy. It had been determined that this specific mutation negatively modulates the sumoylation capability of PTEN. Autophagy was induced in PC3 cells by incubating cells in EBSS for 18 hours and also in U87MG cells by incubating cells in EBSS for 18 hours as well as in Tunicamycin for 24 hours. Autophagic vacuoles stained with MDC in PC3 cells after EBSS starvation displayed 14.88 ± 1.58 % of cells with puncta after PTEN (K254R) transfection as compared to 30.49 ± 1.81 % cells with WT PTEN transfection (Figure 3A, 3B). Similarly, autophagic vacuoles stained with MDC in PTEN (K254R) transfected U87MG

cells after EBSS starvation displayed 22.79 ± 2.32 % of cells with puncta as compared to 45.58 ± 1.07 % cells with WT PTEN transfection (Figure 8A, 8B).

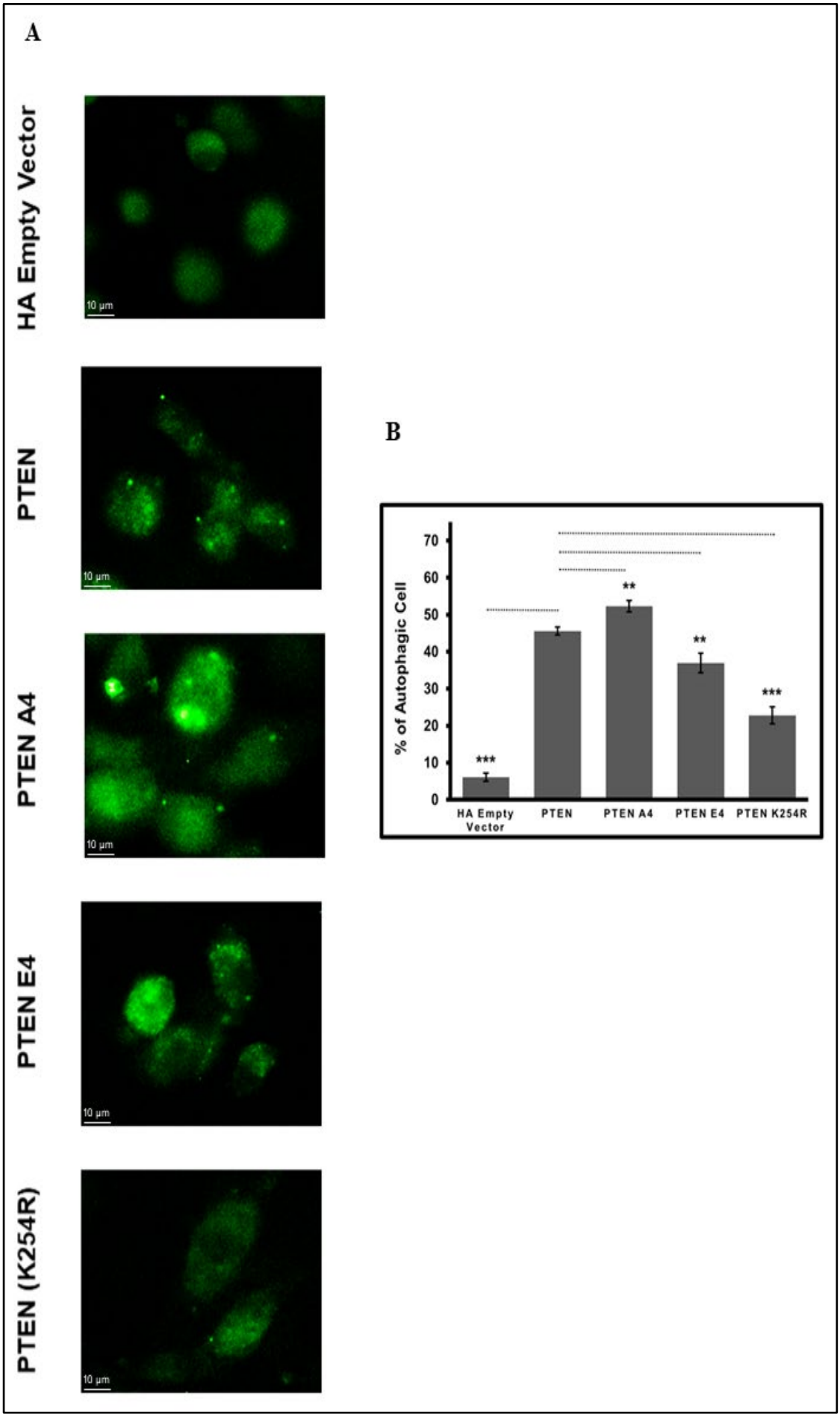

A
HA Empty Vector
PTEN
PTEN A4
PTEN E4
PTEN (K254R)
10 µm
B
% of Autophagic Cell
0
10
20
30
40
50
60
70
**
**
***
***
HA Empty Vector
PTEN
PTEN A4
PTEN E4
PTEN K254R

Figure 2.8

The autophagic (LC3) puncta expression of U87MG cells as a result of EBSS starvation, after transfection with PTEN mutants.

(A) (B) Autophagic (LC3) puncta were expressed in U87MG cells and quantified using MDC labelling. (A) Using a fluorescent microscope (100X magnification), images were taken. Scale bar: 10 μm. (B Percentage of cells that exhibit puncta at 18 hours of starvation with the respective PTEN gene constructs. Over 500 cells were counted. The statistical differences between the values recorded for each mutant and WT PTEN were computed using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

Also, autophagic vacuoles stained with MDC in in PTEN (K254R) transfected U87MG cells after Tunicamycin treatment showed 24.42 ± 2.14 % of cells with as compared to 54.05 ± 1.08 % cells with WT PTEN transfection (Figure 9A, 9B). This showed the significant decrease of autophagic flux in sumoylation deficient mutated form of PTEN (K254R).

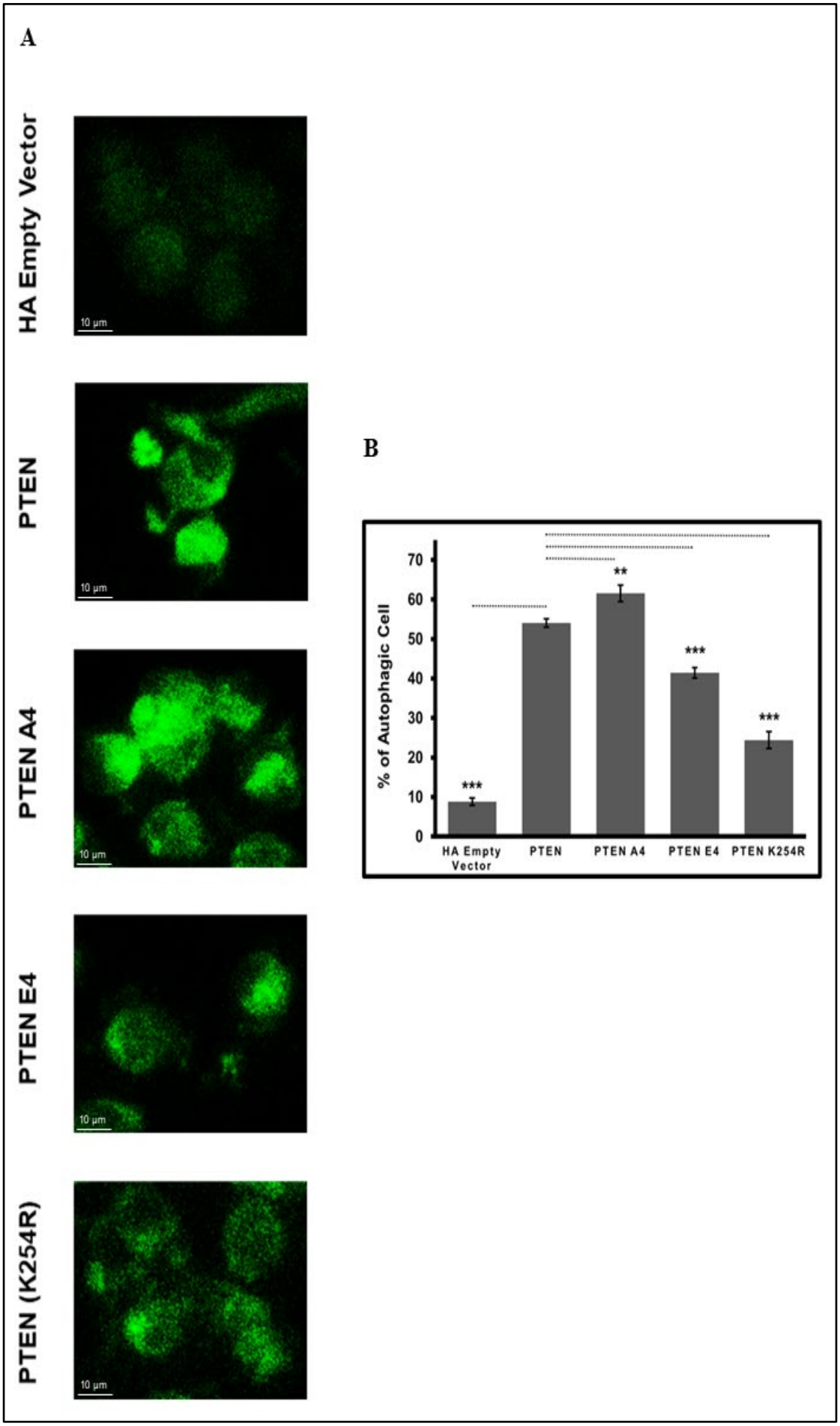

A
HA Empty Vector
PTEN
PTEN A4
PTEN E4
PTEN (K254R)
10 µm
B
% of Autophagic Cell
0
10
20
30
40
50
60
70
**
***
HA Empty Vector
PTEN
PTEN A4
PTEN E4
PTEN K254R

Figure 2.9

The autophagic (LC3) puncta expression of U87MG cells as a result of Tunicamycin mediated ER stress, after transfection with PTEN mutants.

(A) (B) Autophagic (LC3) puncta were expressed in U87MG cells and quantified using MDC labelling. (A) Using a fluorescent microscope (100X magnification), images were taken. Scale bar: 10 μm. (B Percentage of cells that exhibit puncta at 18 hours of starvation with the respective PTEN gene constructs. Over 500 cells were counted. The statistical differences between the values recorded for each mutant and WT PTEN were computed using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

Also, when compared to their WT PTEN transfected counterparts, PTEN (K254R) transfected PC3 and U87MG cells displayed weak fluorescence in LC3B analysis utilizing indirect immunolabelling (Figure 4, 10, 11).

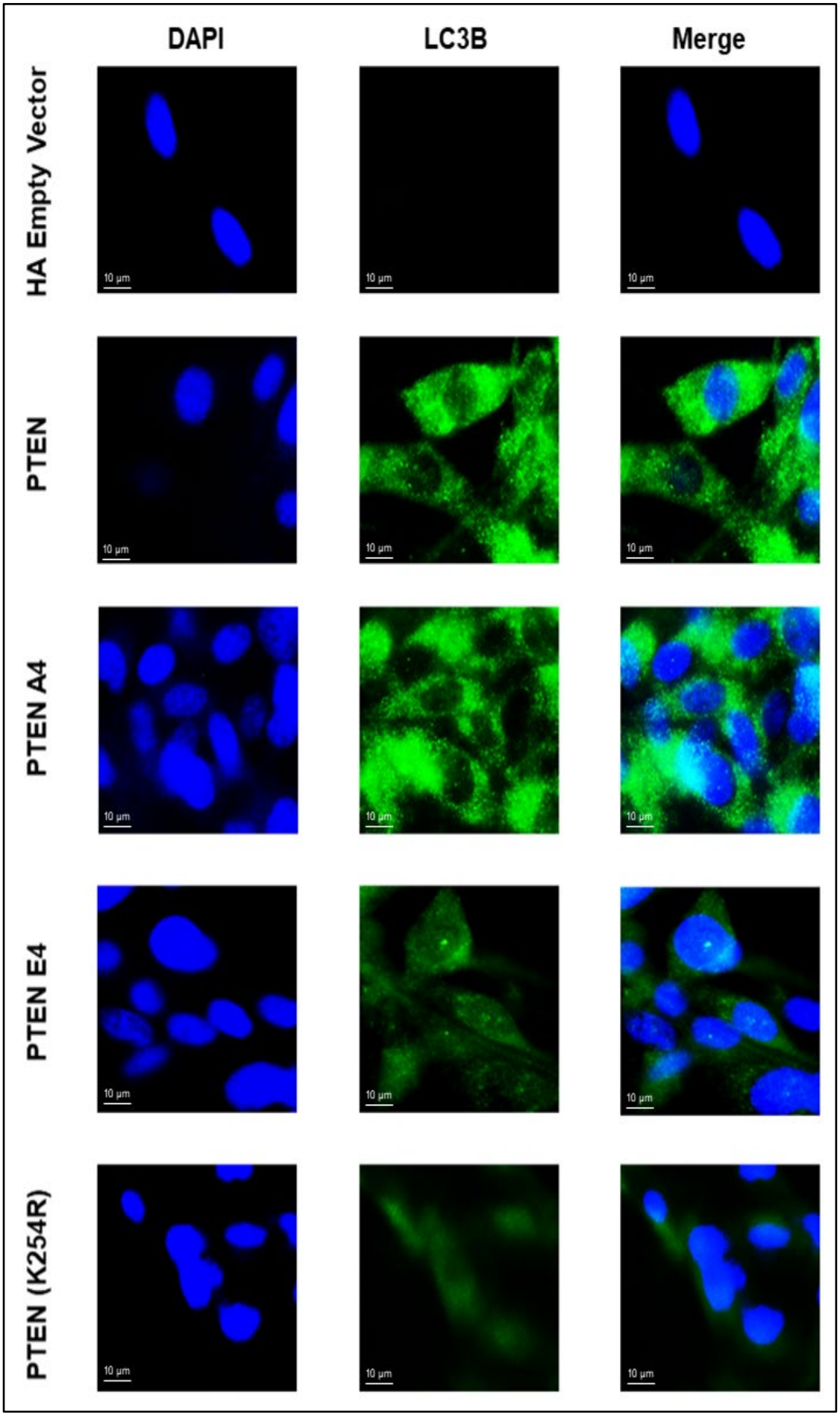

DAPI
LC3B
Merge
HA Empty Vector
PTEN
PTEN A4
PTEN E4
PTEN (K254R)
10 µm

Figure 2.10

U87MG cells' generation of anti-LC3B immunofluorescence following transfection with PTEN mutants and EBSS-mediated starvation.

All the respective gene constructs were used to transfect U87MG cells, and they were starved for 18 hours. They were then examined under a fluorescent microscope (100X magnification) after being immunolabelled with Anti-LC3B and DAPI mounting medium. Scale bar: 10 μm.

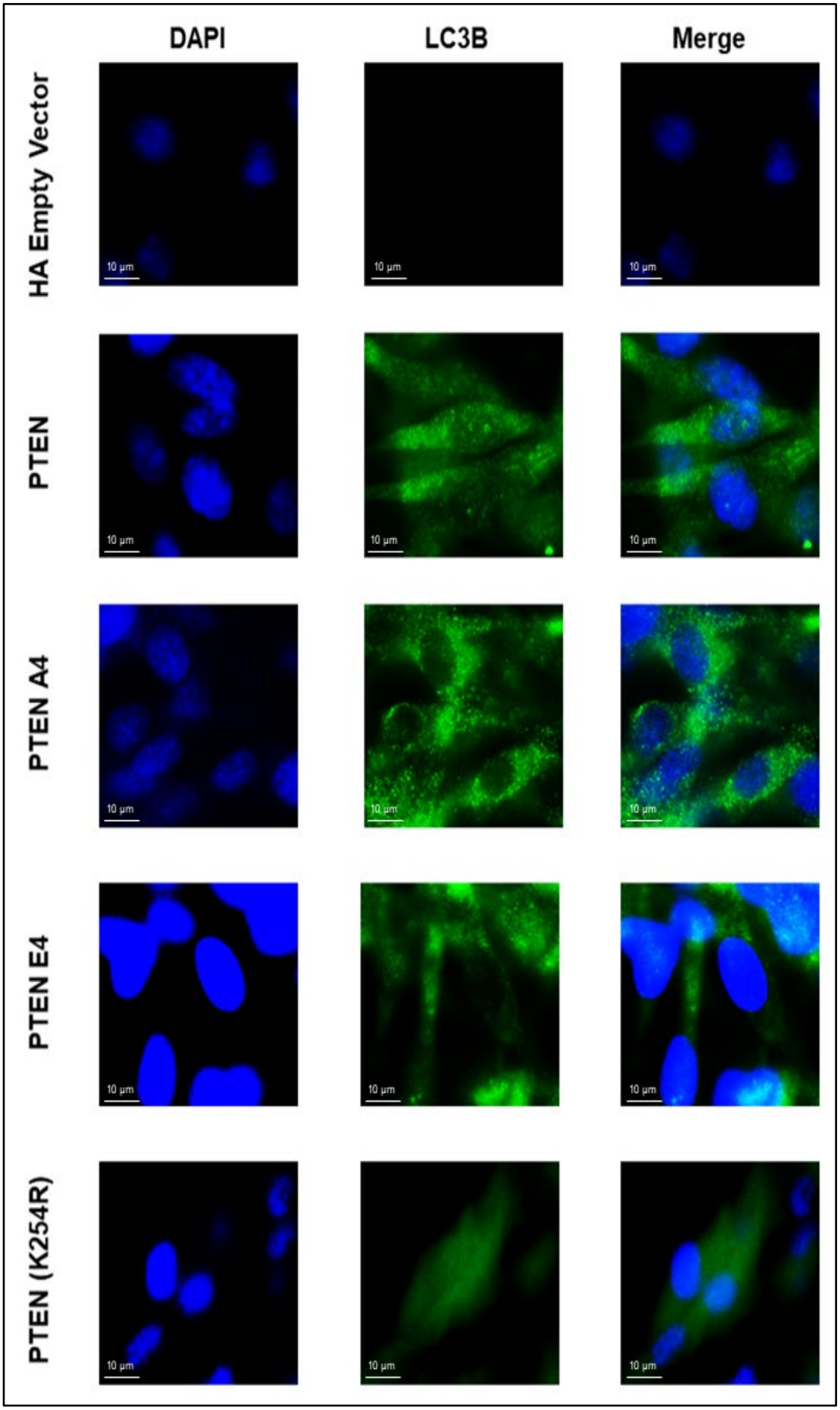

DAPI
LC3B
Merge
HA Empty Vector
PTEN
PTEN A4
PTEN E4
PTEN (K254R)
10 µm

Figure 2.11

U87MG cells' generation of anti-LC3B immunofluorescence following transfection with PTEN mutants and Tunicamycin mediated ER stress.

All the respective gene constructs were used to transfect U87MG cells, and they were starved for 18 hours. They were then examined under a fluorescent microscope (100X magnification) after being immunolabelled with Anti-LC3B and DAPI mounting medium. Scale bar: 10 μm.

Following EBSS starvation, immunoblotting experiments in WT PTEN transfected PC3 cells revealed that the ratios of band intensities of Beclin1/GAPDH; ATG5/GAPDH; ATG7/GAPDH; p62/GAPDH and phospho-mTOR/mTOR were 0.76 ± 0.010; 0.96 ± 0.022; 1.14 ± 0.027; 0.89 ± 0.031 and 0.46 ± 0.019 respectively. For PTEN (K254R) transfected PC3 cells, they were 0.35 ± 0.030; 0.55 ± 0.034; 0.86 ± 0.036; 1.24 ± 0.051 and 0.77 ± 0.021 respectively (Figure 5A, 5B). Similarly, following EBSS starvation, immunoblotting experiments in WT PTEN transfected U87MG cells revealed that the ratios of band intensities of Beclin1/GAPDH; ATG5/GAPDH; p62/GAPDH and phospho-Akt/Akt were 0.92 ± 0.033; 0.70 ± 0.048; 0.80 ± 0.081 and 0.71 ± 0.032 respectively. For PTEN (K254R) transfected U87MG cells, they were 0.48 ± 0.010; 0.35 ± 0.053; 1.57 ± 0.074 and 1.35 ± 0.042 respectively (Figure 12A, 12B).

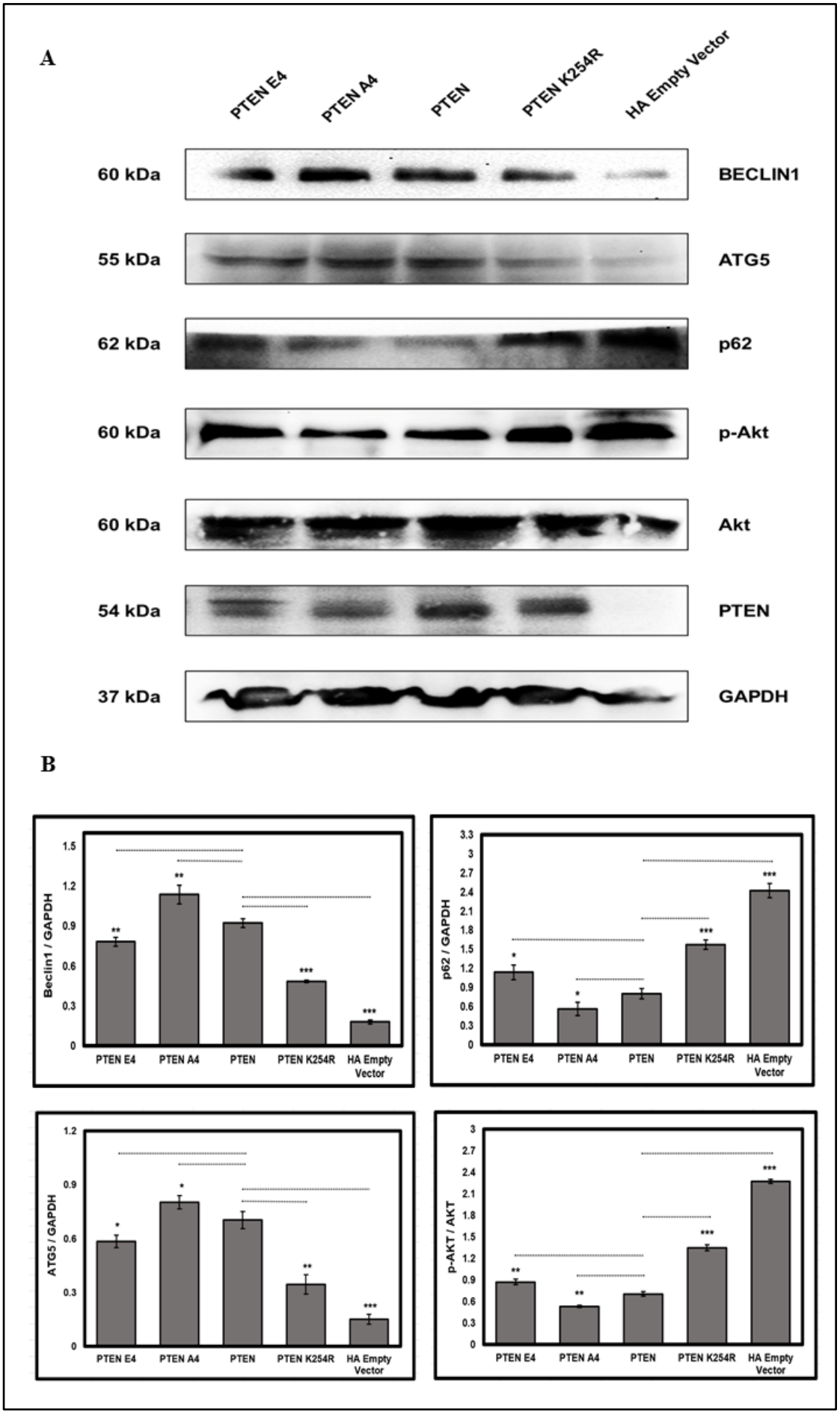

A
PTEN E4
PTEN A4
PTEN
PTEN K254R
HA Empty Vector
60 kDa
BECLIN1
55 kDa
ATG5
62 kDa
p62
60 kDa
p-Akt
60 kDa
Akt
54 kDa
PTEN
37 kDa
GAPDH
B
Beclin1 / GAPDH
p62 / GAPDH
ATG5 / GAPDH
p-AKT / AKT
PTEN E4
PTEN A4
PTEN
PTEN K254R
HA Empty Vector

Figure 2.12

The quantitative assessment of autophagic marker proteins from U87MG cells transfected with PTEN mutants and treated with EBSS (18 h).

(A) Autophagy marker proteins Beclin1, ATG5, p62 and p-Akt were detected by Western Blot analysis of total cellular extracts from U87MG cells. As a loading control, GAPDH was utilized. (B) The ratios of band intensities between respective marker proteins and GAPDH as well as between p-Akt and Akt, were computed using ImageJ software, and were graphically represented. The statistical differences between the recorded values of each mutant and PTEN were determined using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

Also, after Tunicamycin treatment, immunoblotting experiments in WT PTEN transfected U87MG cells revealed that the ratios of band intensities of Beclin1/GAPDH; ATG5/GAPDH; p62/GAPDH and phospho-Akt/Akt were 1.72 ± 0.096; 0.94 ± 0.061; 0.19 ± 0.019 and 0.47 ± 0.016 respectively. For PTEN (K254R) transfected U87MG cells, they were 0.81 ± 0.037; 0.44 ± 0.036; 0.62 ± 0.039 and 1.10 ± 0.057 respectively (Figure 13A, 13B).

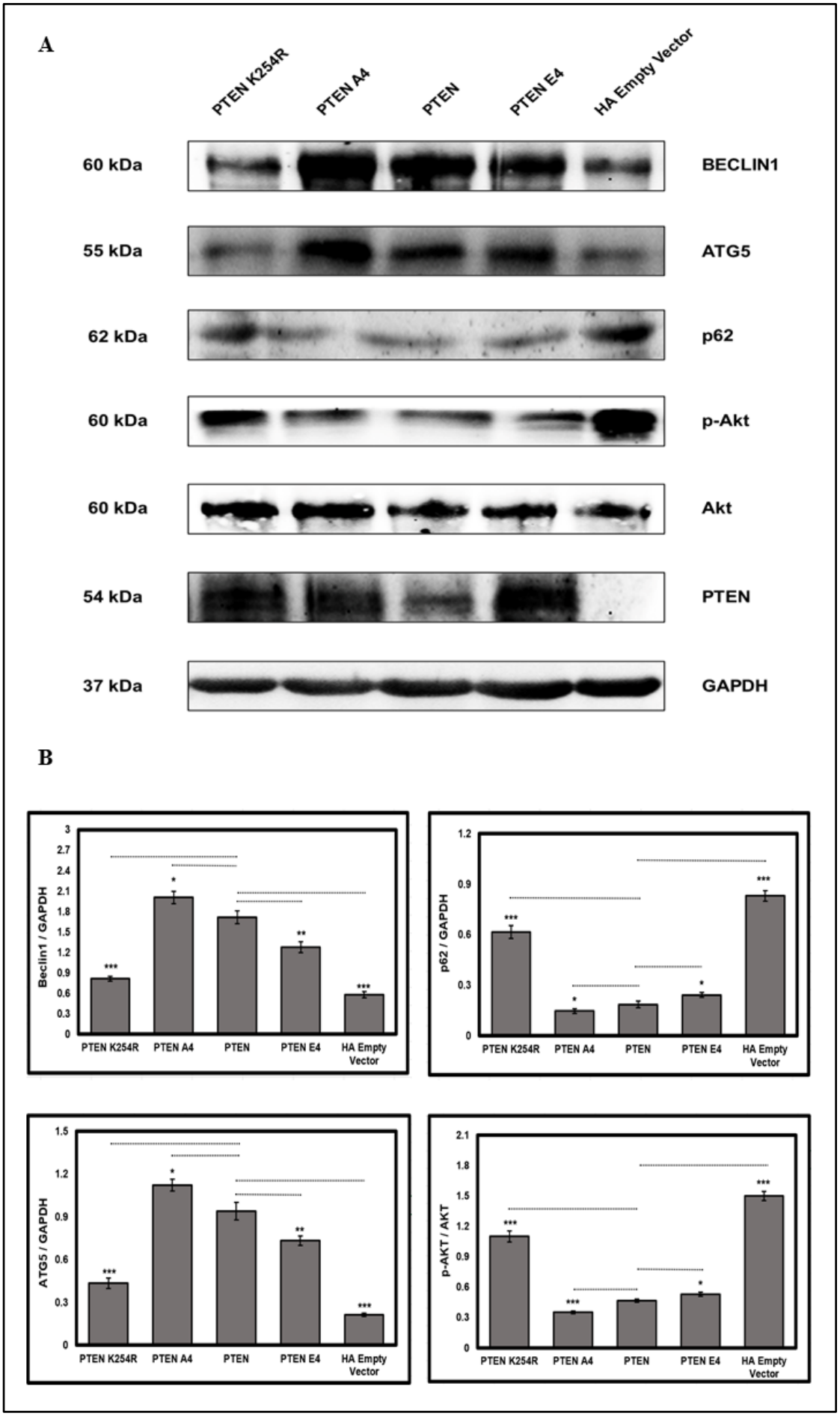

A
PTEN K254R
PTEN A4
PTEN
PTEN E4
HA Empty Vector
60 kDa
BECLIN1
55 kDa
ATG5
62 kDa
p62
60 kDa
p-Akt
60 kDa
Akt
54 kDa
PTEN
37 kDa
GAPDH
B
Beclin1 / GAPDH
p62 / GAPDH
ATG5 / GAPDH
p-AKT / AKT
PTEN K254R
PTEN A4
PTEN
PTEN E4
HA Empty Vector

Figure 2.13

The quantitative assessment of autophagic marker proteins from U87MG cells transfected with PTEN mutants and treated with Tunicamycin (24 h).

(A) Autophagy marker proteins Beclin1, ATG5, p62 and p-Akt were detected by Western Blot analysis of total cellular extracts from U87MG cells. As a loading control, GAPDH was utilized. (B) The ratios of band intensities between respective marker proteins and GAPDH as well as between p-Akt and Akt, were computed using ImageJ software, and were graphically represented. The statistical differences between the recorded values of each mutant and PTEN were determined using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

TEM images, which showed fewer autophagic vacuoles in PTEN (K254R) transfected cells, further supported these findings (Figure 7).

WT PTEN transfected cells were compared to phosphorylation-deficient mutant (A4-S380A, T382A, T383A, S385A) and phosphorylation-mimicking mutant (E4-S380E, T382E, T383E, S385E) transfected cells in order to examine the effects of PTEN-mediated autophagy. It is known that phosphorylation controlled the stability and phosphatase activity of PTEN. PC3 cells were incubated in EBSS for 18 hours, whereas U87MG cells were incubated in EBSS for 18 hours as well as in Tunicamycin for 24 hours to induce autophagy. Autophagic vacuoles stained with MDC in PC3 cells after EBSS starvation displayed 35.46 ± 1.67 % and 26.66 ± .99 % of cells containing puncta after transfection of A4 PTEN and E4 PTEN respectively, as compared to 30.49 ± 1.81 % cells transfected with WT PTEN (Figure 3A, 3B). Similarly, autophagic vacuoles stained with MDC in U87MG cells after EBSS starvation displayed 52.30 ± 1.55 % and 36.94 ± 2.64 % of cells

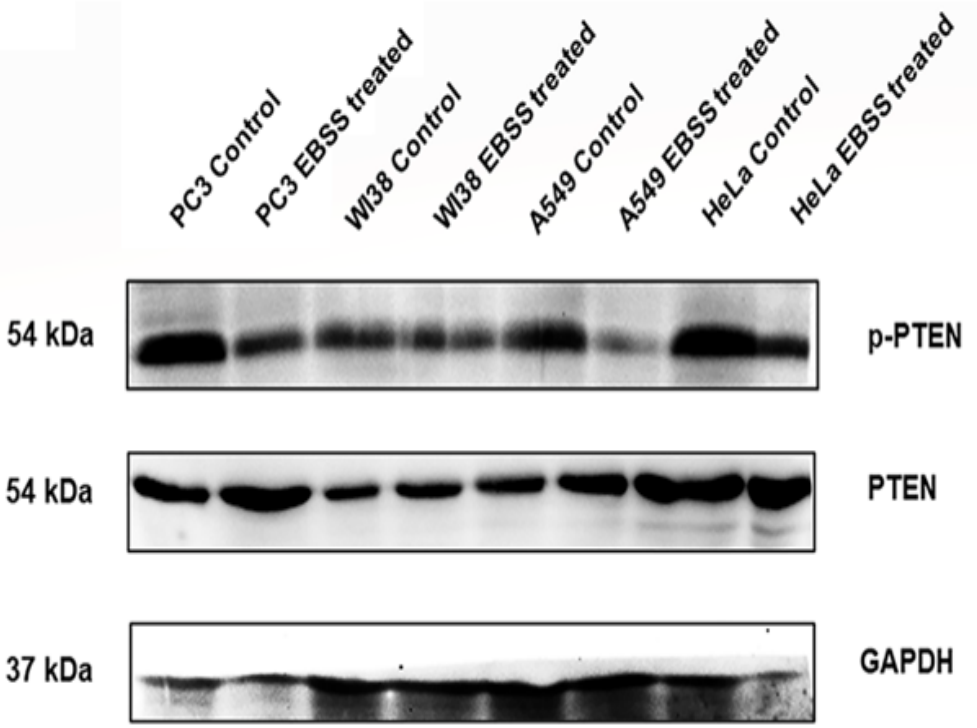
containing puncta after transfection of A4 PTEN and E4 PTEN respectively, as compared to 45.58 ± 1.07 % cells transfected with WT PTEN (Figure 8A, 8B). Also, autophagic vacuoles stained with MDC in U87MG cells after Tunicamycin treatment displayed 61.54 ± 2.07 % and 41.42 ± 1.31 % of cells containing puncta after transfection of A4 PTEN and E4 PTEN respectively, as compared to 54.04 ± 1.08 % cells transfected with WT PTEN (Figure 9A, 9B). When comparing A4 PTEN and E4 PTEN-transfected (PC3 as well as U87MG) cells to WT PTEN-transfected cells, LC3B analysis using indirect immunolabelling displayed strong fluorescence in all three types of tested cells, but with perceptible higher and lower intensity in A4 PTEN and E4 PTEN-transfected cells respectively when compared to WT PTEN-transfected cells (Figure 4, 10, 11). As mentioned earlier, following EBSS starvation, immunoblotting experiments in WT PTEN transfected PC3 cells revealed that the ratios of band intensities of Beclin1/GAPDH; ATG5/GAPDH; ATG7/GAPDH; p62/GAPDH and phospho-mTOR/mTOR were 0.76 ± 0.010; 0.96 ± 0.022; 1.14 ± 0.027; 0.89 ± 0.031 and 0.46 ± 0.019 respectively. Similarly, for A4 PTEN-transfected cells they were 0.97 ± 0.043; 1.16 ± 0.049; 1.32 ± 0.029; 0.56 ± 0.055 and 0.34 ± 0.015 respectively and for E4 PTEN-transfected cells, 0.62 ± 0.022; 0.81 ± 0.030; 1.03 ± 0.017; 1.07 ± 0.058 and 0.60 ± 0.035 respectively (Figure 5A, 5B, 6A, 6B). Similarly, following EBSS starvation, immunoblotting experiments in WT PTEN transfected U87MG revealed that the ratios of band intensities of Beclin1/GAPDH; ATG5/GAPDH; p62/GAPDH and phospho-Akt/Akt were 0.92 ± 0.033; 0.70 ± 0.048; 0.80 ± 0.081 and 0.71 ± 0.032 respectively. Similarly, for A4 PTEN-transfected cells they were 1.14 ± 0.070; 0.80 ± 0.037; 0.56 ± 0.010 and 0.53 ± 0.016 respectively and for E4 PTEN-transfected cells, 0.78 ± 0.032; 0.59 ± 0.035; 1.14 ± 0.012 and 0.87 ± 0.039 respectively (Figure 12A, 12B). Also, in WT PTEN transfected U87MG

cells after Tunicamycin treatment, the ratios of band intensities of Beclin1/GAPDH; ATG5/GAPDH; p62/GAPDH and phospho-Akt/Akt were 1.72 ± 0.096; 0.94 ± 0.061; 0.19 ± 0.019 and 0.47 ± 0.016 respectively. Similarly, for A4 PTEN-transfected cells they were 2.01 ± 0.093; 1.12 ± 0.042; 0.15 ± 0.015 and 0.35 ± 0.014 respectively and for E4 PTEN-transfected cells, 1.28 ± 0.077; 0.73 ± 0.032; 0.24 ± 0.015 and 0.53 ± 0.020 respectively (Figure 13A, 13B). The results were corroborated by TEM images, which showed that the transfected cells with A4 PTEN and WT PTEN had more autophagic vacuoles than the transfected cells with E4 PTEN (Figure 7).

2.3 When autophagy is induced, PTEN's phosphorylation declines and its sumoylation gets elevated.

In this phase, we investigated PTEN-positive cancer cells A549, HeLa, and normal human lung fibroblast cell WI-38 in addition to WT PTEN transfected PC3. The cells were subjected to an 18-hour EBSS-mediated starvation before being compared to their untreated counterparts. Using phospho-PTEN antibody, both starved and unstarved cells were immunoblotted to measure the phosphorylation status of PTEN during EBSS-induced autophagy. The ratios of band intensities of Phospho-PTEN/Total PTEN for starved cells were 0.53 ± 0.009; 0.93 ± 0.029; 0.50 ± 0.046 and 0.44 ± 0.050 for PTEN transfected PC3, WI-38, A549 and HeLa respectively. Similarly, the ratios of unstarved cells were 0.90 ± 0.033; 1.15 ± 0.047; 1.08 ± 0.036 and 0.69 ± 0.043 for PTEN transfected PC3, WI-38, A549 and HeLa cells respectively (Figure 14A, 14B).

A


PC3 Control
PC3 EBSS treated
WI38 Control
WI38 EBSS treated
A549 Control
A549 EBSS treated
HeLa Control
HeLa EBSS treated
54 kDa
p-PTEN
54 kDa
PTEN
37 kDa
GAPDH

B

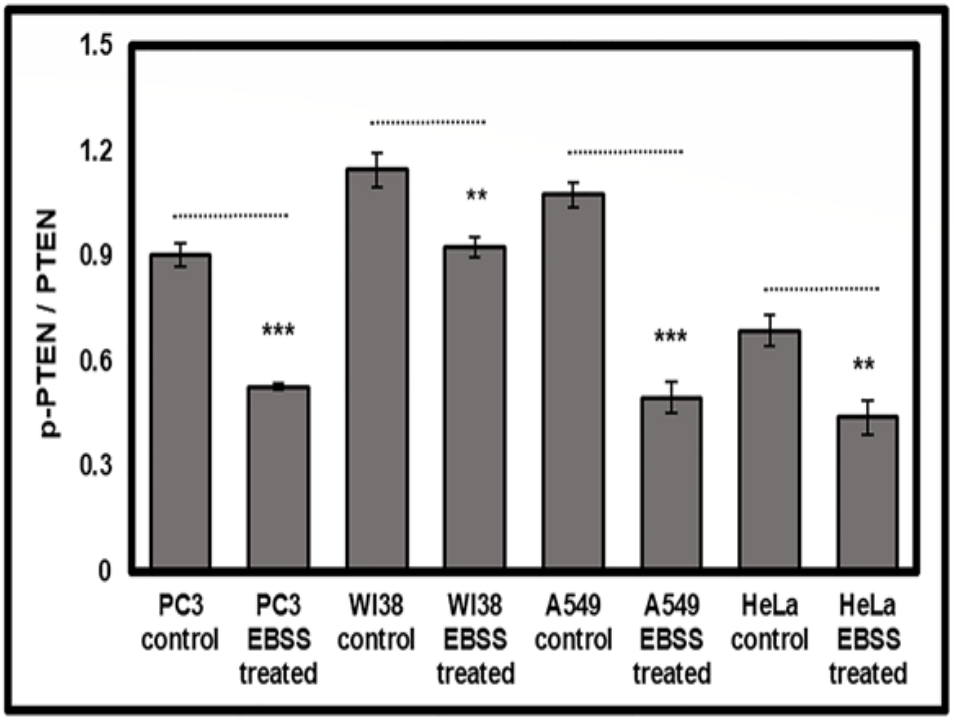

p-PTEN / PTEN
1.5
1.2
0.9
0.6
0.3
0
***
**
***
**
PC3 control
PC3 EBSS treated
WI38 control
WI38 EBSS treated
A549 control
A549 EBSS treated
HeLa control
HeLa EBSS treated

Figure 2.14

The level of PTEN phosphorylation in WI-38, A549, HeLa, and PTEN transfected PC3 cells as a result of autophagy during starvation (18 h).

(A) Total cell extracts from WI-38, A549, HeLa and PTEN transfected PC3 were collected, and using Western blot the levels of p-PTEN following starvation were compared. (B) The ratios between p-PTEN and PTEN band intensities as a result of autophagy are shown graphically. PTEN served as a loading control. ImageJ software was used to calculate the band intensities. The statistical differences between the calculated values of EBSS-treated starved cells and their unstarved counterparts were computed using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

Similarly, PTEN transfected U87MG cells were starved with EBSS for 18 h. The ratio of band intensities of Phospho-PTEN/Total PTEN for starved cells were 1.06 ± 0.017 whereas the ratio of unstarved cells were 1.32 ± 0.067 (Figure 15A). Also, PTEN transfected U87MG cells and WI-38 cells were treated with Tunicamycin for 24 h. The ratio of band intensities of Phospho-PTEN/Total PTEN for treated cells were 0.90 ± 0.035 and 0.82 ± 0.054 for U87MG cells and WI-38 respectively whereas the ratio of unstarved cells were 1.53 ± 0.031 and 1.28 ± 0.026 (Figure 15B 15C).

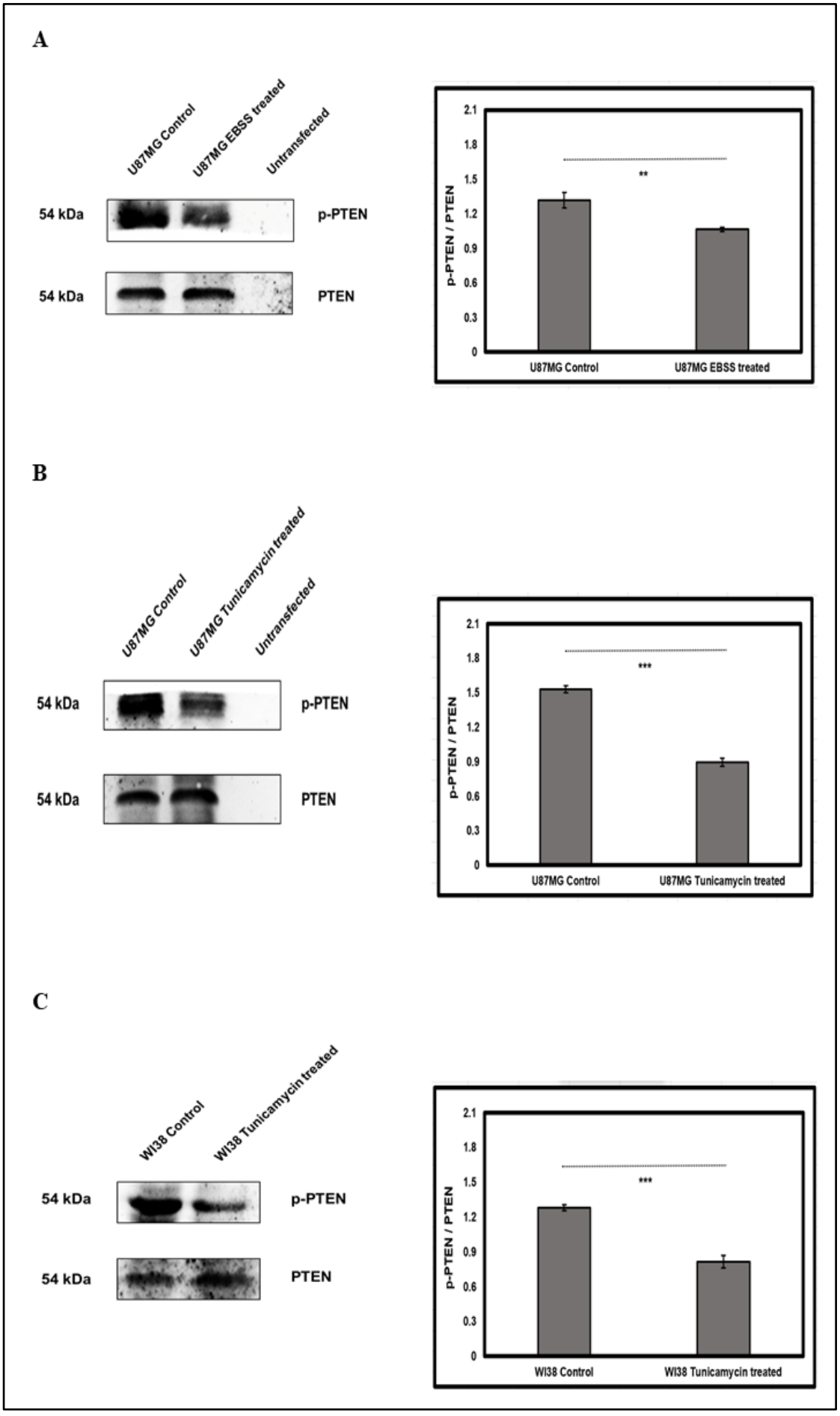

A
U87MG Control
U87MG EBSS treated
Untransfected
54 kDa
p-PTEN
54 kDa
PTEN
p-PTEN / PTEN
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
**
U87MG Control
U87MG EBSS treated
B
U87MG Control
U87MG Tunicamycin treated
Untransfected
54 kDa
p-PTEN
54 kDa
PTEN
p-PTEN / PTEN
***
U87MG Control
U87MG Tunicamycin treated
C
WI38 Control
WI38 Tunicamycin treated
54 kDa
p-PTEN
54 kDa
PTEN
p-PTEN / PTEN
***
WI38 Control
WI38 Tunicamycin treated

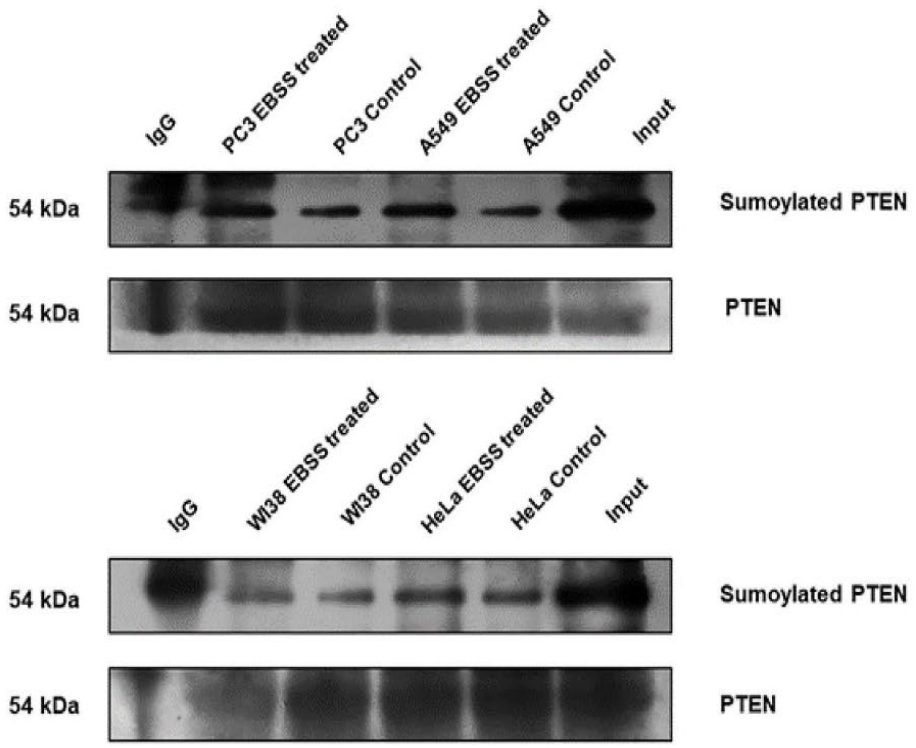
Figure 2.15

The level of PTEN phosphorylation as a result of autophagy in WI-38 after Tunicamycin mediated ER stress (24 h) and PTEN transfected U87MG cells after EBSS-mediated starvation (18 h) as well as Tunicamycin mediated ER stress (24 h).

(A) (B) (C) Total cell extracts from WI-38 and PTEN transfected U87MG were collected, and using Western blot the levels of p-PTEN following autophagy induction were compared. The ratios between p-PTEN and PTEN band intensities as a result of autophagy are shown graphically. PTEN served as a loading control. ImageJ software was used to calculate the band intensities. The statistical differences between the calculated values of treated cells and their untreated counterparts were computed using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

Total PTEN was immunoprecipitated from the cell extract and subsequently immunoblotted with SUMO2/3 antibody in order to measure the sumoylation status of PTEN under EBSS-induced autophagy. The ratios of band intensities of SUMOylated PTEN/Total PTEN for starved cells were 0.62 ± 0.037; 0.45 ± 0.025; 0.63 ± 0.042 and 0.52 ± 0.023 for PTEN transfected PC3, WI-38, A549 and HeLa cells respectively. Similarly, the ratios of unstarved cells were 0.48 ± 0.021; 0.37 ± 0.020; 0.41 ± 0.016 and 0.43 ± 0.013 for PTEN transfected PC3, WI-38, A549 and HeLa cells respectively (Figure 16A, 16B).

A


IgG
PC3 EBSS treated
PC3 Control
A549 EBSS treated
A549 Control
Input
54 kDa
Sumoylated PTEN
54 kDa
PTEN
IgG
WI38 EBSS treated
WI38 Control
HeLa EBSS treated
HeLa Control
Input
54 kDa
Sumoylated PTEN
54 kDa
PTEN

B

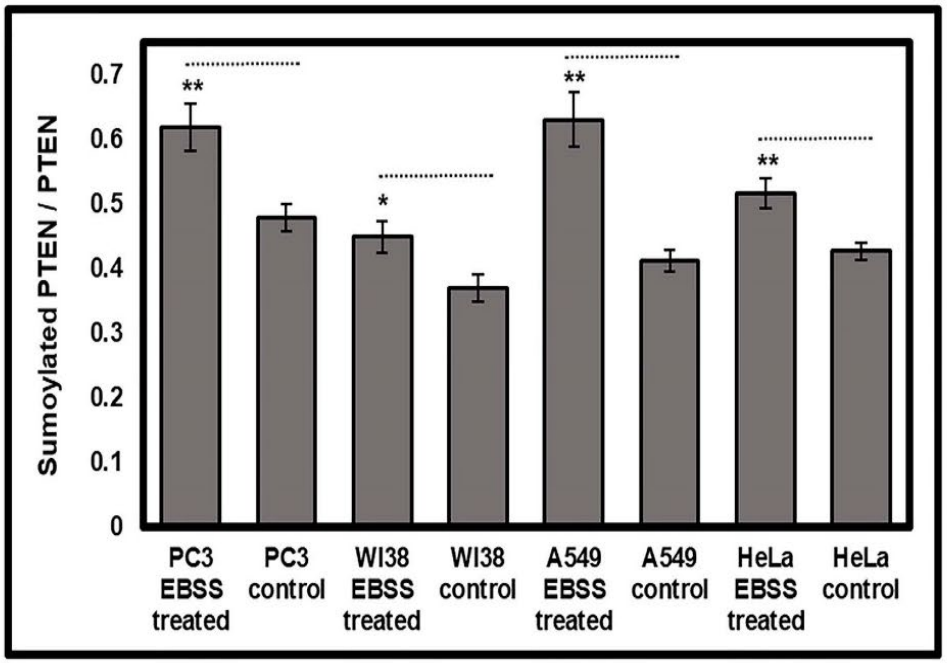

Sumoylated PTEN / PTEN
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
*
**
**
PC3 EBSS treated
PC3 control
WI38 EBSS treated
WI38 control
A549 EBSS treated
A549 control
HeLa EBSS treated
HeLa control

Figure 2.16.

The level of PTEN sumoylation in WI-38, A549, HeLa and PTEN transfected PC3 cells as a result of autophagy during starvation (18 h).

(A) Using Immunoprecipitation, total PTEN was collected from WI-38, A549 and HeLa and PTEN transfected PC3, and by using Western blot the levels of SUMOylated PTEN were compared between EBSS-treated and untreated cells. (B) The ratios between SUMOylated PTEN and PTEN band intensities as a result of autophagy are shown graphically. ImageJ software was used to calculate the band intensities. The statistical differences between the calculated values of EBSS treated starved cells with their unstarved counterparts were computed using the Student's t-test method. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Mean ± SD, N=3.

Additionally, the ratios of band intensities of SUMOylated PTEN/Total PTEN were measured for PTEN transfected U87MG cells treated with both EBSS as well as Tunicamycin and WI-38 cells treated with Tunicamycin (Figure 17A, 17B, 17C).

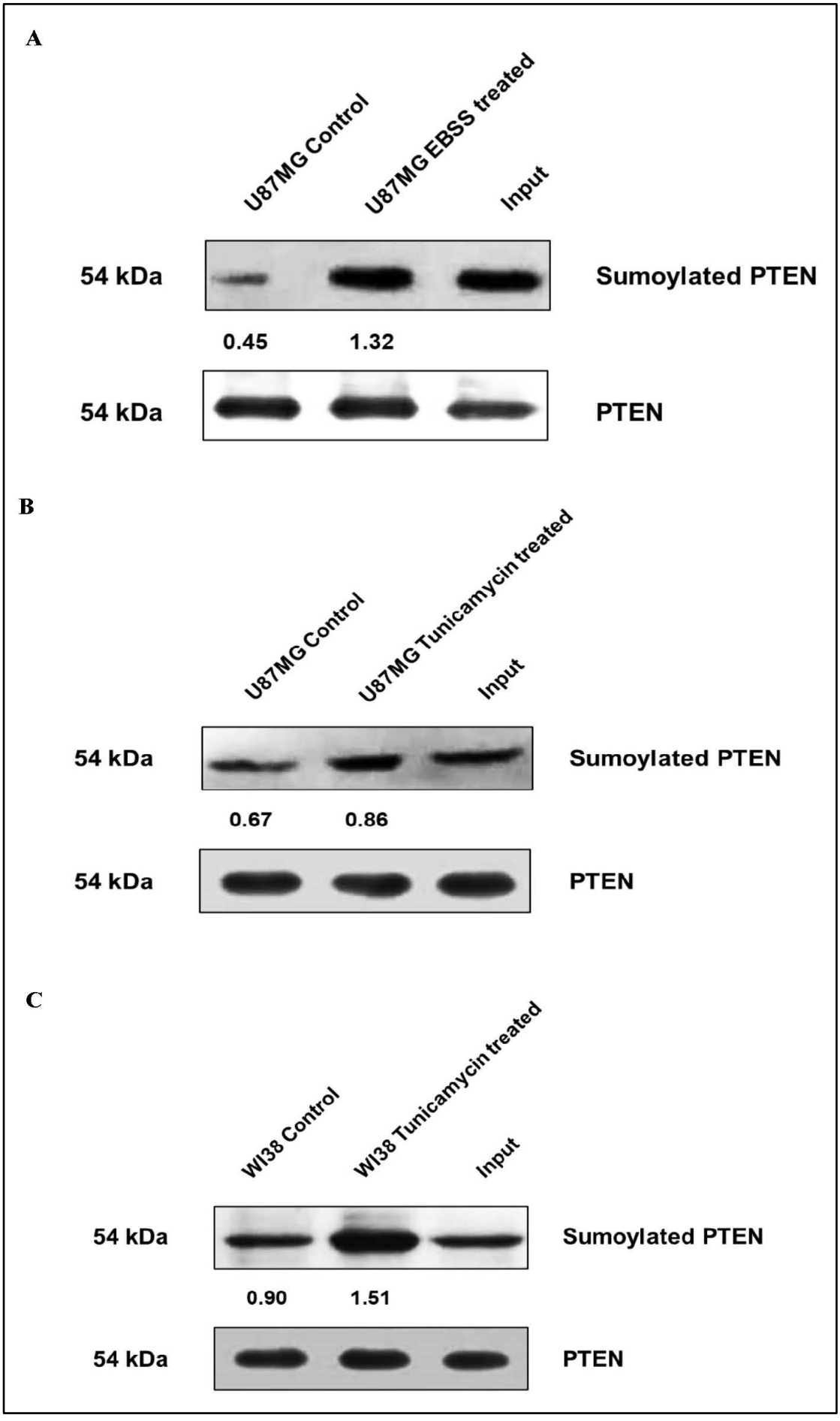

A
U87MG Control
U87MG EBSS treated
Input
54 kDa
Sumoylated PTEN
0.45
1.32
54 kDa
PTEN
B
U87MG Control
U87MG Tunicamycin treated
Input
54 kDa
Sumoylated PTEN
0.67
0.86
54 kDa
PTEN
C
WI38 Control
WI38 Tunicamycin treated
Input
54 kDa
Sumoylated PTEN
0.90
1.51
54 kDa
PTEN

Figure 2.17

The level of PTEN sumoylation as a result of autophagy in WI-38 after Tunicamycin mediated ER stress (24 h) and PTEN transfected U87MG cells after EBSS-mediated starvation (18 h) as well as Tunicamycin mediated ER stress (24 h).

(A) (B) (C) Using Immunoprecipitation, total PTEN was collected from WI-38 and PTEN transfected U87MG, and by using Western blot the levels of SUMOylated PTEN were compared between treated and untreated cells. The ratios between SUMOylated PTEN and PTEN band intensities as a result of autophagy are mentioned. ImageJ software was used to calculate the band intensities.

2.4 PTEN's cell membrane localization is aided and hindered by PTEN's sumoylation and phosphorylation respectively.

PTEN negatively regulates the PI3K/Akt signaling pathway, which consequently leads to its positive regulation of autophagy. The cell membrane is primarily where this downregulation takes place. PIP2 is catalysed to become PIP3 after PI3K is activated. Akt attaches itself to PIP3 at the cell membrane where PDK1 can interact with it, in order to phosphorylate and consequently activate it. The primary downstream target of Akt which plays an active role in reducing autophagy is mTOR which in turn gets phosphorylated. This inhibition of autophagy is reversed when PTEN suppresses the PI3K/AKT pathway by dephosphorylating PIP3 to PIP2. Hence, we tagged both PC3 as well as U87Mg cells with an anti-PTEN antibody and observed them under a confocal microscope. This was done following autophagy induction in order to identify the presence of PTEN in terms of subcellular localization. In transfected WT PTEN

and A4 PTEN cells, we observed strong fluorescence intensity along the cell membranes, in E4 PTEN it was comparatively weaker, and in PTEN (K254R) cells it was completely scattered in the cytoplasm (Figure 18, 19). This is indicative of the previously mentioned fine tuning of PTEN by its phosphorylation and sumoylation in terms of localization and accordingly its function.

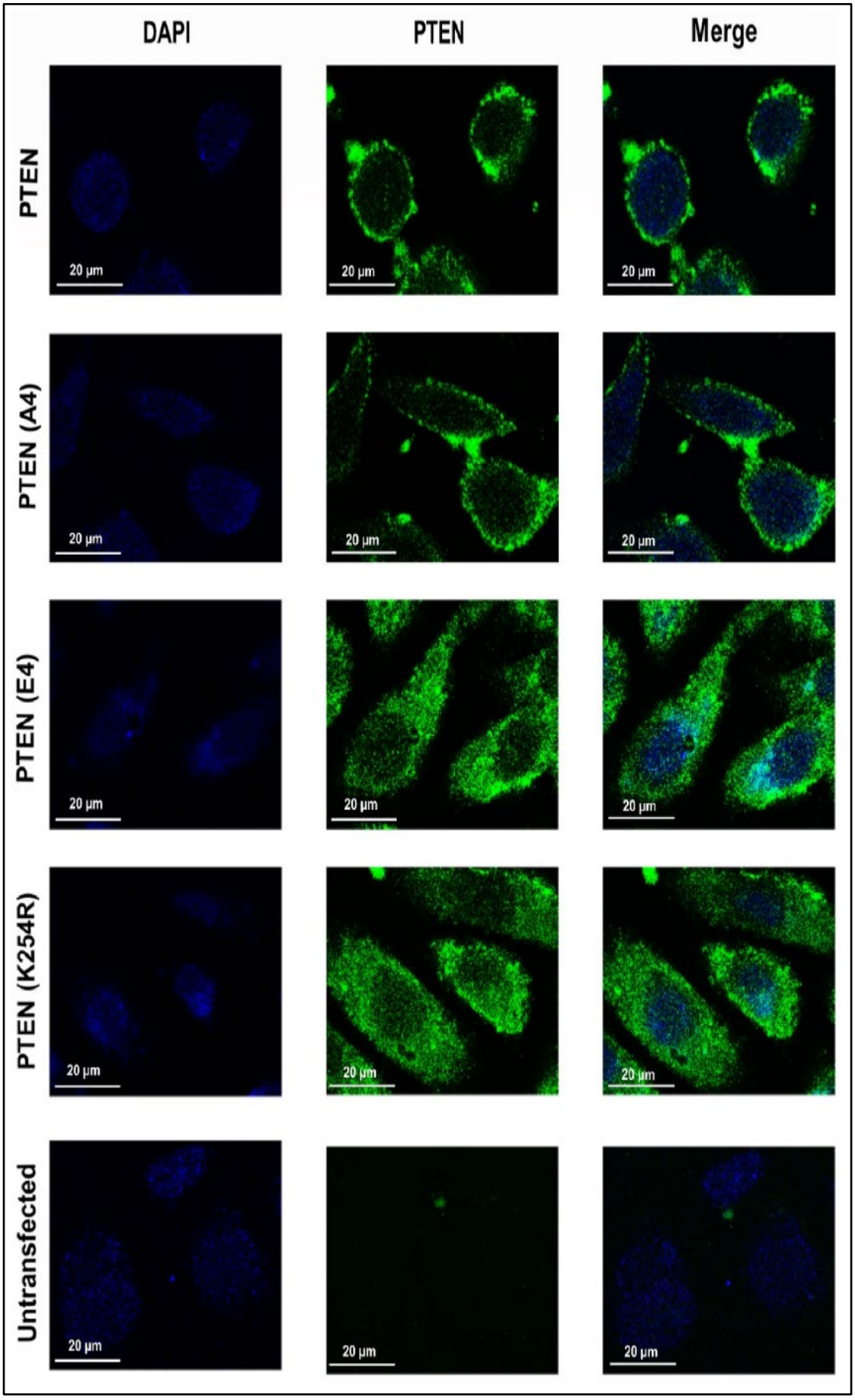

DAPI
PTEN
Merge
PTEN
PTEN (A4)
PTEN (E4)
PTEN (K254R)
Untransfected
20 µm

Figure 2.18

Following transfection of PTEN mutants in PC3 cells, visualization of PTEN's cell membrane localization using confocal microscopy.

Indirect immunolabelling was used to detect PTEN in transfected PC3 cells. After transfecting cells with PTEN gene constructs, they were starved for 18 hours. Finally, they were examined under a confocal microscope (63X magnification) after being immunolabelled with Anti-PTEN and DAPI mounting medium. Scale bar: 20 μm.

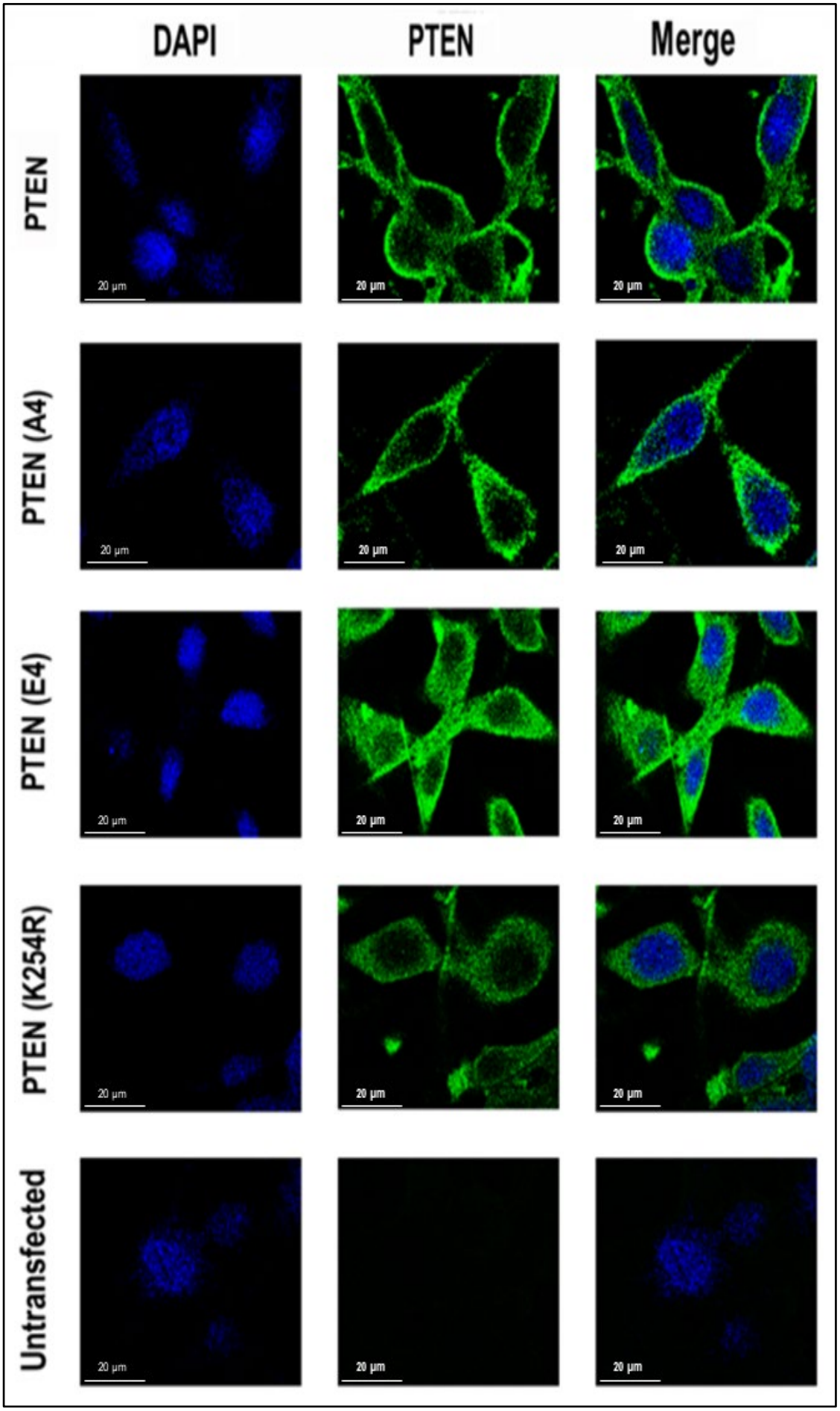

DAPI
PTEN
Merge
PTEN
PTEN (A4)
PTEN (E4)
PTEN (K254R)
Untransfected
20 µm

Figure 2.19

Following transfection of PTEN mutants in U87MG cells, visualization of PTEN's cell membrane localization using confocal microscopy.

Indirect immunolabelling was used to detect PTEN in transfected U87MG cells. After transfecting cells with PTEN gene constructs, they were put under Tunicamycin treatment for 24 h. Finally, they were examined under a confocal microscope (63X magnification) after being immunolabelled with Anti-PTEN and DAPI mounting medium. Scale bar: 20 µm.

Chapter 3

Discussion

Our whole study is centred around the regulation of autophagy by PTEN via PI3K/AKT/mTOR pathway. As have been mentioned previously, PI3K with the help of PIP3 switches on Akt, which in turn activates mTOR [80][81][82][83]. As mTOR plays a major role in curtailing autophagy, PTEN's dephosphorylating PIP3 to PIP2 will induce autophagy [84][85]. So, it goes without saying that autophagy will be almost non-existent in PTEN null cells.

PC3 cells are isolated from bone metastasis of prostatic adenocarcinoma while U87MG cells are isolated from malignant glioblastoma, and they both have inactivated PTEN [157]. We had transfected WT PTEN gene construct along with designated mutants of PTEN constructs into PC3 and U87MG cells and recorded alteration in autophagy levels. This was carried out by several methods such as cell staining by MDC, immunolabelling, immunoblot and visualisation of cells under TEM. Cells with HA empty vector constructs' transfection or cells without any transfection were used as control setup. We had induced autophagy via two distinct approaches namely nutrient deprivation as well as ER stress by EBSS and tunicamycin respectively. Autophagy can be effectively triggered in cells when they are deprived of nutrients. When starved of nutrients like amino acids, autophagy is activated to provide the cell with energy and essential building blocks [158]. EBSS is a saline solution having physiological pH and it induces autophagy by amino acid starvation (we used it because withdrawal of amino acids is seen to be more potent in inducing autophagy than glucose starvation in vitro) [159][160]. ER stress is a condition where the accumulation of unfolded or misfolded proteins in the ER triggers a cellular response to restore homeostasis, thereby going ahead with autophagy [121]. Tunicamycin is an antibiotic that

specifically aggravates ER stress by multiple ways like inhibiting N-glycosylation [138]. Using different activators of autophagy serves both fundamental research purposes as well as potential therapeutic motives.

We observed in our study that transfection of WT PTEN activated autophagy, and a continuous increase in the number of autophagic cells was observed as the time of starvation increased. The untransfected cells and HA empty vector cells showed similar levels of autophagy implying that they can be both used as a control set for further experiments. When PTEN mutant (C124S) were transfected inside the cells, the induction of autophagy was seen to be lowered. This mutant is phosphatase deficient, which failed to negatively regulate the AKT pathway due to its inability to convert PIP3 to PIP2, as previously described. Also, this observed lack of catalysis coincides with the fact that dysregulated PTEN is often coincident with elevated PIP3 levels in the cell membrane [80][81][82][83]. Thus, we can conclude that for autophagy initiation, lipid phosphatase activity, being the canonical function of PTEN, is primarily needed.

Out of several markers used in the quantification of autophagic flux, we naturally selected the ones that are deemed to be the most crucial in the whole process. Beclin1 is integral in the initial nucleation process and the subsequent autophagosome maturation, being part of signalling complexes that recruit indispensable effector proteins [91]. ATG5 is the component of the ATG5 ATG12 ATG16 complex that is required for both phagophore formation and elongation [88][95]. LC3B is necessary for both hemifusion of the autophagosome membrane as well as detection of the cargo [88][95]. ATG7 acts as an E1 enzyme in the formation of both ATG5 ATG12 ATG16 complex as well as LC3 PE conjugation [88][95]. p62 acts like an adaptor protein in cherry-picking desirable

cargo while getting degraded in the process [88][95]. So, it is generally observed that barring p62 which decreases with increase in autophagy, all of the mentioned markers increase with increase in autophagic flux [162][163]. Additionally, both Akt as well as mTOR negatively regulates autophagy [83][84]. So, expression of p-Akt and p-mTOR can be gauged to determine autophagy, where their levels are seen to be decreasing with increase in autophagic flux [164]. Our observations had also been similar to these established molecular markers.

Our principal investigation was then pursued, which was to look for a connection between the two most pertinent PTM and the change in autophagic flux.

As we previously discussed, PTEN's substrate PIP3 is found on the inner leaflet of the cell membrane, and its subsequent dephosphorylation by PTEN causes the PI3K/AKT pathway to be downregulated [80][81][82][83]. Hence, this regulatory node dictates the overall activation of autophagy by PTEN [84][85]. PTEN typically exhibits higher selectivity for the plasma membrane compared to the nuclear membrane because the inner leaflet of plasma membrane is composed of a larger amount of lipids [162]. PTEN’s C2 domain is made up of clustered cationic residues that naturally interacts non-specifically with the membrane [28][29][30][165][166]. According to molecular study, PTEN undergoes a necessary realignment after the subsequent electrostatic association with the membrane [85]. This reorientation is regulated by the C2 domain as well [85]. So, to cut a long story short, C2 domain not only assists in PTEN’s recruitment to the cell membrane but also in orientation of the catalytic phosphatase domain [166][167][168][169]. Functionally, this is of utmost importance as the exactness of PTEN’s orientation as well as penetration within the membrane determines its consequent dephosphorylation action [166][167][168][169].

The PTM sumoylation dictates the position of cellular substrates inside the cell as mentioned before [77]. PTEN is accordingly directed to the cytoplasm by decreased sumoylation, as observed in sumoylation mutants [78][170][171]. PTEN is sumoylated at the K254 location in the C2 domain, among other places [78][170][172]. PTEN's association with the plasma membrane is facilitated by sumoylation according to the principle of electrostatic interaction [170][173]. This is due to the fact that PTEN's net positive charge on the C2 domain has increased due to its covalent modification from sumoylation and is consequently drawn towards the existing negatively charged inner layer of the cell membrane [170][174]. These covalent modifications may additionally keep PTEN in an open conformation and further enhance membrane attachment by negatively regulating PTEN's interaction with its C-terminal tail [78][170]. According to reports, PTEN (K254R) is less prevalent in cell membrane fractions than transfected WT PTEN [170]. PTEN (K254R) transfected cells thus show decreased phosphatase activity and prolonged AKT phosphorylation, both of which are essential for the negative control of autophagy [170]. This was consistent with our finding that, as compared to WT PTEN, PTEN (K254R) transfected cells had lower quantity of autophagic vacuoles as well as reduced levels of autophagic molecular markers. Through an intricate system of phosphorylation and dephosphorylation, the 380–385 Ser/Thr cluster and other phosphorylation sites function as a spontaneous electrostatic switch of membrane association [74][165][175][176][177]. The C-terminal phosphorylation acts as a molecular clamp on the C2 domain, resulting in PTEN to adopt a compact and condensed shape [177]. Its membrane binding surface is unable to effectively make contact with the cell membrane due to this particular "closed" shape [175][177][178]. When it is dephosphorylated, the tail's

tight binding is reversed, making space for its "open" conformation and the subsequent membrane recruitment of its surface cationic residues [175][177][178]. This is comparable to the idea of an enzyme, in which the "closed" shape functions as a stable proenzyme that is prepared for subsequent activity [178]. Additionally, the phosphorylated C-terminal tail functions as a pseudosubstrate and engages in competition with the cell membrane's phospholipids for binding to the C2 domain [74][178]. Another theory is that the C-terminal tail in general interacts with PDZ domain-containing proteins, whereas its phosphorylation masks the PDZ domain binding site [74][175]. As a result of this, protein-protein interactions with the membrane, that are dependent on the PDZ domain and are required for membrane recruitment, is further intercepted. [74][175]. While PTEN (E4) transfected cells showed decreased membrane affinity, PTEN (A4) transfected cells showed WT PTEN like affinity for POPC/POPS (8:2) vesicles (which imitate the inner lipid leaflet of the cell membrane) [165]. The dianionic phosphoryl groups produced by phosphorylation are replaced by single anions in this phospho-mimicking mutant due to glutamic acid alterations. It suggests that there is a further elevation of electrostatic shielding in actual situations [165]. Our results were similarly consistent with these reported findings. PTEN (E4) and PTEN (A4) transfected PC3 as well as U87MG cells, showed a decrease and an increase in autophagy respectively, in response to the phosphorylation dephosphorylation switch.

Also, we wanted to check whether the converse nature of these experiments is affirmative or not. This means whether there is a significant difference in the sumoylation and phosphorylation status of PTEN positive cells on induction of autophagy, are visible or not. For this, we studied normal (WI-38) and cancer

(A549, HeLa) cells which are not PTEN null by nature, alongside PTEN transfected PC3 and U87MG cells. Sumoylation and phosphorylation of PTEN were found to be elevated and reduced respectively on autophagy induction, which further supported our earlier experiments.

The above mentioned sumoylation and phosphorylation mediated alteration in autophagy was seen to be similar in cases of both EBSS and tunicamycin treatment, signifying nutrient deprivation and dysregulated proteostasis converging to a similar line of regulatory action.

This whole work was influenced by several of our previous research which all suggested the importance of PTEN's PTM and its relation to the PI3K/Akt pathway in cell physiology. We had earlier observed how PTEN's phosphorylation and hyperactivation of Akt was implicated in Hep G2 cells' resistance towards etoposide-induced toxicity where they influenced multinucleation [179][180]. Similarly, how they acted in sync to affect genomic stability by downregulation of Rad51 in HEK 293T cells [181]. We had also observed how PTEN's phosphorylation negatively affected PTEN's DNA repair activity and the role it played in replication fork progression [182][183]. Also, we had found out that loss of PTEN's sumoylation and phosphorylation resulted in elevated chromosomal damage [184][185]. The current discourse linking PTEN's PTM to autophagy is a further step in understanding the intrinsic value of this tumour suppressor gene.

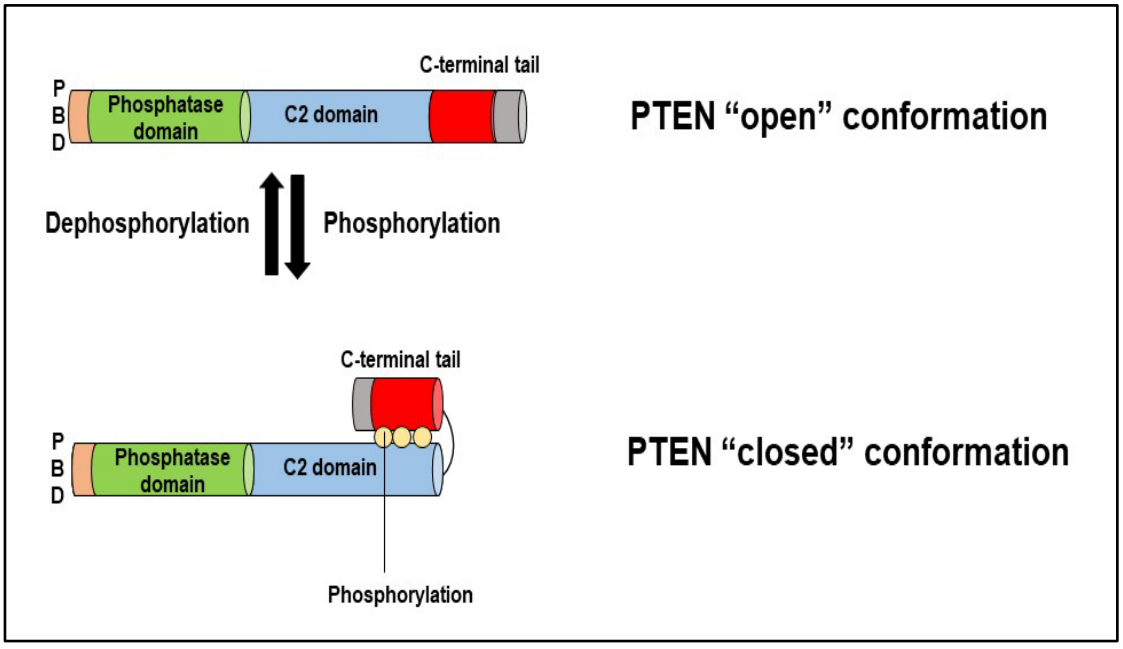


Figure 3.1

Conformational regulation of PTEN.

Phosphorylation of PTEN's C-terminal enhances contact between the tail and C2 domain, resulting in a closed conformation that conceals membrane binding. Dephosphorylating PTEN converts it from a closed to an open conformation, allowing it to bind to membrane and PDZ domain proteins.

Adapted from Lee, Y. R., Chen, M., & Pandolfi, P. P. (2018). The functions and regulation of the PTEN tumour suppressor: new modes and prospects. Nature reviews. Molecular cell biology, 19(9), 547–562. https://doi.org/10.1038/s41580-018-0015-0

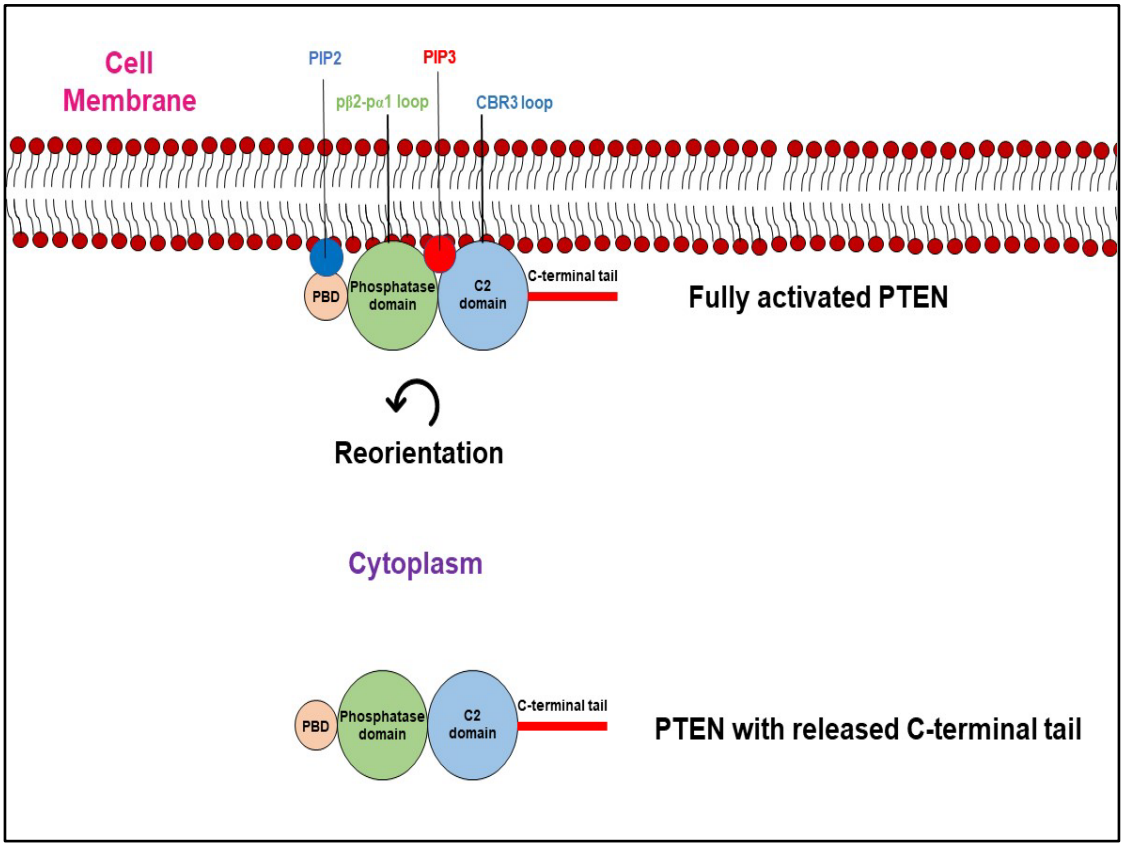


Figure 3.2

Membrane localization of PTEN.

The CBR3 loop and the pβ2-pα1 loop (Arginine loop) are the major motifs involved in the association of PTEN with the cell membrane. Our results were also in line with this established theory where dephosphorylated as well as sumoylated PTEN helped to maintain C-terminal tail in this released state, allowing proper attachment to the membrane and reorientation, which consequently elevated autophagy.

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

Materials and Methods

4.1 Plasmids

Gene-constructs pSG5L-HA-WT-PTEN (WT PTEN) encodes hemagglutinin tagged wild type PTEN protein. pSG5L-HA-A4-PTEN (Ser380Ala, Thr382Ala, Thr383Ala, Ser385Ala) (PTEN A4) encodes dephosphorylation mimicking PTEN. pSG5L-HA-E4-PTEN (Ser380Glu, Thr382Glu, Thr383Glu, Ser385Glu) (PTEN E4) encodes phosphorylation mimicking PTEN. These were gifts from Dr. William R. Sellers, Harvard Medical School. pSG5L-HA-C124S-PTEN (Cys124Ser) (PTEN Pd) encodes phosphatase deficient PTEN. pSG5L-HA-K254R-PTEN (Lys289Arg) (PTEN Sd) encodes sumoylation deficient PTEN. These were generated by Site-directed Mutagenesis according to "Stratagene Kit" and confirmed by sequencing. pSG5L-HA, used as a control empty vector was purchased from "Bio Bharati Life Science Pvt. Ltd.".

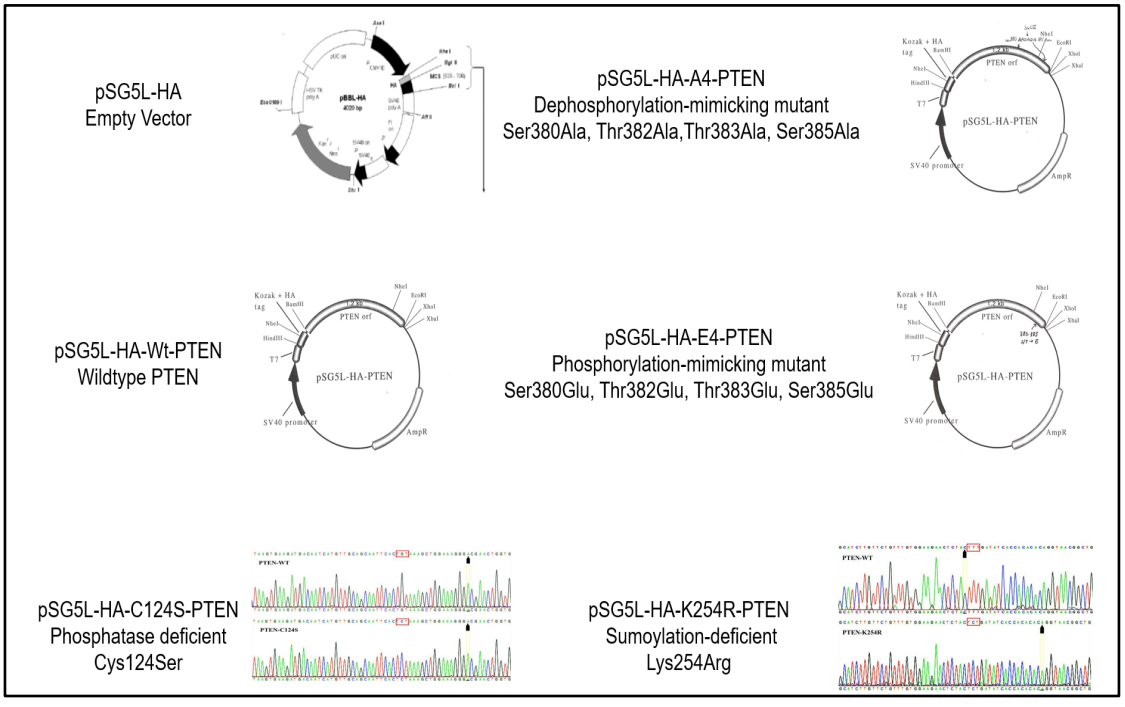


Figure 4.1

Various types of plasmids used in the experiment.

4.2 Cell Culture

Prostate adenocarcinoma (PC3), Glioblastoma (U87MG), Lung carcinoma (A549), Cervical adenocarcinoma (HeLa) and Lung normal fibroblast (WI-38) cells were cultured (CO_2 incubator-Thermo Fisher Scientific). The temperature was maintained at 37°C, CO_2 concentration at 5% and Relative Humidity at 95%. Different culture media (HiMedia) RPMI 1640, DMEM and MEM were used for different cells with 10% Fetal Bovine Serum FBS) (Gibco). The antibiotic and antifungal used were Penicillin/Streptomycin (HiMedia), Amphotericin-B (HiMedia) respectively.

4.3 MDC staining

PC3 cells were cultured on 18 mm coverslips rested on 35 mm plates overnight. Cells were transfected (Lipofectamine 3000 (Thermo Fisher Scientific)) with HA empty vector, WT PTEN and incubated overnight. Untransfected PC3 cells were also used as control. The following day after transfection, cells were treated with EBSS (Gibco) for different time points (4 hours, 8 h, 18 h) and subsequently washed with 1X PBS (HiMedia) followed by incubation with 50 mmole/L MDC (Sigma-Aldrich) for 15 min at 37°C. After subsequent steps of washing with 1X PBS, cells were mounted with glycerol on slides (Merck) and observed under fluorescence microscope (Leica).

PC3 cells were cultured and transfected with HA empty vector, WT PTEN, PTEN A4, PTEN E4, PTEN Pd, PTEN Sd. The following day, cells were treated with EBSS for 18 h and according to the aforementioned protocol, incubated with

MDC. Cells were mounted with glycerol on slides and observed under fluorescence microscope.

U87MG cells were seeded and transfected with gene constructs HA empty vector, WT PTEN, PTEN A4, PTEN E4, PTEN Pd, PTEN Sd. Next day cells were starved with EBSS for 18 hours as well as treated with 1 µg/ml Tunicamycin (Sigma-Aldrich) for 24 hours in separate experiments. Cells were mounted with glycerol on slides and observed under fluorescence microscope.

4.4 Indirect immunolabeling

PC3 cells were cultured on 18 mm coverslips rested on 35 mm plates overnight. Cells were transfected with HA empty vector, WT PTEN. Next day, cells were treated with EBSS for 18h. Next day, cells were fixed and permeabilized in 4% paraformaldehyde (HiMedia) (15 min) and 0.2% Triton X-100 (MP Biomedicals) (10 min) respectively. For the blocking step, 5% FBS in 1X PBS was used where cells were incubated for 60 min. Finally, they were kept overnight with Anti-LC3B tagged antibodies (Abcam) in wash buffer (0.05% Tween 20 (Amresco) and 0.5% FBS in 1X PBS) at 4°C. Cells were subsequently treated with FITC conjugated antibody (Cell Signaling Technology) for 1 hour at RT, after washing with wash buffer. Again, after washing, coverslips were mounted with DAPI (Vector laboratories) and seen under fluorescence microscope.

PC3 cells were cultured and transfected with HA empty vector, WT PTEN, PTEN A4, PTEN E4, PTEN Pd, PTEN Sd. Next day, cells were treated with EBSS for 18 h. The following day, cells were put through the same steps of fixation, permeabilization, incubation in blocking solution and ultimately incubation with Anti-LC3B antibodies overnight. They were subsequently treated with FITC

conjugated anti-rabbit antibody. DAPI was used as mounting medium to visualise cells under fluorescence microscope.

U87MG cells were cultured and transfected with HA empty vector, WT PTEN, PTEN A4, PTEN E4, PTEN Pd, PTEN Sd. Next day, cells were treated with EBSS for 18 h as well as treated with 1 μg/ml Tunicamycin for 24 h in separate experiments. The following day, cells were put through the same steps of fixation, permeabilization, incubation in blocking solution and ultimately incubation with Anti-LC3B antibodies overnight. They were subsequently treated with FITC conjugated anti-rabbit antibody. DAPI was used as mounting medium to visualise cells under fluorescence microscope.

PC3 and U87MG cells were cultured and transfected with WT PTEN, PTEN A4, PTEN E4, PTEN Sd along with untransfected cells as control. Next day, PC3 cells were treated with EBSS for 18 h and U87MG cells were treated with 1 μg/ml Tunicamycin for 24 h. The following day, cells were put through the same steps of fixation, permeabilization, incubation in blocking solution and ultimately incubation with Anti-PTEN tagged antibodies (Santa Cruz Biotechnology) overnight. They were subsequently treated with FITC conjugated anti-rabbit antibody. DAPI was used as mounting medium to visualise cells under confocal microscope (Zeiss).

4.5 Western Blot

PC3 cells were cultured on 60 mm plates overnight and subsequently transfected with HA empty vector, WT PTEN. The following day, cells were treated with EBSS for 4 h, 8 h, 18 h. Whole-cell lysates were generated from cells through steps of centrifugation and using lysis buffer (150 mM NaCl (Merck), 0.1% SDS

(HiMedia), 1% NP-40 (Sigma), 1 mM EDTA (HiMedia), 50 mM Tris (pH 8.0) (HiMedia), 0.5% Sodium deoxycholate (HiMedia), (phosphatase and protease inhibitors) (Genetix Biotech). The lysates were further incubated in protein loading dye (BioBharati LifeScience) and were boiled. Bradford assay (Sigma) was used to ascertain protein concentrations of respective whole-cell lysates and accordingly they were run on 10% SDS-PAGE gel (Bio-Rad Laboratories). This was followed by transferring the proteins from the gel to methanol activated PVDF membranes (Merck). TBS comprising of 5% skim milk (Himedia) and 0.1% Tween-20 was used to 'block' the membranes (TBST). The membranes were ultimately incubated overnight with Anti-GAPDH (BioBharati LifeScience), Anti-PTEN, Anti-Beclin1, Anti-ATG5, Anti-ATG7 (Cell Signaling Technology) antibodies in BSA (Sisco Research Laboratories) at 4°C. The following day, subsequent treatment with HRP conjugated antibodies (Sisco Research Laboratories) was carried out. The resulting protein bands were seen with the help of ECL Substrate Kit (Advansta).

PC3 cells were cultured and were transfected with HA empty vector, WT PTEN, PTEN A4, PTEN E4, PTEN Pd, PTEN Sd. The cells were starved with EBSS (18 h). The generation of whole-cell lysates was completed and the aforementioned process on SDS-PAGE gel was carried out. The proteins were then transferred to PVDF membranes and were ultimately incubated overnight with Anti-GAPDH, Anti-PTEN, Anti-ATG5, Anti p62, Anti-Beclin1, Anti-ATG7, Anti-mTOR, Anti-Phospho-mTOR antibodies. The following day, membranes were incubated with antibodies conjugated to HRP. Finally, the resulting protein bands were seen.

U87MG cells were cultured and were transfected with HA empty vector, WT PTEN, PTEN A4, PTEN E4, PTEN Sd. Transfected cells were starved with EBSS

(18 h) as well as treated with 1 μg/ml Tunicamycin (24 h) in separate experiments. The generation of whole-cell lysates was completed and the aforementioned process on SDS-PAGE gel was carried out. The proteins were then transferred to PVDF membranes and were ultimately incubated overnight with Anti-GAPDH, Anti-PTEN, Anti-ATG5, Anti p62, Anti-Beclin1, Anti-Akt, Anti-Phospho-Akt antibodies. The next day, membranes were incubated with antibodies conjugated to HRP. Finally, the resulting protein bands were seen.

PC3, WI-38, HeLa, A549 cells were cultured and only PC3 cells were transfected with WT PTEN. Next day, one group of each cell line was treated with EBSS for 18 h and the other half was kept untreated. The generation of whole-cell lysates was completed and the aforementioned process on SDS-PAGE gel was carried out. The proteins were then transferred to PVDF membranes and were ultimately incubated overnight with Anti GAPDH, Anti-Phospho-PTEN (Ser380/Thr382/Thr383) (Cell Signaling Technology), Anti-PTEN antibodies. The following day, membranes were incubated with antibodies conjugated to HRP. Finally, the resulting protein bands were seen.

U87MG and WI-38 cells were cultured and U87MG cells were transfected with WT PTEN. One group of U87MG was treated with EBSS for 18 h as well as treated with 1 μg/ml Tunicamycin for 24 h in separate experiments. Their counterpart groups were kept untreated. Similarly, one group of WI-38 cells was treated with 1 μg/ml Tunicamycin for 24 h and its counterpart group was kept untreated. The generation of whole-cell lysates was completed and the aforementioned process on SDS-PAGE gel was carried out. The proteins were then transferred to PVDF membranes and were ultimately incubated overnight with Anti GAPDH, Anti-Phospho-PTEN, Anti-PTEN antibodies The following

day, membranes were incubated with antibodies conjugated to HRP. Finally, the resulting protein bands were seen.

For loading control in all immunoblot experiments, anti-GAPDH antibody was used. ImageJ software was used to estimate the resulting band intensities.

4.6 Immunoprecipitation

PC3, U87MG, WI-38, HeLa and A549 cells were cultured on 60 mm sterile plates overnight. PC3 and U87MG cells were transfected with WT PTEN. Transfected cells were incubated overnight and the next day, one group of each cell type was treated with EBSS for 18 h and other half was kept without any treatment. Also, one batch of U87MG and WI-38 cells was treated with 1 μg/ml Tunicamycin for 24 hours and other half was left untreated. Lysis buffer (150 mM NaCl, 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM EDTA, 0.5% Sodium deoxycholate, 1% NP-40, phosphatase and protease inhibitors) was used to produce whole-cell lysates. Protein A/G agarose bead was used to clear the generated lysates and the following incubation was done overnight at 4°C with Anti-PTEN antibody (Cell Signaling Technology). The subsequent incubation was done for another 2 h at 4°C after a repeated addition of the protein A/G agarose bead. This is followed by a series of steps comprising of centrifugation as well as washing with lysis buffer. 2X protein loading dye was used for the elution of the protein with subsequent boiling. Finally, western blot was performed according to the previously mentioned protocol using Anti-PTEN, Anti-SUMO1, Anti-SUMO2/3 antibody (Cell Signaling Technology). IgG (Genei) was used as a negative control.

4.7 Transmission Electron Microscopy

PC3 cells were cultured on 60 mm sterile plates overnight. Cells were transfected with gene constructs WT PTEN, PTEN A4, PTEN E4, PTEN Pd, PTEN Sd along with untransfected cells as control. Cells were treated with EBSS for 18 h. 3 % 0.1 M sodium cacodylate buffered glutaraldehyde was used for primary fixation (4 h). 1 % osmium tetroxide was used for secondary fixation. Subsequently, a series of gradations of acetone was used for dehydration. Agar 100 resin was used for the embedding which was thereupon polymerised at 60°C. Ultramicrotome was used for generating ultrathin sections. 0.2 % lead citrate and 2 % aq. uranyl acetate were used for staining the sections. Sections were then observed under TEM (Jeol)

4.8 Statistical analyses

For ascertaining the statistical differences between data groups, two-tailed Student's t-test method was utilised. P-value ($p < 0.05$, $p < 0.01$, $p < 0.001$) were adjudged statistically significant. The p-value, standard deviation (SD) and mean were calculated from Microsoft Excel software. Error bars depict the mean ± SD for all plots.

Conclusion

PTEN, the tumour suppressor protein, is a classical regulator of the PI3K/AKT/mTOR signalling pathway which in turn modulates autophagy. So, it goes without saying that PTEN null cells like PC3 and U87MG will fail to express autophagy. In our study, we have showed that transfection of wildtype PTEN in such cells can rescue autophagy, stemming from both nutrient deprivation as well as endoplasmic reticulum stress. Now exploring further, we tried to rescue autophagy using a phosphatase deficient mutant of PTEN and we failed to fully recover it. This indicated the importance of PTEN's lipid phosphatase activity in the regulation of autophagy. Similarly, using other relevant mutants we demonstrated that sumoylation and phosphorylation of PTEN positively and negatively regulates autophagy respectively. These post-translational modifications mediated tuning of autophagy was also found to be existent in PTEN positive normal (WI-38) as well as cancer (A549, HeLa) cells. While searching for a reason behind such observed occurrences, we found out that it was all related to PTEN's association with the cell membrane where it acts on the PI3K/AKT/mTOR pathway. A comprehensive database of PTM disease associations with respect to PTEN would be extremely beneficial for fundamental and clinical research. For example, it could help us to categorize each PTM of PTEN with its associated tumour phenotype, similar to a disease-gene network. This would help us to gauge the actual prominence of each PTM of PTEN in relation to a particular disease. Additionally, it would open a window towards understanding the varied crosstalk between the PTM which are implicated in a disease. Ours results along can aid in this entire procedure. On a similar note, our work can be extended to repurpose existing drugs (both autophagy inducing or

blocking drugs in cancer, as previously mentioned). Also, as PTEN can be delivered via exosomes for therapeutic purpose (we are currently working on that), we can study the effects of mutated PTEN delivery on distant target cells after by differential expression of PTEN.

Out of hundreds post-translational modifications and thousands tumour suppressor proteins, we have selected such representatives for our research, that are most pursued and pivotal in protein biology. Alongside, we linked autophagy, a phenomenon that is considered as the pillar of homeostasis maintenance.

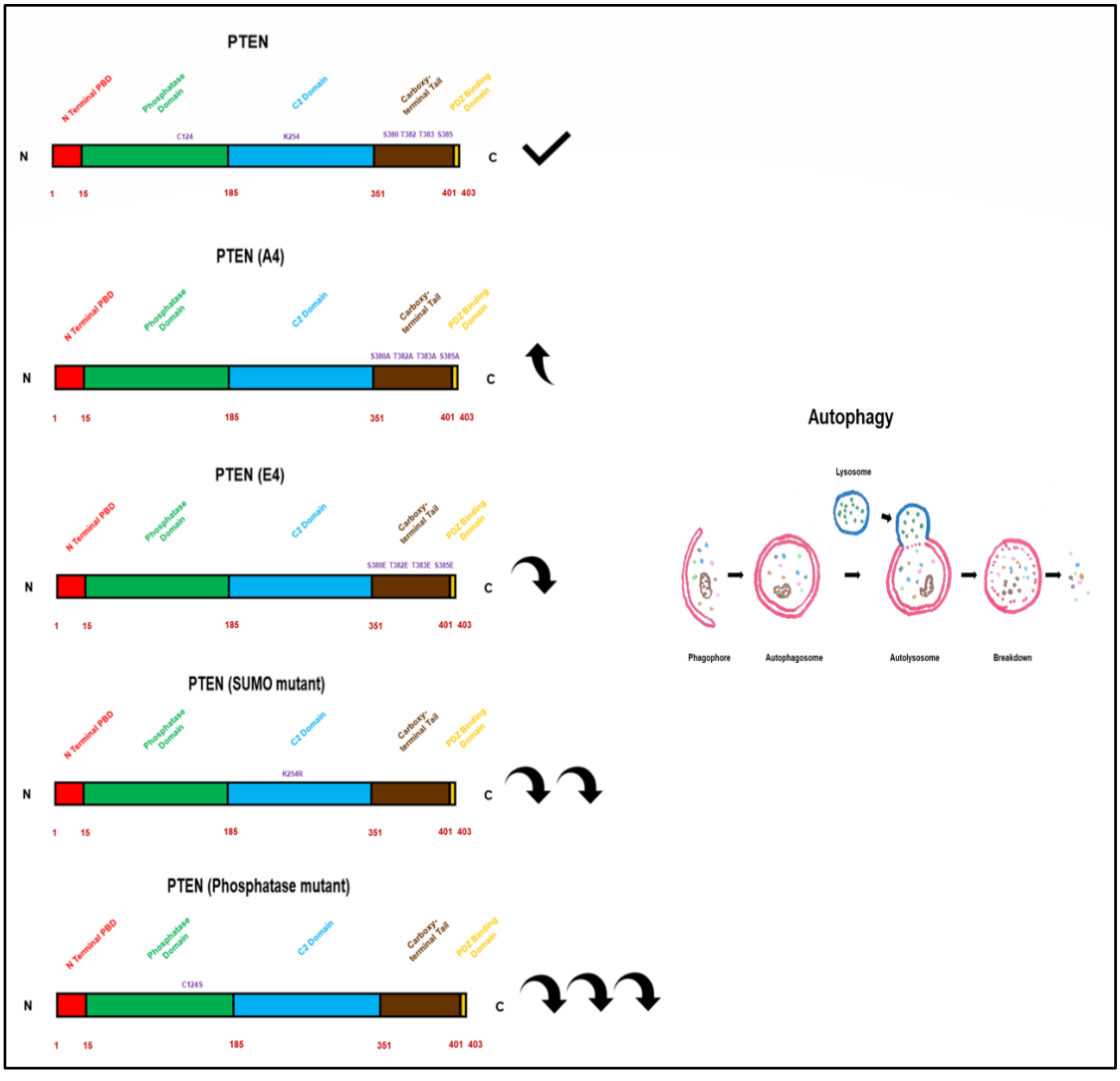


Figure 5.1

Summary of the entire research work.

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- Das, S., Bera, D., De, D., Mondal, D., Karmakar, P., Das, S., & Dey, A. (2022). Thymoquinone incorporated chitosan-sodium alginate/psyllium husk derived biopolymeric composite films: A comparative antibacterial and anticancer profile. European Polymer Journal, 180, 111608. https://doi.org/10.1016/j.eurpolymj.2022.111608
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- Das, S., De, D., Bera, D., Mondal, D., Karmakar, P., Das, S., & Dey, A. (2024). Oxidative stress-generated antibacterial and anticancer activities of piperine incorporated guar gum and psyllium husk derived biopolymeric nanocomposite. (Communicated).

List of Conference presentations

- 2023 ICRIB 2023, International Conference on Recent Innovations in Biotechnology, Sathyabama University, India.
- 2023 Biospectrum 2023, International Conference on Biotechnology and Biological Sciences, UEM University, India.