Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress

by

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Abstract

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Loss of function of the phosphatase and tensin homolog (PTEN) tumor suppressor is frequently found in many human malignancies. PTEN antagonizes the Phosphatidylinositide 3-kinase (PI3K) pathway (*1*) by dephosphorylating the 3 position of phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] (PIP3). PIP3 serves as a second messenger whose levels in the plasma membrane are elevated following cell stimulation with growth factors, mediated by the activity of PI3Ks. Proteins containing pleckstrin homology (PH) domains physically interact with PIP3, bringing them into close proximity with other PH domain-containing proteins and facilitating further functional interactions that serve to propagate the membranous signal.

Although the cytoplasmic role of PTEN in antagonizing PI3K signaling has been well studied, PTEN also resides in the nucleus, where its function remains poorly understood. Here, I demonstrate that SUMOylation (SUMO) of PTEN controls its nuclear localization. Consistent with its restricted cytoplasmic localization, a SUMO-deficient PTEN mutant is fully competent for regulating PI3K signaling. Upon exposure to genotoxic stress, SUMO-PTEN is rapidly excluded from the nucleus in an ATM-dependent manner, identifying a connection between

these two major tumor suppressors. Further, ATM phosphorylated PTEN on threonine 398, and a PTEN mutant that cannot be phosphorylated at this position (PTEN T398A) resist nuclear exclusion following genotoxic stress. Judging by various readouts of the DNA damage response, cells lacking nuclear PTEN are hypersensitive to DNA damage and display impaired homologous recombination-mediated repair of double-strand DNA breaks. Moreover, unlike cells with Wt PTEN, PTEN-deficient cells are susceptible to killing by a combination of genotoxic stress and a small molecule inhibitor of PI3K both *in vitro* and *in vivo*. The synergistc effect of genotoxic stress and PI3K inhibition on PTEN-null cells is dependent on the simultaneous inhibition of both p110α and p110β isoform. Further *in vivo* studies revealed that effective inhibition of PI3K pathway can be achieved with a discontinuous administration schedule in order to reduce the adverse effects associated with this treatment. My findings have considerable implications for individualizing therapy for patients with PTEN-deficient tumors.

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Finally, I would like to thank my wife, Chiara. Companion, lover, friend, she was always in my corner cheering me up and stood by me through the good times and bad. Her and little Diego really fill my life.

« "O frati," dissi, "che per cento milia
perigli siete giunti a l'occidente,
a questa tanto picciola vigilia

d'i nostri sensi ch'è del rimanente
non vogliate negar l'esperïenza,
di retro al sol, del mondo sanza gente.

Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza". »

(Dante Alighieri, Divina Commedia, Inferno canto XXVI, 116-120)

'O brothers!' I began, 'woe to the west
'Through perils without number now have we reach'd;
'To this the short remaining watch, that yet

'Our senses have to wake, refuse not proof
'Of the unpeopled world, following the track
'Of Phoebus.

Call to mind from whence ye sprang:
'Ye were not form'd to live the life of brutes,
'But virtue to pursue and knowledge high.'

(Dante Alighieri, The Divine Comedy, Inferno canto XXVI, 116-120)

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List of acronyms

Acronym	Definition
Aos1	SUMO-activating enzyme subunit aos-1
APC/C	Anaphase-Promoting Complex, also called cyclosome
ATM	Ataxia Telangiectasia Mutated
ATR	ATM- and RAD3-related
BAD	Bcl2-associated agonist of cell death
BER	Base Excision Repair
BMI1	Polycomb complex protein BMI-1, encoded by B cell-specific Moloney murine leukemia virus integration site 1 gene
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA1/2	Breast Cancer Gene 1 and 2
C/EBPs	CCAAT-enhancer-binding proteins
CBP	CREB-binding protein
c-Cbl	Mouse Casitas B-lineage Lymphoma gene
CDH1	cadherin 1
CDK	Cyclin Dependent Kinase
cDNA	Complementary DNA
CENP-C	Centromeric protein C
ceRNAs	competing endogenous RNAs
CHK1/2	Checkpoint kinase 1 and 2
CK2	Casein kinase 2

Acronym	Definition
DDR	DNA damage response
dHJ	double-Holliday Junction
Dlg1	Disc large homolog 1
Dmc1	disrupted meiotic cDNA
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-dependent protein kinase
DR-GFP	Direct recombination GFP
DSB	Double-Strand Break
E2F1	E2F transcription factor 1
EGF	Epidermal Growth Factor
EGR1	Early growth response protein 1
eIF2α	eukaryotic translation initiation factor 2α
eIF2αK2	eukaryotic translation initiation factor 2α kinase 2
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
ErbB2	erythroblastic leukemia viral oncogene homolog 2
ES	Embryonic Stem
FAK	Focal Adhesion Kinase
FOXO	Forkhead box O
GAB1/2	GRB2-associated binding protein 1 and 2
GSK3β	Glycogen synthase kinase *3*
GST	Glutathione S-transferase

Acronym	Definition
HEK293	human embryonic kidney 293
HER2	Human Epidermal Growth Factor Receptor 2
HERC2	HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 2
HES1	Hairy and Enhancer of Split 1
HR	Homologous recombination
HSC	Hematopoietic Stem Cells
IC50	half maximal inhibitory concentration
IP3Rs	inositol 1,4,5-trisphosphate receptors
IR	Ionizing radiation
IRS-1/2	Insulin receptor substrate 1and 2
KAT2B	K(Lysine) Acetyltransferase 2B
KDa	Kilo Dalton
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LOH	Loss Of Heterozygosity
MAGI	Membrane-Associated Guanylate Kinase Inverted
MAMs	mitochondria-associated membranes
Man2C1	Mannosidase, Alpha, Class 2C, Member 1
MAP	Mitogen Activated Protein
MCRS1	Microspherule Protein 1
MDM2	Mouse Double Minute 2
MEC	Mammary epithelial cells
MEFs	Mouse embryonic fibroblast

Acronym	Definition
mES	Mouse embryonic stem (cells)
miRNA	microRNA
MMAC1	Mutated in Multiple Advanced Cancers
MMR	Mismatch Repair
MMTC	Mouse mammary tumor cells
MMTV	Mouse Mammary Tumor Virus
MRN	Mre11, Rad50 and Nbs1 complex
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
mTORC1/2	Mechanistic target of rapamycin 1 and 2
MVP	Major Vault Protein
NAD	Nicotinamide Adenine Dinucleotide
NBs	Nuclear bodies
NEDD4-1	Neural Precursor Cell Expressed, Developmentally Down-Regulated 4
NEM	N-ethylmaleamide
NER	Nucleotide Excision Repair
NHEJ	Nonhomologous End Joining
NLS	Nuclear Localization Signal
PARP	Poly ADP-Ribose Polymerase
PBD	PIP2 binding domain
PCAF	p300/CBP associated factor
PDGF	Platelet-Derived Growth Factor

Acronym	Definition
PDGFR	Platelet-Derived Growth Factor Receptor
PDK1	3-Phosphoinositide Dependent Protein Kinase 1
PDX	Patient derived xenograft
PEST	proline (P), glutamic acid (E), serine (S), and threonine (T)
PH	Pleckstrin homology
PHLPP1	PH Domain And Leucine Rich Repeat Protein Phosphatase 1
PI3K	Phosphatidylinositol 3-Kinase
PIAS	Protein Inhibitor Of Activated STAT
PIP2	phosphatidylinositol 4,5-biphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
pIpC	polyinosine–polycytidine
PKB	Protein Kinase B
PLA	Proximity ligation assay
PML	Promyelocytic Leukemia
PRAS40	Proline-Rich AKT1 Substrate
PRDX1	Peroxiredoxin 1
PREX2a	Phosphatidylinositol-3,4,5-Trisphosphate-Dependent Rac Exchange Factor 2 alpha
PSD95	post synaptic density protein
PtdIns	Phosphatidilinositol
PTEN	Phosphatase And Tensin Homolog
PTENP1	Phosphatase And Tensin Homolog Pseudogene 1

Acronym	Definition
PTP1B	Protein tyrosine phosphatase, non receptor type 1
RanBP2	RAN Binding Protein 2
RanGAP1	Ran GTPase Activating Protein 1
RBPJ	recombining binding protein suppressor of hairless
RNA	Ribonucleic Acid
RNF168	Ring Finger Protein 168
RNF8	Ring Finger Protein 8
ROCK	Rho-Associated, Coiled-Coil Containing Protein Kinase 1
ROS	Reactive Oxygen Species
RPA70	Replication Protein A1
RTKs	Receptor tyrosine kinase
RTqPCR	Real time quantitative polymerase chain reaction
S6/RPS6	Ribosomal Protein S6
S6K1	Ribosomal Protein S6 Kinase, 70kDa, Polypeptide 1
SAE1/2	SUMO1 Activating Enzyme Subunit 1 and 2
SENP1/2	SUMO1/Sentrin Specific Peptidase 1
shRNA	Short hairpin RNA
SIM	SUMO interacting motif
siRNA	Short interfering RNA
ssDNA	Single strand DNA
STAT	Signal Transducer And Activator Of Transcription
SUMO1/2/3	Small Ubiquitin-Related Modifier 1, 2 and 3

Acronym	Definition
TEP1	Tensin like phosphatase 1
TGI	Tumor inhibition factor
TSC2	Tuberous Sclerosis 2
Ubc9	Ubiquitin-Conjugating Enzyme E2I
UV	Ultra violet
WAP	Whey Acidic Protein
WT	Wild Type

List of Publications

Estrogen controls the survival of BRCA1-deficient cells via a PI3K-NRF2-regulated pathway.
Gorrini C, Gang BP, **Bassi C**, Wakeham A, Baniasadi SP, Hao Z, Li WY, Cescon DW, Li YT, Molyneux S, Penrod N, Lupien M, Schmidt EE, Stambolic V, Gauthier ML, Mak TW.
Proc Natl Acad Sci U S A. 2014 Mar 25;111(12):4472-7.

PTEN, here, there, everywhere.
Bassi C, Stambolic V.
Cell Death Differ. 2013 Dec;20(12):1595-6.

Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress.
Bassi C, Ho J, Srikumar T, Dowling RJ, Gorrini C, Miller SJ, Mak TW, Neel BG, Raught B, Stambolic V.
Science. 2013 Jul 26;341(6144):395-9.

Epigenome microarray platform for proteome-wide dissection of chromatin-signaling networks.
Bua DJ, Kuo AJ, Cheung P, Liu CL, Migliori V, Espejo A, Casadio F, **Bassi C**, Amati B, Bedford MT,Guccione E, Gozani O.
PLoS One. 2009 Aug 26;4(8):e6789.

Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive.
Guccione E, **Bassi C**, Casadio F, Martinato F, Cesaroni M, Schuchlautz H, Lüscher B, Amati B.
Nature. 2007 Oct 18;449(7164):933-7.

Chapter 1 Introduction

1.1. Introduction to cancer

Cancer is a disease of pathological hyperplasia in which cells acquire a limitless capacity to divide (*2*). This aberrant, uncontrolled cell division creates masses (in solid tumors) that invade organs and destroy normal tissues. Hippocrates was the first one to use the word cancer, *karkinos* ("crab") in Greek, to describe a tumor, with its clutch of swollen blood vessels around it, reminding him of a crab dug in the sand with its legs spread outwards (*3*). In 400 BC, cientific thought, including the fields of medicine and pathology, was heavily influenced by the theory of fluid mechanics. Cancer was thought of as a humoral disease, an imbalance of the four cardinal fluids (red, black, yellow and white) that composed the body. It wasn't until the 1900s that the more modern definition of cancer emerged. The lack of success of early radical surgeries, led to the realization that tumors can spread from one site to another, causing dissemination of the disease (metastasis) to distant sites, such as the bone, the brain, or the lung.

Early work on the biology of cancer cells suggested that it is a clonally evolving disease that leads to the emergence of cells that uncontrollably divide. The discovery of proto-oncogenes (*4*) suggested that some cancers can be driven genetically, leading to the development of a genetic theory of cancer. Subsequent work led to the realization that cancer is a genetic disease resulting from damaged and mutated genes within a normal cell. DNA mutations occur routinely during a cell's life cycle as a consequence of replication errors, internal and external exposure to damaging agents, as well as high energy radiation. To counter these

threats, sophisticated and partially redundant mechanisms have evolved to detect and repair the DNA mutations. Regardless of how accurate and tightly controlled these processes are, some mutations remain unrepaired. Fortunately, not all mutations have malignant consequences, and cancer arises only when unrepaired mutations occur in specific genes. These key genes are generally grouped into three categories: proto-oncogenes, tumor suppressor and DNA repair genes.

Growth promoting genes (called proto-oncogenes) participate in the control of cell growth, survival and division. Following malignant activation, the protein encoded by the proto-oncogene (which formally becomes an oncogene) acquires growth promoting properties, be it by driving proliferation, apoptosis evasion or escape from senescence. Growth inhibiting genes (called tumor suppressor genes) whose normal function, in antithesis with the proto-oncogenes, is to negatively regulate cell growth, survival and division. Mutations that result in the loss of a tumor suppressor gene function remove their inhibitory influence, altering the complex physiological control of proliferation, and leading to uncontrolled growth. The final group comprises genes whose function is to repair damage to DNA (called DNA repair genes). Their alterations compromise genome stability, leading to increased frequency of mutations in the proto-oncogenes and tumor suppressor genes.

It is important to point out that cancer does not occur from a mutation in a single gene. Instead, the development of cancer involves multiple mutations including mutations in proto-oncogenes, tumor suppressor genes, and DNA repair genes often acting together to cause disease. Accumulating mutations in several

genes are thought to occur over many years, offering an explanation why cancer is more frequently seen in older individuals.

1.2. PTEN discovery

Large deletions involving regions of chromosome 10 occur at high frequency in a variety of advanced cancers (*5*). In the central nervous system, loss of 10q is found in up to 70% of glioblastomas and is associated with the most aggressive grade of astrocytic tumors (*6*). In human prostate cancer, LOH at 10q23 has been associated with cancer progression in 30–60% of cases with increasing frequency correlating with stage and grade of tumors (*7*). In 1997, a defined region of chromosome 10q23 that contained a tumour suppressor gene was identified, and the gene named Mutated in Multiple Advanced Cancers (MMAC1) (*8*). At the same time, a protein encoded by the same region at chromosome 10q23-24 was shown to share sequence homology with the protein tyrosine phosphatase (PTPase) superfamily and tensin, a cytoskeletal protein that links integrins to the actin cytoskeleton at sites of adhesion and named Phosphatase and Tensin homolog deleted on chromosome Ten (PTEN) (*9*). Independently, another group cloned and characterized a novel human protein tyrosine phosphatase mapping to 10q23, corresponding to the same gene, and named it Tensin-like Phosphatase (TEP1) (*10*). While all three names can be found in the literature, by far the most commonly used name is PTEN, and used herein.

1.3. PTEN genetic aberrations

1.3.1. Germline mutations

Germline mutation of one allele of *PTEN* results in pathologies collectively known as *PTEN* hamartoma tumor syndromes, characterized by the development of benign tumors (hamartomas) throughout the body (*11, 12*). These include Cowden syndrome (CS), Bannayan–Riley–Ruvalcaba syndrome (BRRS, which include three conditions formerly recognized as separate disorders, Bannayan-Zonana syndrome, Riley-Smith syndrome, and Ruvalcaba-Myhre-Smith syndrome), *Proteus* syndrome, and *Proteus*-like syndrome. The substantial overlap of PTEN mutations in CS and BRRS suggests that they are not two distinct diseases, but rather a complex group of clinical symptoms resulting from variable penetrance.

Cowden syndrome is an autosomal-dominant disease usually diagnosed by macrocephaly, multiple hamartomas and unusual skin and facial defects, accompanied by predisposition for developing carcinomas of the breast, the thyroid and the endometrium. *PTEN* germline mutations have been detected in up to 90% of CS patients and LOH is frequently found in their tumors and hamartomas (*13*). Dysplastic cerebellar gangliocytoma, also known as Lhermitte–Duclos disease (LDD), is believed to be a hamartomatous growth within the granule cell layer of the cerebellum, which occurs in a subset of CS patients and is the distinctive criteria for its diagnosis. Macrocephaly and multiple hamartomas also define the Bannayan–Zonana syndrome, which, unlike CS, does not confer increased cancer susceptibility (*14*). The mutation spectrum of CS and BZS reveals various loss of

function mutations, with point mutations, insertions and/or deletions and splice site mutations focused around exon 5 of the *PTEN* gene, encoding its phosphatase domain.

Proteus syndrome is a highly variable, rare and complex hamartomatous disorder. To date, only about 120 cases of PS have been documented characterized by rapidly progressing overgrowth of many different tissues, often in a mosaic pattern (*15*). The phenotypic features are frequently congenital and persist or progress throughout postnatal life. They include malformations and hamartomatous overgrowths of multiple tissues, connective tissue nevi, epidermal nevi, and hyperostosis. While tumors and malignancies are not commonly associated with PS, a few rare tumor types (cystadenoma of the ovary, subset of testicular tumors, central nervous system tumors, as well as parotid monomorphic adenomas) have been observed in a subset of patients (*15*).

1.3.2. Somatic mutations

Somatic loss of function mutations of *PTEN* are found in a variety of human cancers including breast, endometrial carcinoma, glioblastoma multiforme, skin and prostate cancers (*16*). Somatic *PTEN* mutations arise as a result of large genomic rearrangements, as well as predominantly monoallelic point mutations scattered along the entire gene (*17*). The *PTEN* region encoding the catalytic domain represents a mutatonal hot spot, while other mutations mostly lead to premature termination of transcription or translation.

1.4. Transcriptional regulation of PTEN

1.4.1. Transcription factors that regulate PTEN

A number of transcription factors have been proposed to regulate PTEN expression, basal and inducible, while others have been shown to operate only in specific cell types. Basal *PTEN* transcription is regulated by the early growth response protein (EGR1) and peroxisome proliferator-activated receptor gamma (PPARγ) (*18*). Investigation of the human genomic *PTEN* locus revealed a p53 binding element directly upstream of the *PTEN* gene. Deletion and mutation analyses showed that this element is necessary for inducible transactivation of *PTEN* by p53, whereas a p53-independent element controlling constitutive expression of PTEN was also identified (*19*). *PTEN* transcription can also be repressed by the polycomb complex protein BMI1 during epithelial–mesenchymal transition (EMT) of nasopharyngeal epithelial cells, or through β-catenin/transcription factor (TCF)-mediated downregulation of EGR1 in ovarian cancer cells (*20, 21*). Regulation of PTEN expression by Notch signaling appears to be tissue-specific and works indirectly by either inhibiting one of the PTEN downregulators, the recombining binding protein suppressor of hairless (RBPJ, commonly known as CBF-1) (*22, 23*) or by activating a PTEN downregulator, the transcription factor hairy and enhancer of split 1 (HES1) (*24*).

1.4.2. Epigenetic regulation of PTEN expression

Another way of regulating *PTEN* gene expression is through epigenetic silencing, a mechanism that is particularly relevant in tumors such as melanoma (*25*), anaplastic thyroid cancer (*26*) and gastric carcinoma (*27*). Hypermethylation of the *PTEN* promoter was first shown in a subset of prostate cancer lines (*28*). Subsequently, epigenetic *PTEN* silencing was shown to be a major mechanism in hematological malignancy cell lines, where approximately 30 to 40% of these lines had a genomic alteration (deletion or mutation), 50% had no transcript, and up to 70% had no PTEN protein (*29*). Epigenetic silencing by methylation in *PTEN* promoter CpG cluster is also thought to be responsible for the loss of PTEN in up to 20% of breast cancers (*30*).

PTEN expression is also influenced by other post-transcriptional mechanisms. At least three microRNAs (miRNAs), miRNA-21, miRNA-22 and miRNA-26a, have been reported to target *PTEN* mRNA (*31-33*). Though this mechanism looks to be part of the normal biological regulation of PTEN, often these miRNAs are found overexpressed in a variety of malignant conditions.

Recently, another layer of sophistication in the regulation of PTEN levels by miRNAs has been unveiled (*34*). Transcripts produced from the PTEN pseudogene *PTENP1* (called competitive endogenous RNAs or ceRNAs) apparently can regulate the effects of miRNAs by competing with the *PTEN* gene for miRNA binding.

1.5. PTEN and the PI3K pathway

1.5.1. PTEN function

PTEN contains an N-terminal phosphatase domain that can dephosphorylates Phospho-Tyrosine, Phospho-Serine and Phospho-Threonine within highly acidic substrates, although with weak catalytic activity. The real breakthrough in the study of PTEN function came from the realization that PTEN's main substrate is an acidic nonproteinaceous molecule, a component of the lipid cellular membrane, phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$ or PIP3) (*1*). By dephosphorylating the D3 position of PIP3, PTEN antagonizes the Phosphatidylinositide 3-kinase (PI3K) pathway (*1, 35*). In cells, PIP3 acts as a second messenger, whose levels in the plasma membrane are elevated following cellular stimulation with growth factors, leading to the activation of the PI3K-PKB/Akt pathway, a highly conserved signaling cascade (Figure 1.1).

1.5.2. PI3K pathway

The PI3K pathway regulates diverse cellular processes, including cell metabolism, survival, proliferation, apoptosis, growth, and migration. These fundamental cellular processes, when deregulated, can contribute or drive a malignant phenotype. Indeed, somatic mutations in cancers target the PI3K pathway at different levels, resulting in increased activity of the pathway itself and the associated phenotypes.

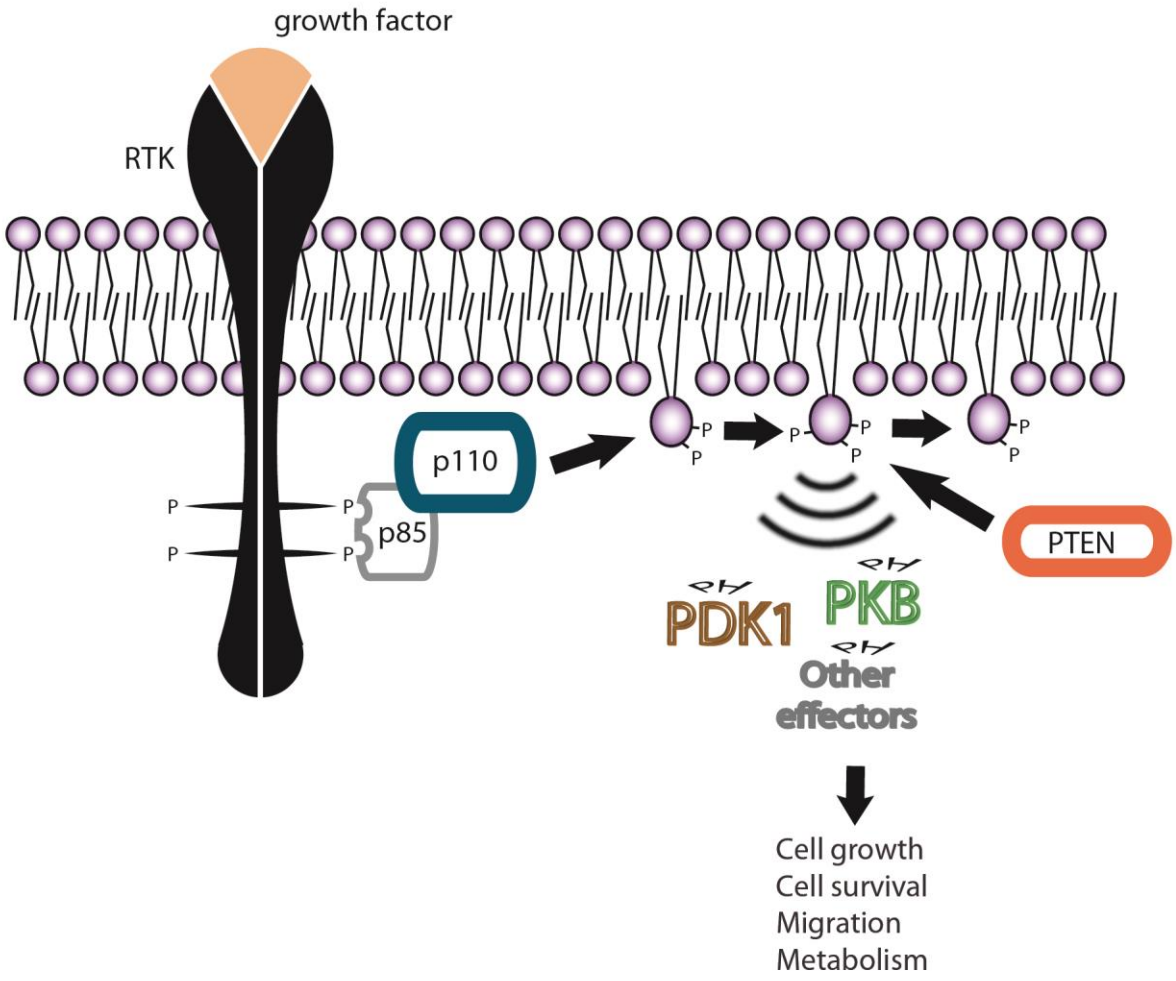

Figure 1.1 Overview of the PI3K pathway

The PI3K pathway is activated at the membrane, by a variety of signals, including receptor tyrosine kinases, integrins, B and T cell receptors, cytokine receptors and G-protein-coupled receptors. Stimulation induces the production of PIP3 by class 1A PI3Ks, heterodimers comprised of a p85 regulatory subunit and a p110 catalytic subunit, in a process that usually involves other regulatory subunits or adapter molecules such as c-Cbl, GAB1, GAB2, IRS-1 and IRS-2 (*36*). Proteins containing pleckstrin homology (PH) domains physically interact with PIP3, bringing them into close proximity with other PH domain-containing proteins and facilitating further functional interactions that serve to propagate the membranous signal. A protein motif of approximately 100 amino acid residues, initially recognized in *p*latelet and *l*eukocyte *C k*inase sub*str*ate prote*in* (pleckstrin) (*37*), PH domains have been identified in a number of signal-transducing proteins, such as β-spectrin (*38*), dynamin (*39*), *p*hospho*l*ipase *C*-δ_1 (PLC-δ_1) (*40*), *S*on *o*f *s*evenless (Sos) (*41*), and *B*ruton's *t*yrosine *k*inase (Btk) (*42*). The most prominent example is PKB/Akt, which, by virtue of its PH domain, binds to PIP3 at the plasma membrane, allowing another PH domain-containing protein, PDK1, to access it and phosphorylate PKB/Akt on T308 within its “activation loop”, leading to partial PKB/Akt activation (*43*). Phosphorylation of Akt at Ser473 within the carboxy-terminal hydrophobic motif, either by mTORC2 (*44*) or by DNA-PK (*45*), stimulates full Akt activity. PKB/Akt, in turn, activates the mammalian target of rapamycin complex 1 or mechanistic target of rapamycin complex 1 (mTORC1), a protein complex that functions as a nutrient/energy/redox sensor and controls protein synthesis (*46, 47*). mTORC1 substrates include the eukaryotic translation initiation factor 4E binding

protein 1 (4EBP1), and ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (S6K1), which, in turn, phosphorylates the ribosomal protein S6 (S6/RPS6), promoting protein synthesis and cellular proliferation (*46*). In addition of being one of the main regulators of protein translation and ribosome biogenesis, AKT phosphorylates as many as 100 substrates thereby modulating a variety of cellular functions (*48*).

Full activation of Akt exerts a strong antiapoptotic effect through the phosphorylation and inhibition of key pro-apoptotic proteins, such as BAD, MDM2 and FOXO proteins. AKT activates cell proliferation by inactivating p27 (*49*) and suppressing glycogen synthase kinase 3 (GSK3)-mediated Myc and cyclin D1 inhibition (*50*).

1.6. Other PTEN functions

Although the canonical function of PTEN is associated with regulation of 3' phosphorylated phosphatidylinositols, other, lipid phosphatase activity-independent roles, have been proposed. PTEN can dephosphorylate protein substrates such as the focal adhesion kinase (FAK) (*51*).

Further, the eukaryotic translation initiation factor 2α kinase 2 (eIF2αK2; also known as PKR)–eIF2α phosphorylation cascade or the microspherule protein 1 (MSP58; also known as MCRS1)-mediated cellular transformation are regulated by PTEN independently of its phosphatase activity (*52, 53*). Recent studies demonstrate that nuclear PTEN interacts with Anaphase-Promoting Complex, also called cyclosome APC/C, promotes its association with CDH1, and enhances the tumor-suppressive activity of the APC-CDH1 complex. Importantly, because this is

impaired by nuclear exclusion but not phosphatase inactivation, PTEN activates APC-CDH1 in a phosphatase-independent manner (*54, 55*).

1.7. PTEN structure

The PTEN crystal structure revealed that the protein is organized in two main parts: an N-terminal half that includes the phosphatase domain and a C-terminal half that is largely composed of a C2 domain (Figure 1.3) (*56*). Three other PTEN functional domains have been identified (Figure 1.2). An amino terminal PIP2-binding (PBD) domain precedes the phosphatase domain whereas the C2 domain is followed by a carboxy-C-terminal tail that includes two PEST repeats and a C-terminal PDZ binding domain. PDZ is an acronym combining the first letters of three proteins — post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1) — which were first discovered to share the domain architecture and bind C-terminal sequences in various proteins (*57*). The PBD module, with its ability to bind PIP2, its thought to contribute to PTEN's recruitment to the plasma membrane (*58*).

The phosphatase domain contains the $C(X)_5R$ motif characteristic of the protein tyrosine phosphatase (PTPase) superfamily (*59*). Within this catalytic signature, the Cysteine (C) residue is essential for catalysis whereas the Arginine (R) binds the phosphoryl group of the substrate positioning it for the attack by the cysteine thioanion. PTEN's catalytic pocket is comparatively larger than that found in other Tyrosine specific PTPs and has a basically charged active site, consistent with its ability to dephosphorylate acidic substrates and in particular PIP3, which is

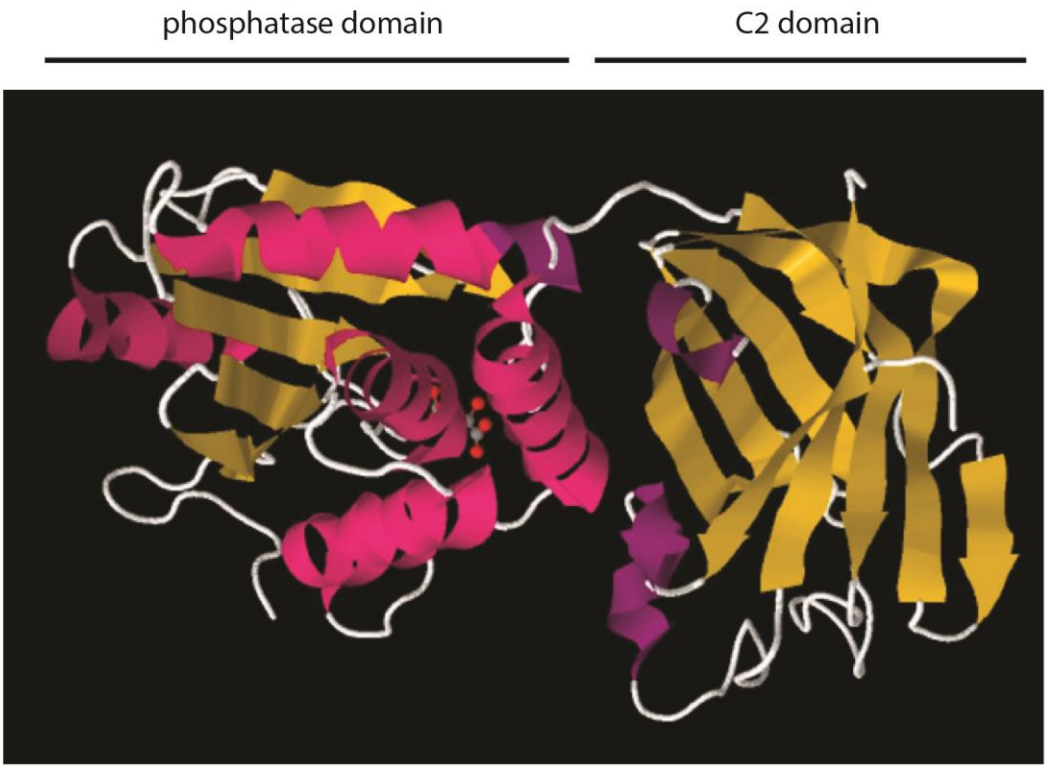

Figure 1.2 PTEN crystal structure

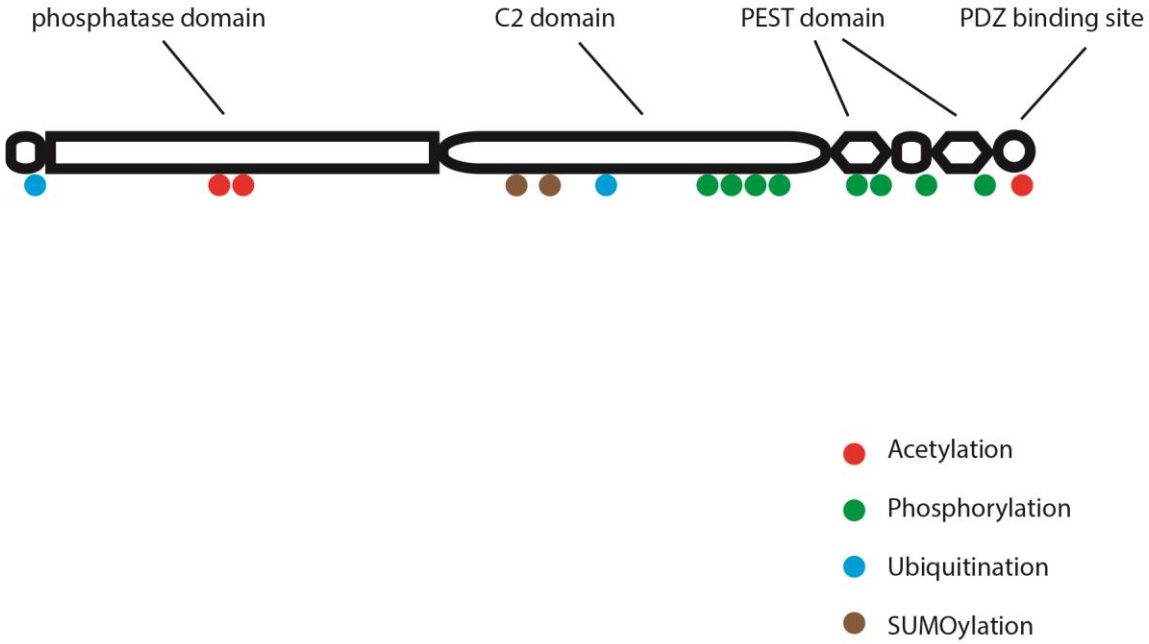

Figure 1.3 PTEN protein structure and major post-translational modifications

bulkier than phospho-tyrosines (*56*). PTEN phosphatase activity has also been tested on other D3-phosphorylated phosphoinositides. PTEN catalytic acitivity is 200-fold greater towards PIP3 than that for PtdIns(3,4) P_2 and PtdIns(3,5)P_2, and is 1000-fold greater than that for PtdIns3*P* (*60*). Structural analysis of PTEN (*56*) suggested that Histidine 93 and Lysine 128 make contact with the D-5 phosphate group explaining the 75% reduction in phosphatase activity of a PTEN mutant (Histidine 129 to Alanine, H83A) frequently observed in tumors (*60*). Another mutation, Glycine 129 to Aspartic acid (G129E, found in Cowden Syndrome patients and sporadic tumors), restricts the width of the PTEN active site, effectively abolishing its phosphatase activity toward lipids while maintaining its phospho-Tyrosine phosphatase activity (*61*). Conversely, PTEN Y138L (mutation of Tyrosine position 138 into Leucine) is thought to preserve activity against lipids, but not proteins (*62*). The specific conformation of its catalytic pocket, directs PTEN towards highly acidic proteinacious Tyr-, Ser- and Thr-phosphorylated substrates *in vitro* although with a significantly lower activity compared to another tyrosine phosphatase, PTP1B (*63, 64*).

The C-terminal C2 domain of PTEN is thought to mediate its interactions with the plasma membrane (*56*). Even though it is Ca^{2+}-independent, PTEN C2 domain structure resembles the Ca^{2+}-dependent C2 domains of other lipid-metabolizing enzymes, such as phospholipase A_2, phospholipase C_{d1} and the conventional protein kinase Cs (*65, 66*). A cluster of cationic residues of the β-sandwich, composed of 8 β-strands, on the membrane binding face of PTEN appear to mediate membrane anchoring (*56*). A C2 domain PTEN mutant (M-CBR3) that has

a decreased ability to bind phospholipids shows diminished ability to suppress cell growth compared with wild-type PTEN (*56*). Recent evidence reveals further complexity of these interactions, suggesting that PTEN SUMOylation at Lysine 266 located within the CBR3 loop may affect PTEN membrane association, facilitating the binding of PTEN to the plasma membrane via electrostatic interactions (*67*). Nevertheless, PTEN structural analysis using neutron reflectometry (NR) demonstrates that the CBR3 loop of PTEN's C2 domain, as well as further electrostatic interactions of the phosphatase domain are sufficient for membrane association, independent of SUMOylation (*58*).

PTEN unstructured C terminal, 50 amino acids, represents a hub of regulatory modifications and interactions. Several phosphorylated residues within the carboxyl-terminus play a prominent role in regulation of protein stability (*68*). The PDZ binding site, encompassing the C-terminal three amino acids, has been shown to mediate multiple protein-protein interactions (*69*). Also in this short stretch, another post-translational modification, acetylation, can interfere with the aforementioned PDZ binding domain function (*70*). The following section examines PTEN post-translational modifications and their role in PTEN biology in greater detail.

1.8. Postranslational modifications of PTEN

PTEN is target of various post-translational modifications by small functional groups such as acetate and phosphate and polypeptide such as Ubiquitin and SUMO. These covalently linked modifications govern PTEN regulation by directly

affecting its enzymatic activity, its interaction with other proteins or its subcellular localization.

1.8.1. Phosphorylation

PTEN is phosphorylated on multiple residues within its C2 and C-terminal domains (fig 2). The best studied cluster of PTEN phosphorylation sites encompasses residues 366-385 (*71-73*). GSK3β was found to phosphorylate Threonine 366 and Serine 362 while CK2α targets Serine 370 and 380, Threonine 382 and 383 and Serine 385 (*71-73*). Phosphorylation of S370 by CK2 serves as priming for T366 and S362 phosphorylation by GSK3. According to this model, p-T366 was shown to be important for PTEN's interaction with the oncogenic protein MSP58 (*52*).

Interestingly, the region targeted by these two kinases that are normally active in proliferating cells, coincides with the PTEN region implicated in regulation of its stability. Namely, removal of C-terminal 50 amino acids reduces PTEN stability while increasing its catalytic activity (*68*). The equivalent was achieved by mutating three phospho-sites (S380, T382, and T383) within PTEN C-terminus. Remarkably, PTEN phosphorylation on S380, T382, T383 and S385 results in a conformational change, from open to closed (*74*). It is thought that while the closed conformation stabilizes the protein, it also leads to dissociation of the C2 domain from the lipid bilayer, an interaction considered necessary for the proper alignment of its catalytic domain with the membrane localized PIP3. Moreover, the phosphorylation-dependent conformational change appears to mask the PDZ binding site and prevent PTEN interaction with PDZ domain-containing proteins (see section 1.7).

The existence of PTEN in two different conformational states also raises a possibility that PTEN may auto-dephosphorylate. The closed-sandwich structure predicts the positioning of the phosphatase domain in close proximity with the phosphorylated patch, suggesting of autodephosphorylation of these residues. Based on the affinity of PTEN for very acidic substrates, it has been hypothesized that PTEN dephosphorylation of threonine 383 is mediated by an intramolecular autocatalytic activity (*64*). Moreover, PTEN mutants that lack lipid phosphatase activity but retain protein phosphatase activity exhibit decreased phosphorylation of S380, T382, T383 and S385 *in vivo* (*75*).

In leukocytes stimulated by chemoattractants, the C2 domain of PTEN is phosphorylated at Ser229 and Thr321 by RHOA-associated protein kinase (ROCK), which activates PTEN and targets it to the membrane (*76*). Finally, it was demonstrated that the Rak tyrosine kinase physically interacts with PTEN in MCF7 cells and phosphorylates PTEN on Tyr336. Knockdown of Rak enhanced the binding of PTEN to its E3 ligase NEDD4-1 and promoted PTEN polyubiquitination, presumably leading to PTEN protein degradation (*77, 78*).

1.8.2. Acetylation

PTEN is acetylated in two different regions. K acetyltransferase 2B (KAT2B, also known as PCAF) acetylates PTEN on Lys125 and Lys128 in a growth factor stimulation-dependent manner (*79*). The close proximity of these residues to the catalytic site implies that acetylation may affect PTEN enzymatic activity. PTEN is also acetylated by CBP within its PDZ binding site (K402), which affects the

interaction between PTEN and its PDZ domain-containing partners (*70*). PTEN acetylation is upturned by the activity of deacetylase sirtuin 1 and PTEN is hyperacetylated and excluded from the nucleus in *Sirt1*-deficient mouse embryonic stem (mES) cells (*80*).

1.8.3. Ubiquitination

PTEN protein expression is lost or severely diminished in many cancers not accompanied by a mutation of the *PTEN* gene or hyper-methylation of its promoter, raising an interest in deregulation of ubiquitin-mediated proteasome degradation as potential means of PTEN loss of function. NEDD4-1, an E3 ubiquitin ligase has been suggested as the regulator of PTEN ubiquitination (*81*). In a complex relationship, PTEN polyubiquitylation on K13 and K289 leads to proteasome-mediated degradation, while its monoubiquitination at K289 facilitates its nuclear import (*81*). While PTEN ubiquitination remains a likely mechanism of PTEN control, the notion that Nedd4-1 is implicated in this is dubious. In mice lacking NEDD4-1, PTEN protein levels remain intact and its ubiquitination is still evident (*82*). Morevoer, nuclear localization of PTEN is not impeded in NEDD4-1 deficient cells (*82*). Thus, other E3 ligases may target PTEN. Indeed, WWP2, a NEDD4-like E3 ubiquitin ligase, and X-linked inhibitor of apoptosis protein (XIAP), a member of the inhibitor of apoptosis family of proteins, have been shown to promote PTEN ubiquitination and regulate its protein levels. Both WWP2 and XIAP have been shown to polyubiquitinate PTEN, targeting it for degradation (*81, 83*).

1.8.4. Oxidation

Like for the other PTPs, the PTEN dephosphorylation reaction relies on a catalytic Cysteine nucleophile that can be susceptible to oxidation (*1*). Reactive oxygen species (ROS) in the form of H_2O_2 are endogenously generated in cells exposed to peptide growth factors such as insulin, EGF, or PDGF (*84*). The catalytic activity of PTEN is modulated by ROS through oxidative stress-induced formation of a disulphide bond between Cysteine 71 and 124 (*85*). Further regulation of PTEN oxidation and inactivation seems to be achieved through physical interaction of peroxiredoxin I (PRDX1) predicted to inhibit disulphide bond formation (*86*). Finally, low concentrations of NO results in *S*-nitrosylation of PTEN Cys-83 leading to inhibition of its enzymatic activity (*87, 88*).

1.9. PTEN subcellular localization

PTEN localization to the plasma membrane is a key event involved in countering the PI3K pathway. Multiple PTEN domains are implicated in plasma membrane localization. A basic patch at the N-terminus of PTEN facilitates its binding to membranes by interacting with PIP2 (*89*), whereas the C2 domain promotes membrane recruitment which favors a productive orientation of the phosphatase domain at the membrane surface (*90*). Additional C-terminal interactions with PDZ domain-containing proteins may also contribute to membrane association (*91*).

Post-translational modifications also impact PTEN's plasma membrane localization. PTEN phosphorylation by CK2 on a cluster of C-terminal sites,

including Ser370 and 380, Thr383 and Ser385 causes a conformational change that results in the masking of the PDZ domain-binding site, thereby suppressing PTEN plasma membrane recruitment (*92*). Finally, SUMOylation of PTEN at Lys 266 located within the CBR3 loop may further promote binding of PTEN to the plasma membrane via electrostatic interactions (*67*).

PTEN is also readily found in the nuclei of many cultured cells and tissues, including normal breast epithelium (*93*), proliferating endometrium (*94*), normal pancreatic islet cells (*93*), vascular smooth muscle cells (*95*), follicular thyroid cells (*96*), squamous cell carcinoma (*97*) and primary cutaneous melanoma (*98*). While nuclear phosphatidylinositols have been reported, they are part of distinct, partially detergent-resistant proteolipid complexes that are not dynamically regulated and not likely PTEN substrates (*99*). Various molecular mechanisms responsible for PTEN nuclear localization have been proposed.

PTEN lacks a typical nuclear localization sequence (NLS), but a putative nuclear localization signals within PTEN thought to mediate its interaction with the major vault protein (MVP) have been identified (*100, 101*). Moreover, N-terminal sequences responsible for Ran-mediated nuclear transport (*102*) and a potential PI3K signaling-sensitive, cell cycle-regulated PTEN nuclear export mechanism have been proposed (*103*). PTEN may also have a cytoplasm-retention/nuclear export sequence within its N-terminus (*104*). Interestingly, mutations within this region result in constitutive nuclear localization, precluding PTEN growth-suppressive function at the plasma membrane (*104*).

The possibility that PTEN may have phosphatase activity-independent, nuclear PTEN tumor suppressive roles arose from the identification of familial Cowden syndrome-associated PTEN mutations at a highly conserved lysine (Lys289 in human PTEN) that inhibits its nuclear localization, but not phosphatase activity (*81, 90*). In the nucleus, PTEN was found to participate in maintaining genomic integrity, acting via at least two different mechanisms. Nuclear PTEN associated with the centromeric binding protein CENP-C, which is critical for kinetochore assembly and the Metaphase to Anaphase transition (*105*). Further, acting as a co-factor for the transcription factor E2F1, nuclear PTEN appeared to regulate the expression of Rad51, a key component of the DNA repair machinery (*105*).

A combined proteomic and cell biology approach led to the discovery that PTEN can be both mono- and poly-ubiquitinated at Lys289, as well as at Lys13, and that PTEN ubiquitination status leads to distinctive fates (Figure 1B) (*77, 81*). Based on the proposed model, mono-ubiquitinated PTEN localizes to the nucleus where it is protected from proteosome-mediated degradation, whereas polyubiquitinated PTEN remains in the cytoplasm where it is rapidly degraded (*81*). While this simplistic model is likely not consistent with all physiological scenarios (*82, 106*), it raises an intriguing possibility that ubiquitin or ubiquitin-like posttranslational modifications of PTEN govern its subcellular localization and tumor suppressor function.

Recently, PTEN has also been found in the endoplasmatic reticulum (ER) and mitochondria-associated membranes (MAMs) (*107*). Here, PTEN appears to

regulate the Ca^{2+} release from the ER in a protein phosphatase-dependent manner that counters the PKB/Akt-mediated reduction of Ca^{2+} release via the inositol 1,4,5-trisphosphate receptors (IP3Rs), with which PTEN direcly interacts (*107*). Reduction in the kinetics of Ca^{2+} release from the ER upon PTEN loss may contribute to reduced sensitivity to apoptosis (*107*). Finally, studying a PTEN protein initiated from an alternate translation start site, a secreted PTEN polypeptide has also been discovered (*108*). This 70 KDa, fully phosphatase competent protein named PTEN-long, appears capable of entering cells and regulating PI3K signaling pathway in them.

1.10. PTEN protein interactions

The PTEN three C-terminal amino acids, which constitute a PDZ domain binding site, represent another potential regulatory moiety. PDZ domains are found in many eukaryotic proteins and mediate protein-protein interactions. More than 10 distinct PDZ domain-containing proteins, including several MAGI proteins, hDLG and Bazooka/Par-3, have been identified as candidate PTEN interactors primarily through yeast two-hybrid and proteomic approaches (*109*).

Through the PDZ binding motif, PTEN interacts directly with the NHERF1 and NHERF2 (Na+/H+ exchanger regulatory factor) homologous adaptor proteins. NHERFs interact directly with the platelet-derived growth factor receptor (PDGFR) and PTEN, forming a ternary complex. Ligand stimulation of PDGFR results in increased signaling through PI3K, where NHERF-dependent proteins recruitment of PTEN restricts the activation of the pathway (*110*). Remarkably, NHERF1 also

interacts with the PH domain Leucine-rich repeat of protein phosphatase 1 (PHLPP1) and, acting as a scaffold, modulates AKT in a PTEN-independent manner (*111*).

The PDZ binding site of PTEN is also involved in its recruitment to adherens junctions through binding to MAGI-2, an interaction that is thought to promote PTEN activity (*112*). MAGI are adherens junction scaffold proteins that play critical roles in signal transduction by optimizing the activity and stability of their associated proteins. PTEN interaction with MAGI-2 is enhanced by its phosphorylation on Thr382/Thr383 and results in PTEN stabilization (*112*), raising the possibility that PTEN's inhibitory effect on PI3K signaling may cooperate with adherens junction signaling pathways to cause cessation of cell growth (*113*).

In Schwann cells, discs-large (DLG), a protein with multiple PDZ domains, interacts with the PTEN's PDZ-binding motif, leading its stabilization and reduced PKB/AKT activity. First identified in a yeast two-hybrid screen as a binding partner for PTEN (*92*), it was later demonstrated that mammalian disks large homolog 1 (Dlg1) interacts with PTEN to inhibit axonal stimulation of myelination (*114*).

Through interaction with myosinV, a cargo protein that transports other proteins along the actin filaments into the cell periphery, PTEN is part of a cellular signaling integration that amplifies PI3K pathway function. According to this model, myosinV coordinates transport of PTEN to the membrane to downregulate PI3K signaling. This interaction is dependent on GSK3 phosphorylation of PTEN providing a positive feedback mechanism that also involves CK2 (see section 1.8.1) (*115-118*). MyosinV-mediated transport of PTEN controls PI3K signaling and

neuronal soma size and disruption of this mechanism leads to the development of neurons with increased cell size, reminiscent of brain phenotypes caused by PTEN loss (*117*).

PI3K (type I) consists of a p110 catalytic subunit and a p85 regulatory subunit (*119*). Recent studies found that p85 binds and stimulates PTEN, providing a novel, antagonistic way for p85 to regulate both positively and negatively the PI3K pathway (*120*). Other proteins have been shown to interact with PTEN and negatively regulate its tumor suppressor activity by a wide variety of mechanisms. Phosphatidylinositol-3,4,5-trisphosphate RAC Exchanger 2a (P-REX2a) interacts with PTEN and inhibits its lipid phosphatase activity. P-REX2a interaction with PTEN results in an increase in PIP3 levels, subsequent AKT activation and increased phosphorylation of downstream effectors (*121*).

Man2C1 and Sharpin bind directly to PTEN and inhibit its lipid phosphatase activity (*122, 123*). DJ-1 is an oncogene whose expression is associated with increased levels of activated PKB and poor clinical outcome in various cancer types. DJ1 binds directly to PTEN under oxidized conditions resulting in decreased PTEN activity (*124*).

1.11. Mouse models of PTEN loss

Tumor suppressor gene knockouts in mice can provide great insight into their physiological function. Homozygous *PTEN* inactivation ($PTEN^{-/-}$) is embryonically lethal (*125*) while mice bearing a single allele inactivation of *PTEN* ($PTEN^{+/-}$)

develop tumors which include lymphomas, breast and endometrial hyperplasia and cancers as well as malignancies of the prostate and adrenal glands, partially recapitulating the spectrum of tumors found in Cowden desease (*126*). The high penetrance and early onset of these tumors represents an impediment for the study of PTEN loss in other organs, which prompted the generation of conditional *PTEN* deletion murine models.

1.11.1. PTEN knockout mice

Different strategies were used to target the genomic region that encodes the phosphatase domain of PTEN by either deleting exons 4, 5 and 6 (*127*), 3, 4 and 5 (*125*) or 5 alone (*128*). Suzuki et al. were able to characterize the embryonic lethality of the *PTEN* $^{-/-}$ embryos. At E8.5, *PTEN*$^{-/-}$ mutants embryos are relatively smaller than their wild-type littermates, and show defective patterning of the cephalic and caudal regions. The embryonic lethality, which occurs by E9.5, has been linked to an imbalance between growth and patterning which results in defective development.

The phenotype of the heterozygous mice generated by all three strategies extensively overlaps with Cowden disease, even if the genetic background of PTEN$^{+/-}$ mice is a strong determinant of susceptibility to specific tumour types (*129*). *PTEN*$^{+/-}$ animals develop a broad range of tumors, including mammary, thyroid, endometrial and prostate cancers, as well as T-cell lymphomas (*125*). These tumors show elevated phosphorylation of protein kinase B (PKB, also known as Akt kinase) linking the malignant phenotype to hyperactivated PI3K pathway, as

consequence of *PTEN* haploinsufficiency (*125*) or *PTEN* LOH at the *PTEN* locus (*130*).

The most aggressive phenotype observed in *PTEN*$^{+/-}$ mice is lymphoid hyperplasia that eventually progresses to T-cell lymphoma (*125, 128*). Enlarged lymph nodes in the neck and axilla are detectable as early as at 20 weeks of age, caused by accumulation of B and T cells, macrophages and fibroblasts (*128*). Further examination of these lesions shows that B cells and macrophages of *PTEN*$^{+/-}$ mice have reduced levels of apoptosis compared with wild-type cells (*125*).

Recapitulating the increased risk of developing breast cancer of patients with Cowden disease, 61% of *PTEN*$^{+/-}$ female mice develop mammary tumors by 30–49 weeks of age (*35*), mostly adenocarcinomas or small fibroadenomas. This is not associated with the commonly reported metastases to the lymph nodes and the lung, which are observed in only 6% of the female *PTEN*$^{+/-}$ mice (*35*). In the breast, loss of a single *PTEN* allele has also been shown to cooperatively accelerate tumorigenesis by reducing tumor latency in a mammary tumor mouse model driven by the overexpression of the Wnt oncogene under the control of the mouse mammary tumor virus promoter (MMTV-Wnt) (*131*).

Female *PTEN*$^{+/-}$ mice also develop multifocal endometrial hyperplasia, a lesion that is thought to be a precursor lesion to endometrial carcinoma (*128, 130*). In male mice, *PTEN* haploinsufficiency causes changes in the prostate epithelium, including benign epithelial hyperplasia and intraepithelial neoplasia, as well as rare instances of adenocarcinoma (*128, 130*). Interestingly, it appears that a further subtle decrease in PTEN levels is sufficient to induce pathological changes in the

prostate (*132*). Mice that carry one hypomorphic *PTEN* allele (an allele with normal function, but less than normal gene expression, $PTEN^{+/hyp}$) display massive prostate hyperplasia with complete penetrance by the age of 8 weeks (*132*).

1.11.2. Tissue-specific deletion of PTEN

A number of tissue-specific homozygous deletions of *PTEN* have been generated taking advantage of the Cre-mediated conditional gene-targeting approach (*133*). Conditional deletion of the *PTEN* gene in organs such as breast (*134*) and prostate (*135-138*) has almost invariably led to tumorigenesis and malignant phenotypes, replicating the spectrum of disorders observed in the germline, monoallelic deletion of *PTEN* in the mouse, as well as that of human tumors that feature PTEN loss. Morevoer this approach unveiled novel functions of PTEN in normal development and physiology of various organ systems.

Given the high frequency at which *PTEN* mutations occur in human glioblastomas, multiple studies conditionally deleted *PTEN* in different brain cell populations (*139-141*). Crossing of $PTEN^{flox/flox}$ mice with transgenic mice in which Cre recombinase expression is driven by the glial fibrillary acidic protein (Gfap) promoter inactivates *PTEN* in the granule cells of the cerebellum and partially in the granule cells of the dentate gyrus in the hippocampus (*139-141*). These animals have enlarged brain size, when compared with the brains of wild-type littermates, develop seizures and ataxia early in life and die prematurely. Other features of GfapCre/$PTEN^{flox/flox}$ mutants mice include hydrocephaly, dysplasia of the cerebellar cortex and abnormal neuronal soma size, with *PTEN*-deficient neurons being about

twice the size of wild-type neurons (*139*). Importantly, *PTEN* deletion restricted to neuronal cells in the cerebellum and hippocampus resulted in a phenotype that closely resembles human Lhermitte–Duclos disease (*139*). This condition represents a subset of Cowden disease and is characterized by megaloencephaly, mental retardation, autism, seizures and/or hydrocephalus (*142*).

Soma hypertrophy, macroencephaly and premature death also characterize Nse/Cre*PTEN*$^{flox/flox}$ mice, where expression of Cre under the control of the Nse (neuron-specific enolase) promoter, which deletes *PTEN* from all fully differentiated neurons (*143*). Because of the different cells targeted in this case, these changes were observed mainly in the forebrain and hippocampus.

PTEN deletion from neuronal precursors (nestin-driven Cre expression, NesCre) caused increases in brain size throughout embryonic development, resulting in lethality shortly after birth (*140*). The phenotype could be explained by the ability of *PTEN*-deficient neural stem cells to undergo more self-renewing divisions, pointing to a key role for PTEN in controlling neural stem/progenitor cells cell cycle progression.

It has to be remarked that, although removal of *PTEN* from various central nervous system cell subpopulations in the mouse invariably causes hyperproliferation and hypertrophy, it does not ultimately lead to tumor development. This is in apparent contrast with the notion that *PTEN* is frequently found inactivated in malignant human brain tumors (*144*). However, it has to be considered that, in this context, PTEN loss is probably not sufficient to drive tumorigenesis and additional genetic alterations might be required (*145*).

The evidence that *PTEN*$^{+/-}$ mice mainly develop T-cell, but not B-cell, lymphomas was confirmed by studies performed using selective deletion of PTEN (*146*). Lck/Cre*PTEN*$^{flox/-}$ mice, in which *PTEN* is deleted in all T lineage cells, show lymphadenopathy, splenomegaly and an enlarged thymus at an early age. Tumor masses were found in 10 week old mice, which eventually die of malignant T-cell lymphoma (*146*). Autoimmune disease features arise in these animals as a consequence of defective thymic selection, which results in increased levels of autoantibodies and enhanced T-cell proliferation. Conversely, *PTEN* specific deletion in the B-cell lineage does not lead to development of B-cell malignancies (*147*). Analysis of these mice revealed that PTEN does play an important role in B-cell physiology, in particular B-cell maturation and proliferation following antigen stimulation. The lack of tumor development in the mutant mice may be due to the fact that class switch recombination is defective in *PTEN*-deficient B cells due to decreased levels of activation-induced cytidine deaminase, which, on the other hand, appears to be essential for germinal center B-cell lymphomagenesis (*148*).

The effects of *PTEN* deletion in murine hematopoietic stem cells (HSCs) was studied by using transgenic mice expressing Cre under the control of the polyinosine–polycytidine (pIpC) inducible Mx1 promoter (*149, 150*). *PTEN* removal confers HSCs the capacity to escape quiescence, which causes the rapid depletion of the HSC pool. When transplanted into irradiated recipient mice, and therefore depleted of HSCs, *PTEN*-deficient HSCs failed to replenish hematopoietic cells of all lineages (*149*). Myeloproliferative disorders in these mice were detected within 6 weeks following *PTEN* deletion leading to a rapid progression to acute myeloid

leukemia or acute lymphoblastic leukemia. The phenotype could be related to the function of PTEN in the PI3K pathway, as treatment with rapamycin, an mTOR inhibitor, counteracted the development of myeloproliferative disorders and leukemia (*150*).

Specific deletion of *PTEN* in β-cells of the pancreas (Rip/Cre*PTEN*$^{flox/flox}$) caused an increase in islet cell numbers and total islet mass compared to wild type control mice. Increase in β-cell and progenitor cell proliferation, as well as a decrease in apoptosis, conferred by the hyperactivated PI3K pathway, is thought to be at the origin of this phenotype. RipCre*PTEN*$^{flox/flox}$ mice have improved insulin responsiveness and decreased blood glucose levels (*151-155*).

1.12. PTEN and genomic instability

PTEN loss of function is often associated with genetic instability (*156, 157*). Moreover, genetic deletion of *PTEN* in mouse embryonic fibroblasts (MEFs) causes accumulation of unrepaired DNA double-strand breaks (DSBs) (*105*). PTEN loss is thought to contribute to genome integrity via at least two molecular mechanisms (*81, 158*) (see section 1.9). Nevertheless, subsequent work reported that PTEN deficient cells had no defect in Rad51 expression, and that the genomic instability in PTEN deficient cells could not be attributed to lack of PTEN at centromeres, since no interaction was detected between centromeric DNA and PTEN in wild type cells (*159*). Instead, it was proposed that PTEN deficiency alters multiple cell cycle checkpoints possibly leaving less time for DNA damage repair and/or chromosome segregation (*159*).

This inherent genomic instability makes the tumors that feature PTEN loss vulnerable to the action of drugs that magnifies this deficiency. The rationale of this strategy (also referred to as synthetic lethality) implies that DNA damage is selectively increased in tumor compared to normal tissues by the dysfunction of two DDR pathways. As a consequence, high proliferating tumor cells progressively accumulate an amount of DNA damage incompatible with cell survival. This approach is currently being tested in tumors with breast cancer associated gene 1 and 2 (*BRCA1/2)* defects (which causes defects in the homologous recombination pathway, see following section 1.13) with the use of inhibitors of poly (ADP-ribose) polymerase (PARP) enzymes. PARP family of proteins, of which PARP-1 is the most abundantly expressed member, binds to a variety of DNA structures, including single- and double-strand breaks (*160*). Upon binding to damaged DNA, PARP-1 forms homodimers and catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose to form long branches of ADP-ribose polymers on glutamic acid residues of a number of target proteins including histones and PARP-1 (automodification domain) itself (*161*). Poly(ADP-ribosylation) confers negative charge to histones leading to electrostatic repulsion among histones and DNA, a process implicated in chromatin remodeling, DNA repair, and transcriptional regulation (*162*).

The action of PARP inhibitors on a PTEN-null scenario has been tested on glioblastoma, breast and prostate tumour cell lines (*163*) as well as on cells derived from andometrioid endometrial carcinomas (*164*). These studies proved that homozygous *PTEN* loss or mutations sensitize tumour cells to PARP inhibitors. The same effect could be reproduced by modulating PTEN levels in cells expressing

normal levels of the protein (*164*), establishing a direct link between PTEN status and sensitivity to PARPi. Finally, in a case study, PARPi treatment of a patient diagnosed with PTEN-null metastatic endometrioid endometrial adenocarcinoma lead to reduction in the size of the brain metastases and significant clinical benefits (*165*).

These first encouraging results suggest that PARPi could represent a viable therapeutic intervention for PTEN-deficient tumors and underlines the importance of PTEN role in DDR the maintenance of genomic integrity.

1.13. The DNA damage response

Proper genome maintenance through continual cell division cycles is *condicio sine qua non* for all organisms to ensure normal reproduction, development and prevention of diverse diseases including cancer. DNA damage can arise from endogenous processes such as DNA mismatches occasionally introduced during DNA replication, DNA strand breaks caused by abortive topoisomerase I and topoisomerase II activity or from ROS produced from normal metabolic byproducts that can attack DNA. Exogenous sources mainly include mutagenic chemicals, ultraviolet (UV) and ionizing radiations (IR) (table 1.1). It is worth mentioning that DNA replication is not always halted by unrepaired DNA as some polymerases that do not require a stringent base-pairing can bypass the blockage (*166*).

To counteract this threats, cells have developed complex surveillance mechanisms capable of detecting the DNA lesions and signaling their presence to activate pathways that delay cell cycle progression, repair the DNA lesions, or

Damaging Agents	Type of damage	Repair process
X-rays, Oxigen radicals, Alkylating agents, Spontaneous reactions	Uracil, Abasic sites, 8-Oxoguanine, Single strand break	Base-excision repair (BER)
UV light, Polycyclic aromatic hydrocarbons	(6-4)PP, Bulky adducts, CPD	Nucleotide-excision repair (NER)
X-rays, Anti-tumor agents (cis-Pt, MMC)	Interstrand cross-link, Double-strand break	Recombinational repair (HR, EJ)
Replication errors	A-G mismatch, T-C mismatch, Insertion, Deletion	Mismatch repair

Table 1.1 Common types of DNA damage and repair mechanisms

eliminate the genetically unstable cells by inducing cell death (fig 4) (*167-172*). Many proteins are part of the repair machinery that also relies on post-translational modifications to transduce and spacially localize the signal. The most prominent ones are phosphorylation, ubiquitination and SUMOylation (see section 1.14).

At the core of the DDR-signaling in mammalian cells are the protein kinases Ataxia telangiectasia mutated (ATM) and ATM- and RAD3-related (ATR). ATM and ATR phosphorylate and activate two other kinases, CHK1 and CHK2 which, together with ATM and ATR, are the master regulator of the “cell-cycle checkpoints” (*173*). Targeting cyclin-dependent kinase (CDK) activity, they slow down or arrest the cell-cycle progression at the G_1-S, intra-S and G_2-M phases to allow DNA-damage to be repaired. At the same time, ATM/ATR activity promotes DNA-repair by activating, transcriptionally or post-transcriptionally, proteins involved in this process but also by recruiting repair factors to the damage which will be discussed in detail later. Generally the DDR machinery is able to repair the DNA damage that a cell may accumulate during its life cycle, but if the extent of the damage is deemed too high, cell death by apoptosis or cellular senescence is evoked (*174, 175*). The signaling cascade initiated by ATM/ATR that influences chromatin structure, effect checkpoint activation and promotes DNA repair is delineated by several protein and complexes. A comprehensive review of the DDR is beyond the scope of this thesis, for the topic includes many cellular processes each comprising a vast number of multi-component protein complexes. A general outline of the basic DDR response will be given, with an emphasis on detection and repair of DSBs by HR and NHEJ.

1.13.1. Single-strand damage

The complexity of DNA-lesions that a cell can encounter dictates the existence of specialized DNA-repair mechanisms. Based on the nature of the alteration to the DNA double helix, the lesions can be classified in two main categories: single-strand damage and double-strand break. In single-strand damage, the aberration is present in only one of the two strands whereas the other strand can be used as a template to support the correction of the damaged strand. Single-strand damage could be the results of diferrents alterations and the cells possess a number of repair mechanisms that remove and replace the damaged nucleotide(s).

Mismatches and insertions/deletions are processed by the mismatch repair (MMR) mechanism (*176*). A single-strand incision is created on the DNA double helix where the aberrant base is located which is subsequently acted upon by exonucleases that excise the area surrinding the mismatch. The resulting gap in the DNA strand is filled in by DNA polymerase and finally the two ends are rejoined by a ligase enzyme.

In base-excision repair (BER) - which resolves uracil insertions, abasic sites, 8-oxoguaines and single strand breaks - the damaged base is often recognized by a DNA glycosylase that mediates base removal. Similarly to MMR, nuclease, polymerase and ligase proteins complete the repair (*177*).

The nucleotide excision repair (NER) system recognizes helix-distorting base lesions caused by 6-4 photoproducts, cyclobutane pyrimidine dimers and bulky

adducts. The damaged DNA is excised as a 22-30 base oligonucleotide, leaving a single-stranded DNA (ssDNA) stretch. Also in this case, DNA synthesis by DNA polymerases and subsequent ligation complete the repair process (*167*).

1.13.2. Double-strand break

The DNA DSB is the principle cytotoxic lesion caused by ionizing radiation and radio-mimetic chemicals, but can also arise by stalled replication forks when DNA polymerase encounters a DNA single-strand break. DSBs can also occur as intermediates during biological events, such as V(D)J recombination in the development of lymphoid cells. DSBs are considered the greatest threat to DNA integrity because the damage involves both strands of the double helix leaving no intact complimentary strand to act as a template for the rapair process. If left unrepaired, DSBs can cause genomic rearrangements and translocations, due to the breaking and rejoining of two non-adjacent areas along a chromosome, a course which often lead to malignant transformation (*178*).

For DSB repair, two principal mechanisms are used: non-homologous end-joining (NHEJ) (*179*) and homologous recombination (HR) (*180*). HR is considered an “error free” mechanism for DSB repair because homologous sequences, prevalently from sister chromatids, are used to prime repair synthesis. Conversely, NHEJ is generally considered an error-prone process because it does not involve a DNA template. It has to be noted that the need for a sister chomatid restricts the function of HR mainly to S and G2 phases of the cell cycle, while NHEJ is active during any phase, making it the most common mechanism to repairing DSBs (*181*).

The first step in DSB repair is the detection of the lesion and activation of the relevant pathways aimed at halting the cell cycle and initiation of the DNA repair processes (*182*). Among its several targets, ATM phosphorylates histone H2AX, a variant that represents 10% of histone H2A, localized at the site of damage. It is believed that phosphorylated histone H2AX (γH2AX) acts as a molecular recruitment platform for ATM itself and several other proteins that contribute to the coordinated repair effort (*183*). Among these, two prominent examples are represented by BRCA1 and p53-binding protein (53BP1) because they regulate DSB repair pathway choice between HR and NHEJ, respectively (*184*).

BRCA1 is a large protein with multiple functional domains. Germline mutations of BRCA1 predispose women to breast and ovarian cancers (*185*). The molecular mechanisms underlying BRCA1's roles in DNA damage response are not fully understood, since BRCA1 has been shown to interact directly or indirectly with numerous molecules, including tumor suppressors, oncogenes, DNA damage repair proteins, cell cycle regulators, transcriptional activators and repressors (*186*). It is important to note that loss-of-function mutations of BRCA1 confer a complex phenotype which includes growth retardation, increased apoptosis, defective DNA damage repair, abnormal centrosome duplication, defective G_2/M cell cycle checkpoint, impaired spindle checkpoint, chromosome damage and aneuploidy (*187*). It has been proposed that mutations in BRCA1 do not directly result in tumor formation, but instead they cause genetic instability, subjecting cells to a high risk of malignant transformation.

53BP1 binds to damaged chromatin and carries out several functions. 53BP1 recruits additional DNA double-strand break (DSB) signalling and repair proteins to the site of DNA damage. Also, 53BP1 promotes ataxia-telangiectasia mutated (ATM)-dependent checkpoint signalling, especially at low levels of DNA damage (*188*). Finally, 53BP1 promotes the synapsis of distal DNA ends during NHEJ (*189*).

1.13.3. Non-homologous end-joining

Despite being considered an imperfect repair process, it is estimated that about 85% of DSBs in a cell are processed by NHEJ (*190*) (Figure 1.3). NHEJ relies on DSBs recognition by the Ku proteins (Ku70 and Ku80) that create a scaffold for the assembly of the other NHEJ key enzymes (*181*). Ku70/80 recruits the DNA-dependent protein kinase, catalytic subunit, also known as DNA-PKcs that help capture both ends of the broken DNA molecule and form a molecular bridge that brings the two DNA ends back together (*181*). Because the breakage of the double helix might not be clean and could leave overhanging single strand DNA (ssDNA) ends that are not compatible, the DNA termini are processed by the endonuclease (Artemis) or polymerases (pol μ and pol λ) in order to get the two DNA ends into a ligatable configuration (Figure 1.3) (*181*). The ligase IV/XRCC4 complex catalyses the ligation of the processed DNA ends. The fill-in or removal of base pairs is the step that accounts for nucleotide loss or addition at the rejoining site which creates “genomic scars” that can lead to an accumulation of randomly located mutations over time.

1.13.4. Homologous recombination

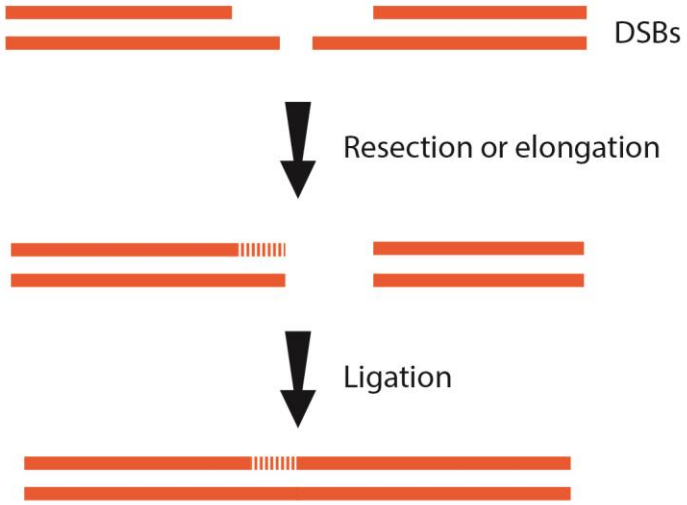

Figure 1.4 Schematic of non-homologous end-joining repair process

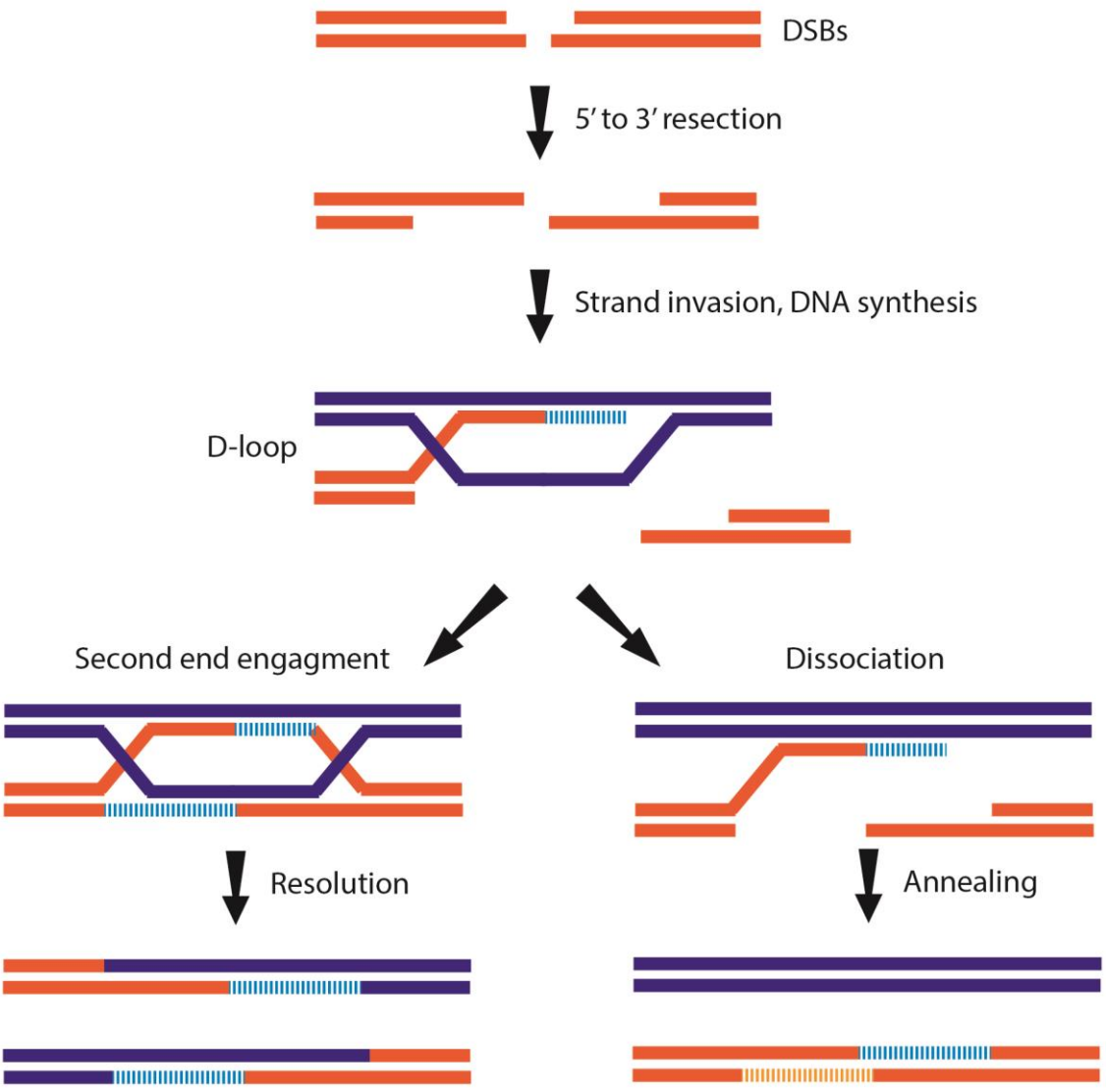

Figure 1.5 Schematic of homologous recombination repair process

HR is generally restricted to S and G_2 because it uses sister-chromatid sequences as the template and therefore is able to engineer more accurate repair (Figure 1.4) (*191*). Instrumental in this process is the MRE11-RAD50-NBS1 (MRN) complex (*182*). MRN complex, in its role as a lesion-specific sensor, has a structural role in stabilizing broken chromosomes, and promotes the assembly of large macromolecular complexes (known as foci) that facilitate efficient DSB responses (*192*).

A fundamental step in HR is represented by the generation of a stretched ssDNA end, accomplished by degrading 5′-strands at DSBs, that is used to search for homology. Subsequently, in a process orchestrated by a protein called RAD51, the ssDNA invades the undamaged homologous duplex DNA template template (*193*). RAD51 is a recombinase, an enzyme that mediates the pairing and shuffling of DNA sequences during HR (*193*). Two recombinases, Rad51 and Dmc1, exist in higher eukaryotes. Rad51 is needed for mitotic HR events such as DSB repair and also for meiotic HR, whereas Dmc1 is only expressed in meiosis (*194*). Rad51 loading onto ssDNA facilitates the formation of a physical connection between the invading DNA substrate and homologous duplex DNA template, leading to the generation of heteroduplex DNA (D-loop) (*195*). The Rad51 helical filament recognizes and pairs with the homologous region of the sister chromatid, creating a template for DNA synthesis (*193*). After the elongation of the invading ssDNA strand, the restoration of the broken DNA helix can be completed following different mechanisms. The invading strand can be simply dissociated from the D-loop, anneal with the complementary strand at the other end of the damage and then

DNA synthesis completes the process as in gap repair (*196*). Alternatively, the second end of DSB can be engaged to stabilize the D-loop structure (second-end capture), leading to the generation of a structure named double-Holliday Junction (dHJ) (*197*). A dHJ is then resolved to produce crossover or non-crossover products. Finally, the D-loop structure can assemble into a replication fork and copy the entire chromosome arm in a process called break-induced replication (BIR) (*198*).

1.14. SUMOylation

Covalent modifications of proteins are the main mechanisms of altering their function and regulating their abundance and subcellular localizations. SUMO (small ubiquitin-related modifier) is a small ubiquitin-like protein (Ubls), with emerging roles in protein regulation.

1.14.1. Conjugation of SUMO to target proteins

SUMOylation often, but not exclusively, occurs on Lysine residues contained within a short consensus sequence ψ-K-X-E/D, where ψ is a large hydrophobic amino acid, generally Isoleucine, Leucine, or Valine; K is the Lysine residue that is modified; X is any residue; and E is a glutamic acid (*199*). The linkage between SUMO and its substrates is an isopeptide bond between the C-terminal carboxyl group of SUMO and the ε-amino group of a lysine residue in the substrate {Johnson, 2004 #14}. Vertebrates have three SUMOs: SUMO-1 (also known as

sentrin, PIC1, GMP1, Ubl1, and Smt3c), SUMO-2 (sentrin-3, Smt3a), and SUMO-3 (sentrin-2, Smt3b). SUMO-2 and -3 share ~95% sequence identity and are presumed to be functionally identical (*200*). They are ~50% identical to SUMO-1 which appears to have partially distinct functions. Cells contain a large pool of free, unconjugated SUMO-2/3 while the majority of SUMO-1 is conjugated to other proteins (*200*). Unlike SUMO-1, conjugation of SUMO-2/3 is strongly induced in response to various stresses, indicative of specific, context dependent functions for SUMO-2/3 (*200*). Similar to ubiquitylation, SUMOylation involves attachment of a ~ 11 kDa polypeptide via a three-step reaction (Figure 1.5) (*201*). The E1 SUMO-activating enzyme is a heterodimer composed by Aos1 (also called SAE1, Sua1) and Uba2 (SAE2) and catalyzes the initial ATP-dependent activation of the SUMO C terminus which is then transferred to the E2 SUMO-conjugating enzyme Ubc9 (*202*). Ubc9, which directly binds the SUMO consensus motif, is thought to be the only SUMO-conjugating enzyme in mammals and serves as the SUMO donor in the final reaction in which SUMO is transferred to the amino group of the substrate Lys (*203*). While E1 and E2 are often sufficient to SUMOylate proteins *in vitro*, *in vivo* the final step of conjugation involves an E3 SUMO-protein ligase (E3) which is thought to enhance specificity. In mammalian cells, three families of SUMO E3 ligases have been identified: the PIAS (protein inhibitor of activated STAT) family (*204*); the second consists of a domain in the large vertebrate nuclear pore protein RanBP2/Nup358 (*205*); and the third is the polycomb group protein Pc2 (*206*).

SUMO can also be attached to Lys residues within SUMO itself, leading to the formation of SUMO chains (polySUMOylation) (*207*). Unlike in SUMO1, a lys

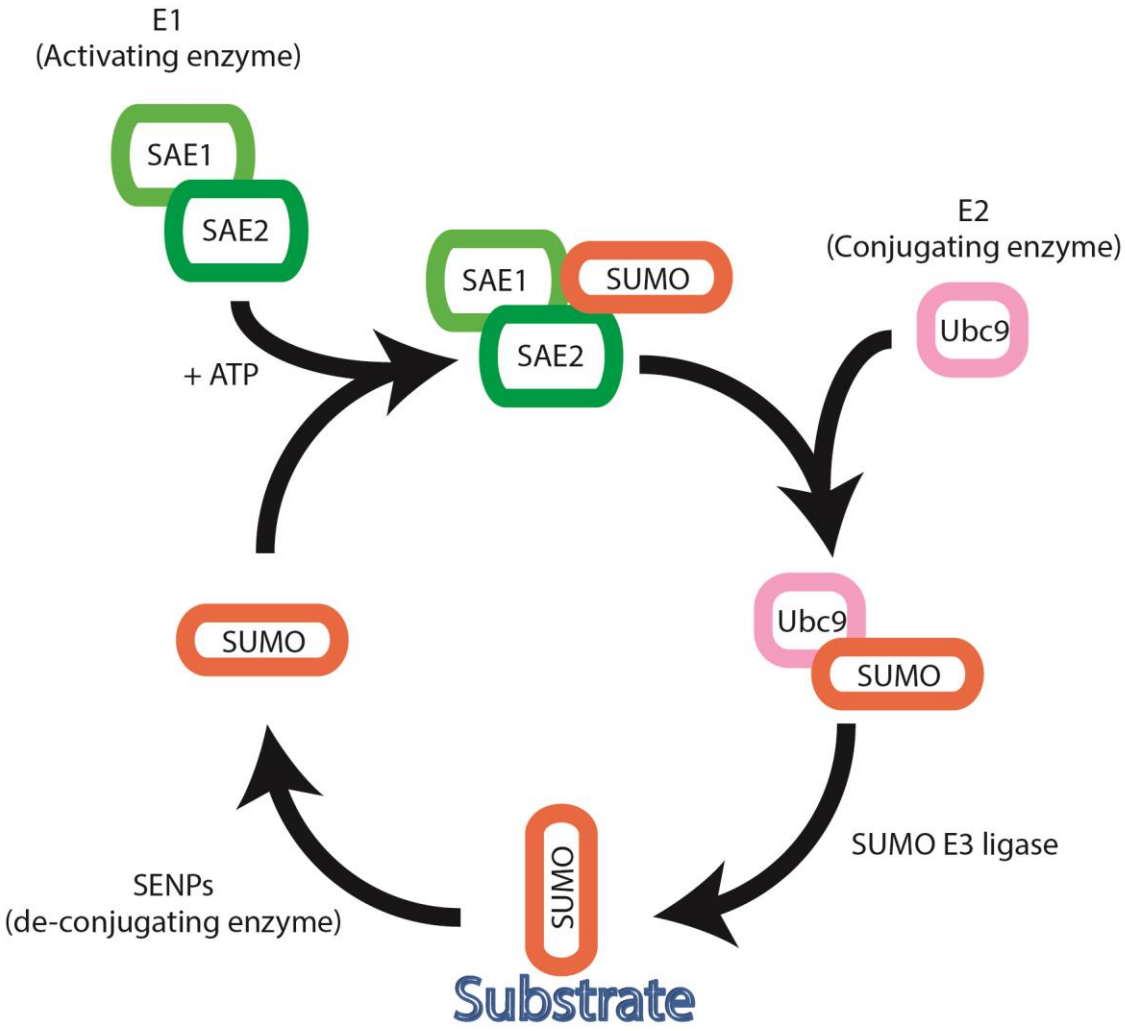

Figure 1.6 Schematic of SUMO conjugation and de-conjugation pathway

residue (K11) that conforms to the SUMO consensus motif, is found within mammalian SUMO-2 and SUMO-3. SUMO-1, on the other hand, can be attached to Lys residues within SUMO-2/3 chains thereby preventing the elongation of such chains.

Similar to Ubiquitination, SUMOylation is a reversible modification. Specific isopeptidases belonging to the Ulp family cleave SUMO moieties from the target proteins and contribute to the dynamics of SUMOylation (*208*). Interestingly, these enzymes also carry out the cleavage of a short C-terminal peptide from newly synthesized pro-SUMO to generate the mature SUMO peptides ready for conjugation (*209*).

1.14.2. SUMO functions

Biochemical detection of SUMOylated proteins is impeded by the action of deSUMOylases which rapidly deSUMOylate target proteins upon cell lysis (*210*). This, combined with the fact that only a fraction of the substrate (often less than 1%) is SUMOylated at any given time, explains why the SUMOylated protein often evade identification. Given the stoichiometry of SUMOylation, mutation of the SUMO attachment sites in proteins often leads to subtle, if detectable changes in protein function.

Early studies of SUMOylation suggested a role in transcription repression (*211*). Nevertheless, subsequent studies revealed SUMO functions in DNA repair, nuclear transport, signal transduction, and cell cycle progression regulation (*212*). SUMO plays a major role in promyelocytic leukaemia nuclear bodies (PML NBs)

which act as storage depots for nuclear factors, site of modification and assembly of transcription factors (*213*). The principal component of PML NBs is the PML protein which is SUMOylated at three sites (*214*). PML SUMOylation is essential for formation of functional NBs and recruitment and assembly of interacting proteins. Thus, control of PML SUMOylation can broadly affect transcription programmes.

Independent of PML NBs, SUMO is directly involved in transcription repression at the promoter level. Studies performed with SUMO-deficient mutants of a number of transcription factors such as Elk-1, Sp-3, SREBPs, STAT-1, SRF, c-myb, C/EBPs, androgen receptor, or the coactivator p300 reported an increase in transcription on responsive promoters, confirming a suppresive role for SUMO in gene expression (*215-218*).

SUMOylation has also been linked to maintenance of higher-order chromatin structure and proper chromosome segregation. SUMO knockout studies in *S. cerevisiae* (*219*) and *D. melanogaster* (*220*) revealed gross defects in chromosome condensation, whereas mammalian cell work confirmed that SUMO localizes at or adjacent to the kinetochore (*221-223*).

The first and most studied SUMOylated protein is RanGAP1, relating to its high abundance compared to other SUMO targets (*224*). RanGAP1 is the GTPase activating protein for the small GTPase Ran that is central to the nucleocytoplasmic transport. SUMOylation is key to RanGAP1 nuclear localization where it interacts with the mitotic spindle and strongly associates with the kinetochores (*222*). RanGAP1 that cannot be SUMOylated does not associate with spindles (*225*). Remarkably RanGap1 E3, RanBP2, resides on the cytoplasmic side of the nuclear

pore complex, while the SUMO isopeptidase SENP2 is on the nucleoplasmic side, suggesting a model in which proteins might be rapidly sumoylated and desumoylated as they cross the nuclear membrane (*224*).

1.14.3. SUMOylation and DNA damage

Even though SUMOylated proteins can be found outside of the nucleus, the most extensive evidence exists for its functions in the nucleus, in particular in DNA repair pathways. In base excision repair (BER), Thymine-DNA glycosylase (TDG) removes thymine or uracil from G:T or G:U mismatches that arise through deamination of 5-methylcytosine or Cytosine, respectively. SUMO1 conjugation within the TDG C-terminus triggers its interactions with SUMO interacting motifs (SIMs, discussed later) found elsewhere within the protein, that alter the biochemical properties and subcellular localization of the protein (*226, 227*). Thus, SUMOylation of TDG promotes its dissociation from the base lesion ensuring progression of the repair process following TDG binding and base hydrolysis (*228*). SUMO may also participate control of the activity of DNA topoisomerase I (TOP1) and topoisomerase II (*229, 230*). This was inferred from the evidence that both are SUMOylated in mammalian cells in response to topoisomerase inhibitors suggesting that SUMOylation might target preferentially the protein in its inactive form.

Working in concert with ubiquitylation, SUMOylation also regulates and coordinates various pathways involved in double strand break (DSB) repair contributing to DNA damage recognition, signaling, and repair. Immunofluorescence

microscopy revealed that SUMO1, SUMO2/3, UBC9, and the PIAS and MMS21 SUMO E3s accumulate at sites of DSBs or stalled replication forks (*231, 232*). Pioneering work in yeast uncovered the fundamentals of these interactions (*233*). In *S. cerevisiae,* SUMOylation protects Rad52, a key homologous recombination (HR) factor, from degradation and regulates its subcellular localization (*234*). Cohesin is a multiprotein complex that promotes equal chromosome distribution during mitosis and accurate repair of DNA damage postreplication (*235*). All subunits of cohesin become SUMOylated in the presence of DNA double-strand break and the modification by SUMO is required for sister chromatid tethering after DNA damage (*236*).

In mammalian cells, SUMO has been implicated in several DNA repair pathways. Depletion of PIAS1 or PIAS4, two members of the PIAS family of SUMO E3 ligases, markedly impairs DSB repair by HR and non-homologous end joining (NHEJ) and causes hypersensitivity toward DSB-generating agents (*237*). Specifically, PIAS1 and PIAS4 mediate the association of RNF168, an ubiquitin E3 ligase, as well as that of 53BP1 and BRCA1 with damage foci (*231, 232*). In response to ionizing radiation, 53BP1 and BRCA1 are SUMOylated in PIAS1/PIAS4-dependant manner (*232*). In addition to 53BP1 and BRCA1, the list of SUMOylated proteins implicated in DDR include HERC2 (*238*), tyrosyl DNA phosphodiesterase (TDP1) (*239*), RPA70 (*240*) and BLM (*241*).

There are considerable interactions between the SUMO and the ubiquitin pathways, including evidence that SUMOylation can “prime” proteins for ubiquitination and proteasomal degradation (*242*). Recently, a new family of

proteins, the small ubiquitin-related modifier (SUMO)-targeted ubiquitin ligases (STUbLs), which directly connect SUMOylation and ubiquitylation, has been discovered (*242*). STUbLs use SUMO-interaction motifs (SIMs) to recognize their SUMOylated targets. STUbLs are global regulators of protein SUMOylation levels, and cells lacking STUbLs display genomic instability and hypersensitivity to genotoxic stress (*242*).

More generally, it appears that SUMOylation serves as a facilitator for the local accumulation of proteins involved in DNA damage response mediated by the interaction of the SUMO-peptide attachment with SUMO-interacting motifs (SIM) on another protein. In fact, while sumoylation of various HR factors is collectively crucial for effective DSB repair, loss of any one individual sumoylation has little effect enforcing the notion of SUMO as a “protein group modification” for elements of the DNA repair pathways.

1.15. Thesis outline

1.15.1. Study rationale

Many cancer-associated *PTEN* mutations have no effect on its phoshatase activity. This suggests that at least some of the residues or regions that are found mutated in tumors contribute to PTEN tumor suppressor function by influencing the protein stability, conformation or localization. In particular, the localization of PTEN in the nuclear compartment is required for its ability in protecting cells from transformation (*243*). While it has been proposed that mono-ubiquitination of PTEN leads to its nuclear localization (*81*), other studies that partially contradict this findings seem to suggest the existence of additional mechanisms (*82*).

Exploring the PTEN immunoreactivity in cell lysates prepared under denaturing conditions from several mammalian cell lines, I identified a novel PTEN protein species that seems to be preferentially localized in the nucleus. I hypothesized that this PTEN form represents a post-translational modifications that governs PTEN subcellular localization and tumor suppressor function. Experiments were performed to identify the nature of the modification and its role in the regulation of PTEN tumor suppressor functions.

Having established that SUMO-PTEN plays a determinant role in homologous recombination and thus cells that do not express PTEN are more sensitive to the combined action of genotoxic stress and PI3K inhibition, in chapter 3 I investigate the molecular and physiological consequences elicited by PTEN nuclear localization.

1.15.2. Thesis objectives

- Characterization of a newly discovered form of PTEN
- Determination of the physiological relevance of newly discovered PTEN post-translational modification
- Explore the sensitivity of PTEN-null cells to different, therapeutically relevant, agents

Chapter 2 Nuclear PTEN controls homologous recombination-mediated DNA repair and sensitivity to DNA damage

A version of this chapter is a published manuscript:

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Author contribution:

C.Bassi and V.Stambolic designed research

C.Bassi performed the majority of experiments

J.Ho, T.Srikumar, R.Dowling, C.Gorrini, and S.J.Miller performed some experiments

C.Bassi., T.Srikumar, B.G.Neel, B.Rought, and V.Stambolic analyzed data

T.W. Mak, B.G.Neel, B.Raught, and V.Stambolic supervised research

C.Bassi, B.G.Neel and V.Stambolic wrote the paper

2.1. PTEN is target of SUMOylation

In the course of analyzing post-translational modifications of PTEN, we noticed that, in addition to the expected PTEN protein of Mw ~55kD, an ~75 kDa PTEN protein species could be detected by immunoblotting with three independent anti-PTEN antibodies in denaturing lysates prepared from multiple mammalian cell lines (Figure 2.1a). Assuming comparable affinity of the anti-PTEN antibodies for both isoforms, this novel species, which we initially termed “PTEN-H,” represented < 10% of the total PTEN in the cell lines tested. Importantly, PTEN-H was absent from mouse embryonic fibroblasts (MEFs) and HCT116 cells with targeted disruption of the *PTEN* gene (Figure 2.1a). Moreover, transfection of a vector expressing HA-epitope-tagged PTEN cDNA gave rise to PTEN and PTEN-H, establishing that PTEN-H arises as a consequence of post-translational modification, rather than alternative initiation or splicing (Figure 2.1b).

Given that PTEN-H had not been reported previously, we explored the experimental conditions that resulted in its detection. Use of mild detergents, such as 0.1% Triton or 0.3% CHAPS, largely precluded PTEN-H detection (Figure 2.2a). By contrast, inclusion of N-ethylmaleamide (NEM), an inhibitor of cysteine-based enzymes, including deSUMOylases and deubiquitinases, during cell lysis, increased the detection of PTEN-H (Figure 2.2b). Mono-sumoylation could explain an ~20kD increase in apparent molecular weight, so we asked whether PTEN-H was a SUMOylated PTEN form by probing PTEN immunoprecipitates from denaturing HEK293 cell lysates with anti-SUMO antibodies. Indeed, while these

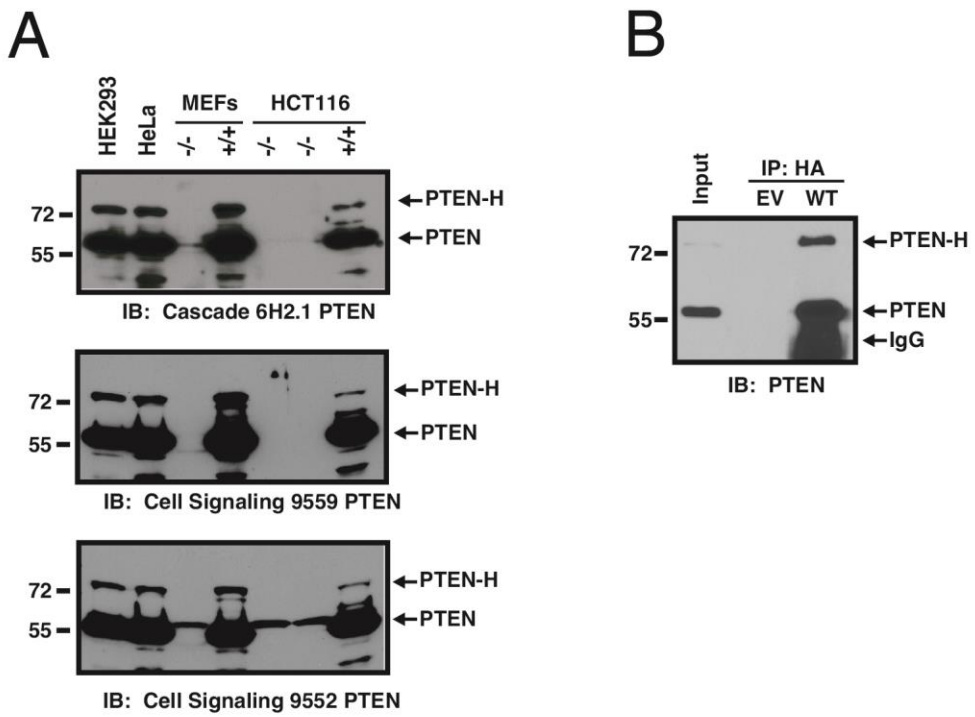

Figure 2.1 A new post-translational modification of PTEN. (A) Multiple cell lines express a 75 kDa form of PTEN. Whole cell lysates were immunoblotted with 3 PTEN antibodies: 6H2.1 mouse mAb (top), rabbit mAb CST #9559 (middle), or a rabbit pAb (bottom) CST #9552. PTEN-H is indicated (arrow). (B) Transfection of minimal PTEN cDNA leads to PTEN-H formation. pcDNA3.1-HA-PTEN (WT) or pcDNA3.1-HA (EV) were transfected into HEK293 cells and HA-immunoprecipitates immunoblotted for PTEN.

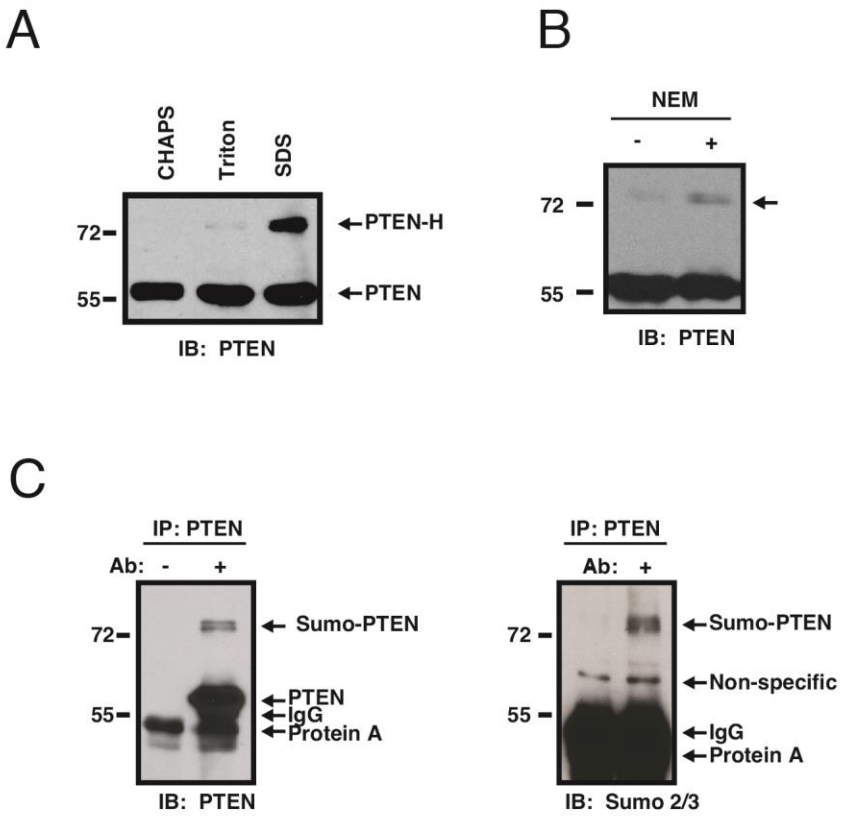

Figure 2.2 Detection and identification of SUMO-PTEN. (A) HEK2939 cells were lysed in buffers containing the indicated detergents and no NEM. Lysates were resolved by SDS-PAGE and immunoblotted with anti_PTEN antibody 6H2.1. (B) Inclusion of N-ethylmaleameide (20 nM) in the lysis buffer improves PTEN-H recovery. HEK293 cells were lysad in a hypotonic/NP-40 buffer either without or with NEM, as indicated. (C) SUMOylation of endogenous PTEN. PTEN was immunoprecipitated from HEK293 lysates with protein A beads alone or PTEN antibody (CST #9559) and immunoblotted with the PTEN 6H2.1 antibody (left) then stripped and re-probed with antibodies to Sumo2/3 (right).

immunoprecipitates contained PTEN and PTEN-H, only the latter reacted with anti-SUMO 2/3 antibodies (Figure 2.2c), indicating that PTEN-H is a SUMOylated form of PTEN (SUMO-PTEN). Consistent with this notion, knockdown of Ubc9, the sole SUMO-conjugating E2 protein (*244*), led to a decrease in SUMO-PTEN abundance (Figure 2.3a), whereas overexpression of the SENP1 or SENP2 deSUMOylases decreased SUMO-PTEN levels in mammalian cells (Figure 2.3b). Furthermore, treatment of PTEN immunoprecipitates with recombinant SENP1 or SENP2 decreased SUMO-PTEN levels in an NEM-sensitive manner (Figure 2.3c).

2.2. Lysine 254 is a major SUMOylation site on PTEN

To determine the site(s) of PTEN SUMOylation, we first subjected a series of PTEN deletion mutations to SUMOylation *in vitro* (Figure 2.4a). Although the N-terminal portion of PTEN was not SUMOylated, several C-terminal PTEN polypeptides were (Figure 2.4b), with the minimal SUMOylated region encompassing amino acids 238-320 (Figure 2.4b). This region of PTEN harbors a strong predicted SUMOylation site at position 254, based on the consensus ΨKxE (where Ψ is a hydrophobic amino acid, K is the predicted site of SUMOylation, x is any amino acid and E is glutamic acid). Indeed, the PTEN (238-320) K254R mutant was not SUMOylated *in vitro* (Figure 2.4c). In parallel, we analyzed *in vitro* SUMOylated PTEN polypeptides by nanoflow liquid chromatography-tandem mass spectrometry (nLC-MS/MS) and SUMmOn pattern recognition software (*245, 246*). These experiments independently identified K254 as a *bona fide in vitro*

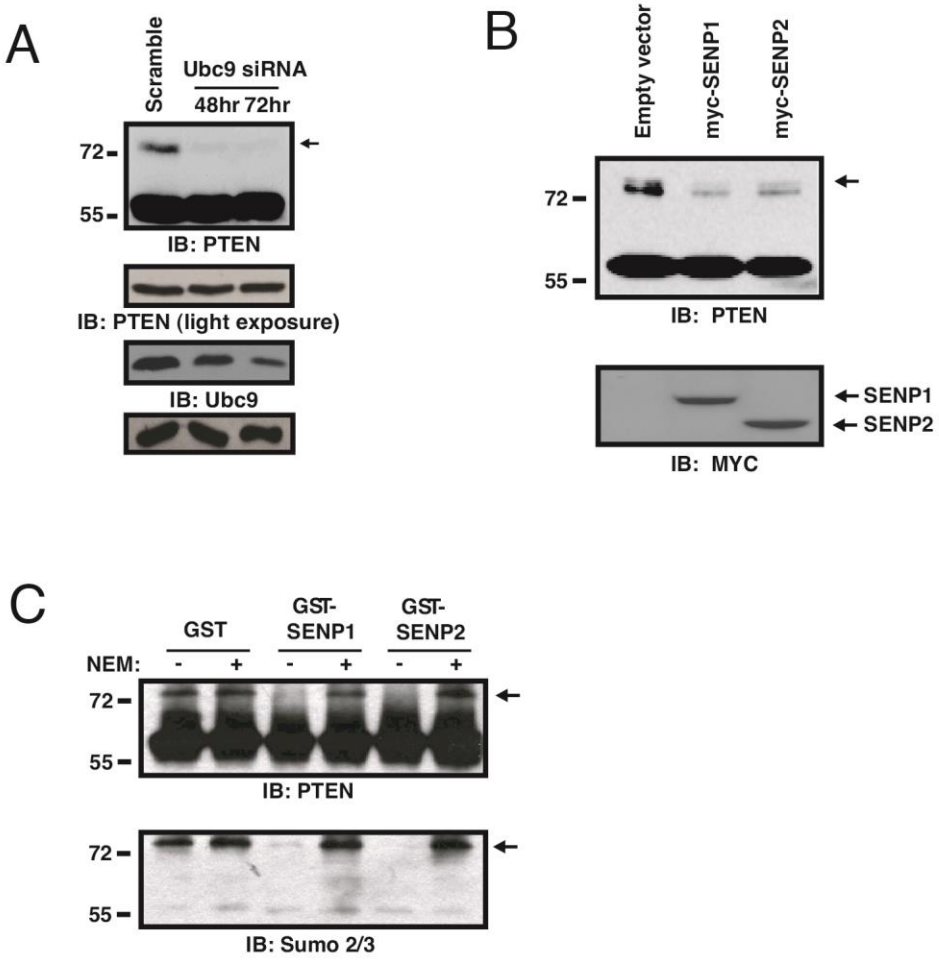

Figure 2.3. SUMO-PTEN levels are regulated by Ubc9 and deSUMOylases. (A) Formation of SUMO-PTEN requires Ubc9. HEK293 cells were transfected with scrambled or Ubc9 siRNAs, and harvested at 48 (middle lane) or 72 (right lane) hrs post-transfection. Lysates were immunoblotted for PTEN, Ubc9 and GAPDH, as indicated. Arrow indicates SUMO-PTEN. (B) HEK293 cells were transfected with empty vector or myc-SENP1 or myc-SENP2 expression vectors, lysed and immunoblotted with the indicated antibodies. (C) Sensitivity of PTEN-H to deSUMOylases. Immunoprecipitated Flag-PTEN was incubated with GST, GST-SENP1 or GST-SENP2, as indicated, in the presence or absence of 20 mM NEM. Reactions were immunoblotted with anti-PTEN 6H2.1 (top panel) or anti-Sumo2 (lower panel) antibodies.

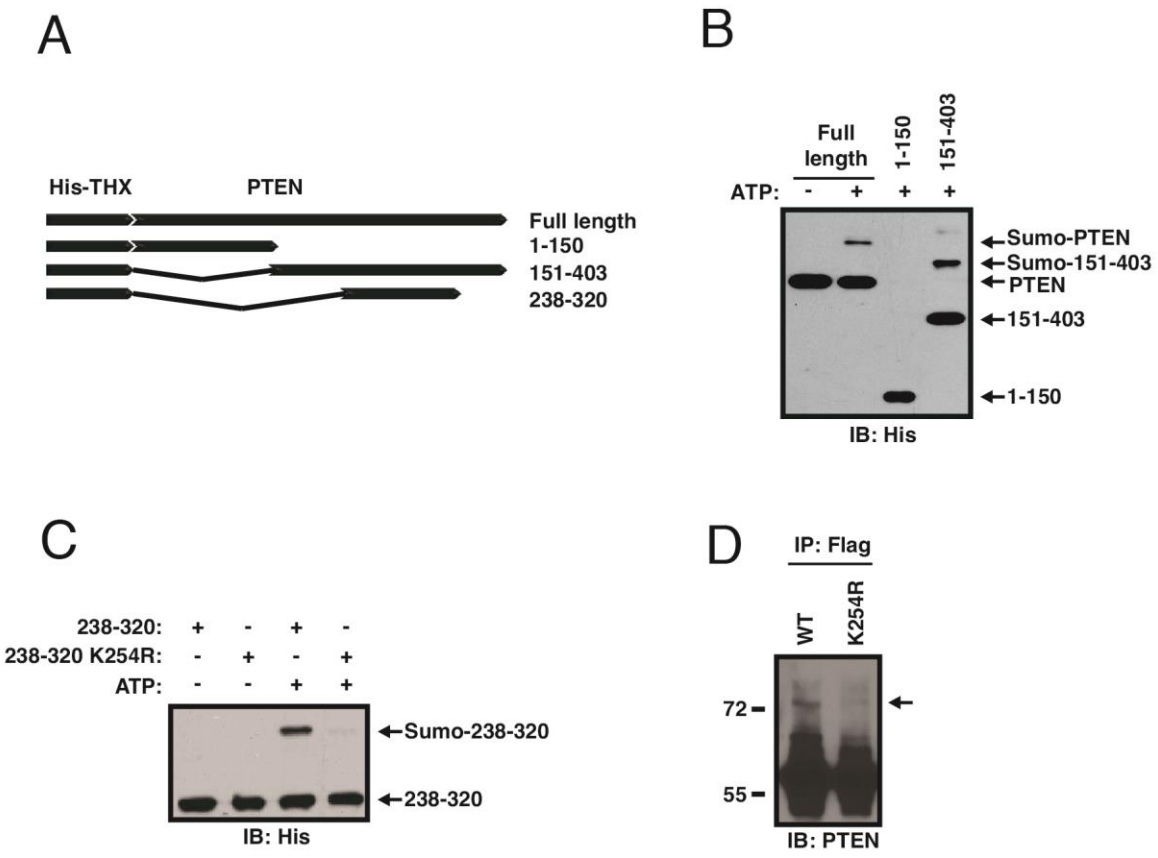

Figure 2.4. In vitro SUMOylation of PTEN deletion mutants. (A) Schematic of recombinant His-Thioredoxin-PTEN fusion constructs used for in vitro SUMOylation reactions. (B) PTEN 151-403 is SUMOylated. Full length PTEN and the indicated truncations were subjected to in vitro SUMOylation reactions, followed by immunoblotting with an anti-His antibody. (C) Mutation of lysine 254 abolishes SUMOylation on PTEN 238-403. PTEN truncation products 238-403 and 238-403 K254R were used as substrates in in vitro SUMOylation reactions. Lysine 254 was the only site which corresponded to the SUMO consensus ΨKxE sequence (where Ψ is a hydrophobic amino acid, K is the predicted site of SUMOylation, x is any amino acid and E is glutamic acid). (D) PTEN is SUMOylated on lysine 254 in vivo. HEK293 cells were transfected with FLAG-PTEN-wt or FLAG-PTEN-K254R, together with His-SUMO2 and myc-His-Ubc9, as indicated. Flag immunoprecipitates were immunoblotted for PTEN.

sumoylation site (Figure 2.5 and 2.6). Consistent with PTEN SUMOylation at this site *in vivo*, when HeLa cells were co-transfected with expression vectors for Ubc9, Sumo2, and Flag-PTEN K254R, the latter was not SUMOylated, whereas PTEN SUMOylation was readily detected in cells co-transfected with FL-PTEN, Ubc9 and Sumo2 (Figure 2.4d). Taken together, our results identify K254 as the major PTEN SUMOylation site *in vitro* and in intact cells.

2.3. Physiological function of SUMO-PTEN and its regulation

2.3.1. SUMO-PTEN lipid phosphatase activity

Expression of Wt PTEN or PTEN K254R in PTEN-deficient U87MG glioblastoma cells (*8*) resulted in comparable decreases in phosphorylation of PKB/Akt, a measure of the capacity of PTEN proteins to antagonize PI3K signals. Likewise, WT and SUMO-deficient PTEN caused similar decreases in phosphorylation of the PKB/AKT target GSK3β (Figure 2.7a). Consistent with these findings, the ability of recombinant, GST-PTEN-K254R mutant to dephosphorylate soluble $PI(3,4,5)P_3$ was indistinguishable from that of GST-Wt PTEN (Figure 2.7b). Thus, the PTEN SUMOylation-site mutant retains the ability to counter PI3K signaling.

Scan	Charge	relPos	modScore	precursor mass	matched ions	TIC	%TIC	BPI
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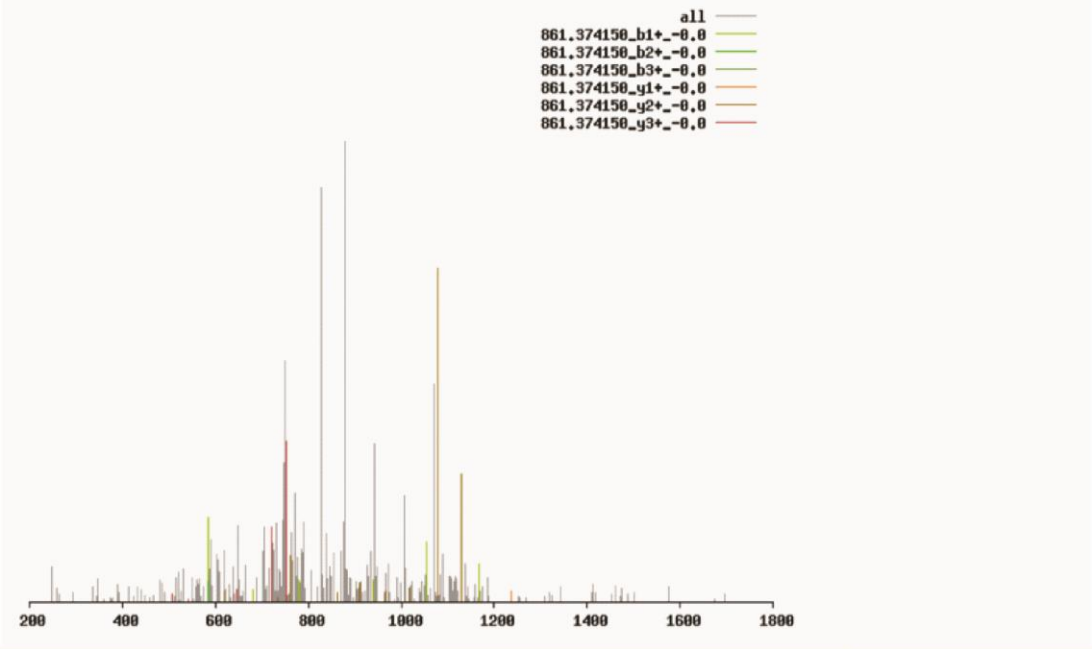

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	F	P	Q	P	L	P	V	C	G	D	I	K(0)	V	E
861.374150_b1+_-0.0	148.184	245.301	373.432	470.549	583.708	680.825	779.957	883.096	940.148	1055.24	1168.4	2157.94	2257.08	
861.374150_b2+_-0.0	74.5962	123.155	187.22	235.778	292.358	340.916	390.483	442.052	470.578	528.122	584.702	1079.48	1129.04	
861.374150_b3+_-0.0	50.0667	82.4389	125.149	157.521	195.241	227.613	260.658	295.037	314.055	352.417	390.137	719.987	753.031	
861.374150_y1+_-0.0		2257.03	2159.91	2031.78	1934.67	1821.51	1724.39	1625.26	1522.12	1465.07	1349.98	1236.82	247.271	148.139
861.374150_y2+_-0.0		1129.02	1080.46	1016.4	967.837	911.258	862.699	813.133	761.564	733.038	675.493	618.914	124.14	74.5733
861.374150_y3+_-0.0		753.016	720.643	677.933	645.561	607.841	575.469	542.425	508.045	489.028	450.665	412.945	83.0956	50.0515
	14	13	12	11	10	9	8	7	6	5	4	3	2	1

Figure 2.5. Identification of a SUMO conjugation site on PTEN using mass spectrometry and the SUMmOn algorithm. In vitro SUMOylated PTEN was subjected to GluC proteolysis, and the resulting peptides were analyzed by LC-ESI-MS/MS. Data were analyzed using the SUMmOn algorithm, as in (25, 26). A PTEN peptide (FPQPLPVCGDIKVE) was identified as a SUMO1 conjugate (the SUMO modified internal lysine residue is underlined). Shown is the SUMmOn screenshot of a CID spectrum highlighting the mixed fragment ion series from the C-terminal fragment of SUMO1 (left) and the fragment ion series from the PTEN target peptide (right). Predicted fragment ion masses from the indicated peptide are listed in the table (for several different charge states) and those that were assigned (i.e. present in the CID spectrum) are colored red (for b-ions) or blue (y-ions). The SUMmOn header also indicates: MS/MS scan number of this CID, precursor charge state, relative scoring position of the assigned charge state as compared to other charge states (relPos), the SUMmOn modification score (modScore), the uncharged precursor mass (M), the number of matched ions, total ion current (TIC), percentage TIC matched (%TIC) by the assigned ions, and base peak intensity (BPI) for the scan. K(0) denotes the modified lysine residue. The SUMmOn target score is also shown (right panel), along with target peptide information and fragmentation ion series. For a more in-depth explanation of the identification of SUMO conjugation sites using mass spectrometry and the SUMmOn scoring algorithm, please see references 25 and 26.

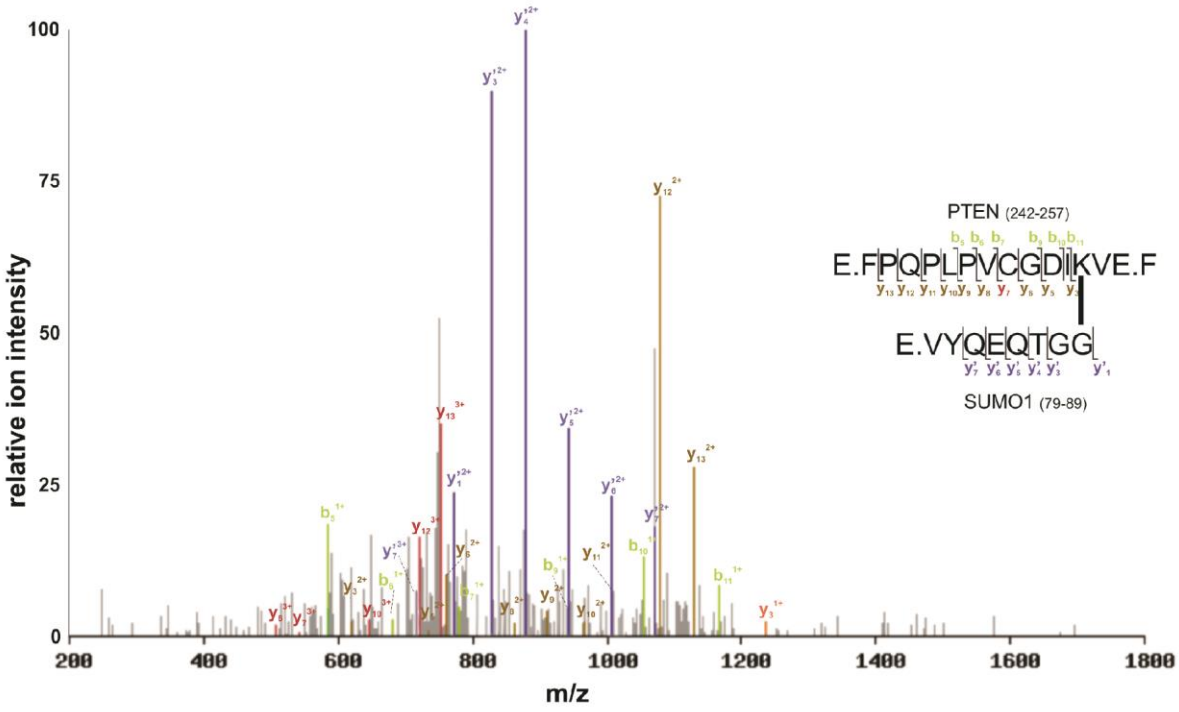

Figure 2.6. Identification of a SUMO conjugation site on PTEN using mass spectrometry and the SUMmOn algorithm. Mass spectrometric analysis identifies lysine 254 as a SUMO conjugation site in PTEN. In vitro SUMOylated PTEN 238-403 was subjected to proteolysis with GluC (a protease that cleaves C-terminal to glutamate residues), and the resulting peptides were subjected to liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). MS/MS data were analyzed with the SUMmOn algorithm (218, 219) to assign SUMO and PTEN peptide fragment ions. Shown is an annotated collision-induced dissociation (CID) MS/MS spectrum of the SUMO-modified PTEN peptide (inset): *blue*, fragment ions derived from the C-terminal SUMO1 remnant (see inset); *green*, b-ions derived from the PTEN peptide; *brown*, y-ions derived from the PTEN peptide. All assigned fragment ions are also indicated in the inset.

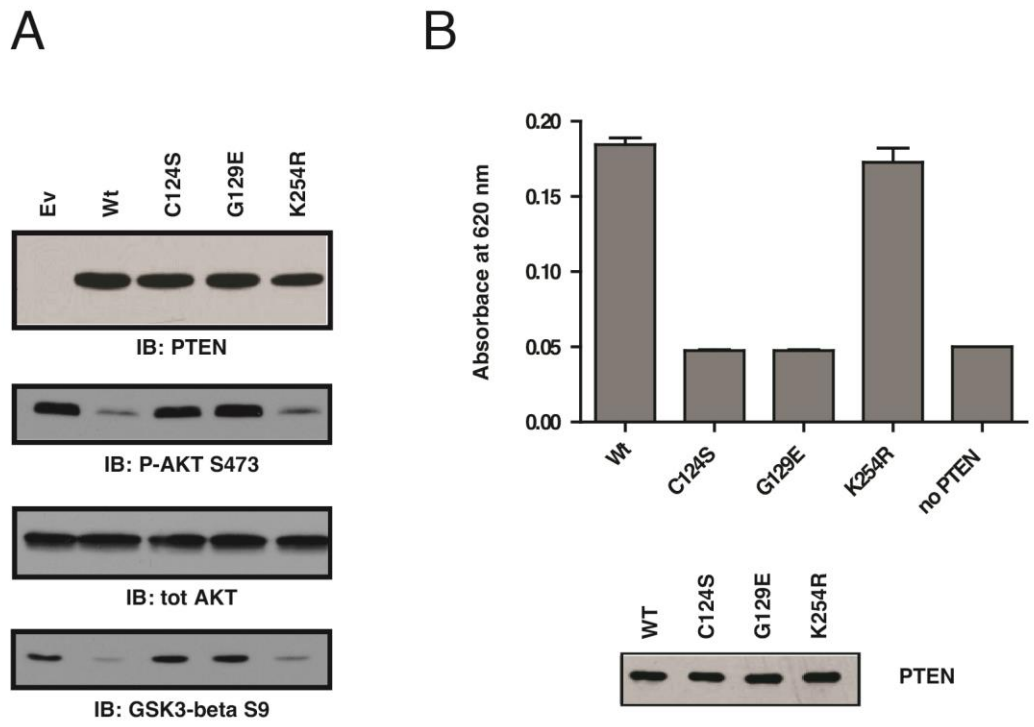

Figure 2.7 K254R mutation does not affect PTEN phosphatase activity. (A) PTEN K254R mutation does not impair its ability to regulate PI3K signaling. U87MG cells were reconstituted with the indicated Flag-PTEN proteins and the status of the PI3K pathway estimated by monitoring the activation –specific phosphorylation of PKB/Akt at S473 and of GSK3β at S9 by immunoblotting. (B) Phosphatase assays with PTEN mutants. One microgram of the indicated recombinant His-THXPTEN proteins (0.5 ug; control for protein loading at bottom) were incubated with soluble PI(3,4,5)P3. Released phosphate was detected using the Malachite green assay.

2.3.2. SUMOylation regulates PTEN nuclear localization during DNA damage stress

Because SUMOylation did not appear to affect PTEN-mediated regulation of the PI3K/Akt pathway, we examined its effects on PTEN sub-cellular localization. Remarkably, unlike Wt PTEN and an unrelated lysine PTEN K289R mutant, PTEN K254R expressed in HEK293 (Figure 2.8a) or U87MG (Figure 2.8b) cells failed to localize to the nucleus, suggesting that SUMOylation promotes PTEN nuclear localization. To better understand the relationship between SUMOylation and PTEN nuclear transport, we treated cells with leptomycin B, an inhibitor of nuclear export (Figure 2.8c). PTEN-K254R localized in the nuclei of leptomycin B-treated cells, indicating that under normal conditions, this mutant enters the nucleus but is not retained there (Figure 2.8c). Remarkably, unlike serum starvation/stimulation or treatments with various cytokines and growth factors, which did not influence PTEN localization (data not shown), ionizing radiation (IR) led to almost complete loss of nuclear PTEN (Figure 2.9a). Judging by fractionation of cellular lysates, SUMOylated PTEN is the predominant nuclear PTEN form and is turned over within hours of exposure to DNA damage (Figure 2.9b).

2.3.3. SUMO-PTEN is required for proper Homologous recombination repair

Prompted by the differential nuclear localization of PTEN following IR, we investigated the DNA damage response of PTEN-deficient U87MG cells engineered to express PTEN, PTEN K254R, mutants of PTEN lacking all phosphatase activity (PTEN C124S) or only lipid-phosphatase (but not protein-phosphatase) (PTEN

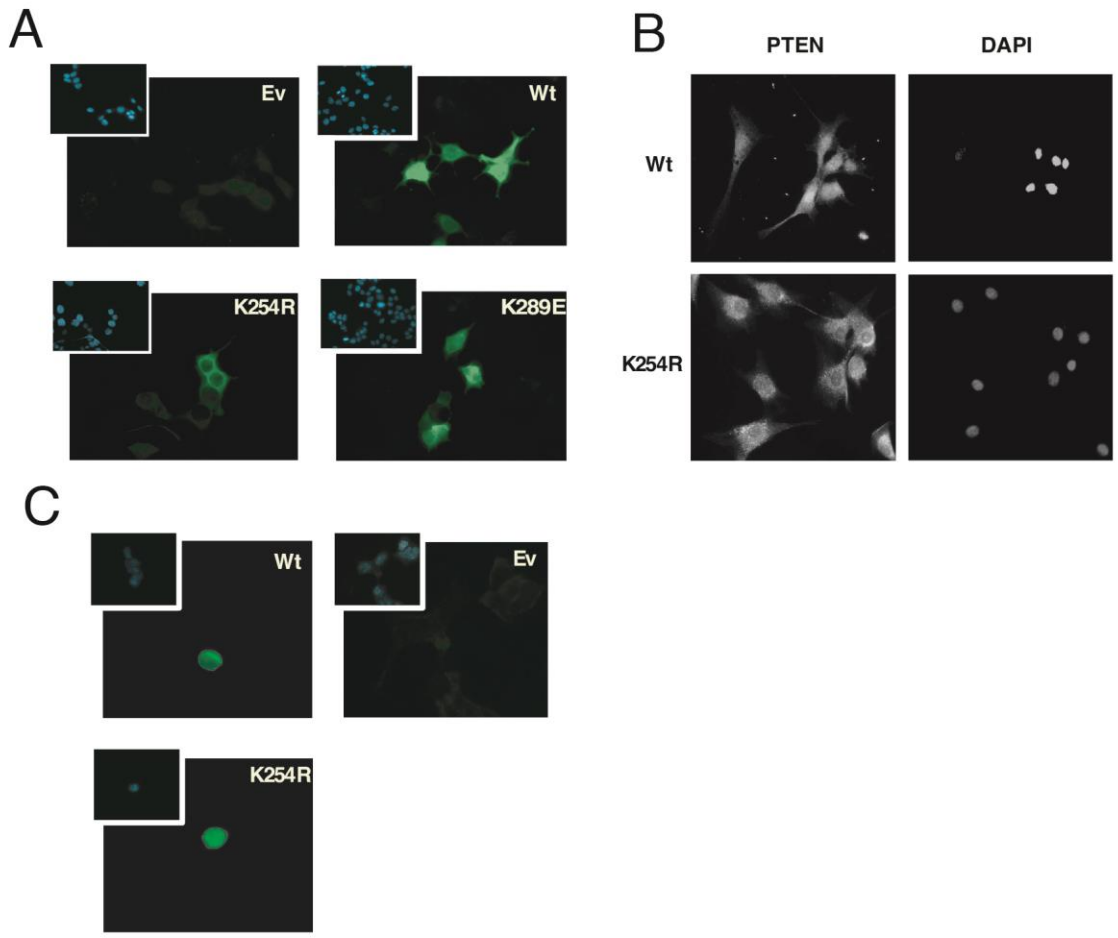

Figure 2.8 PTEN SUMOylation regulates nuclear retention. (A) Exclusion of the SUMO-de_cient mutant PTEN K254R from the nucleus. Flag-FITC immuno_uorescence images of HEK293 cells transfected as indicated. Insets show DAPI staining. (B) U87MG cells were reconstituted with Flag- PTEN-wt or -K254R, _xed with 4% paraformaldehyde and immunostained for PTEN. (C) Nuclear retention of SUMOylated PTEN. Immunofuorescence as in (A). Cells were treated with 10 ng/ml of leptomycin B for 4 hours.

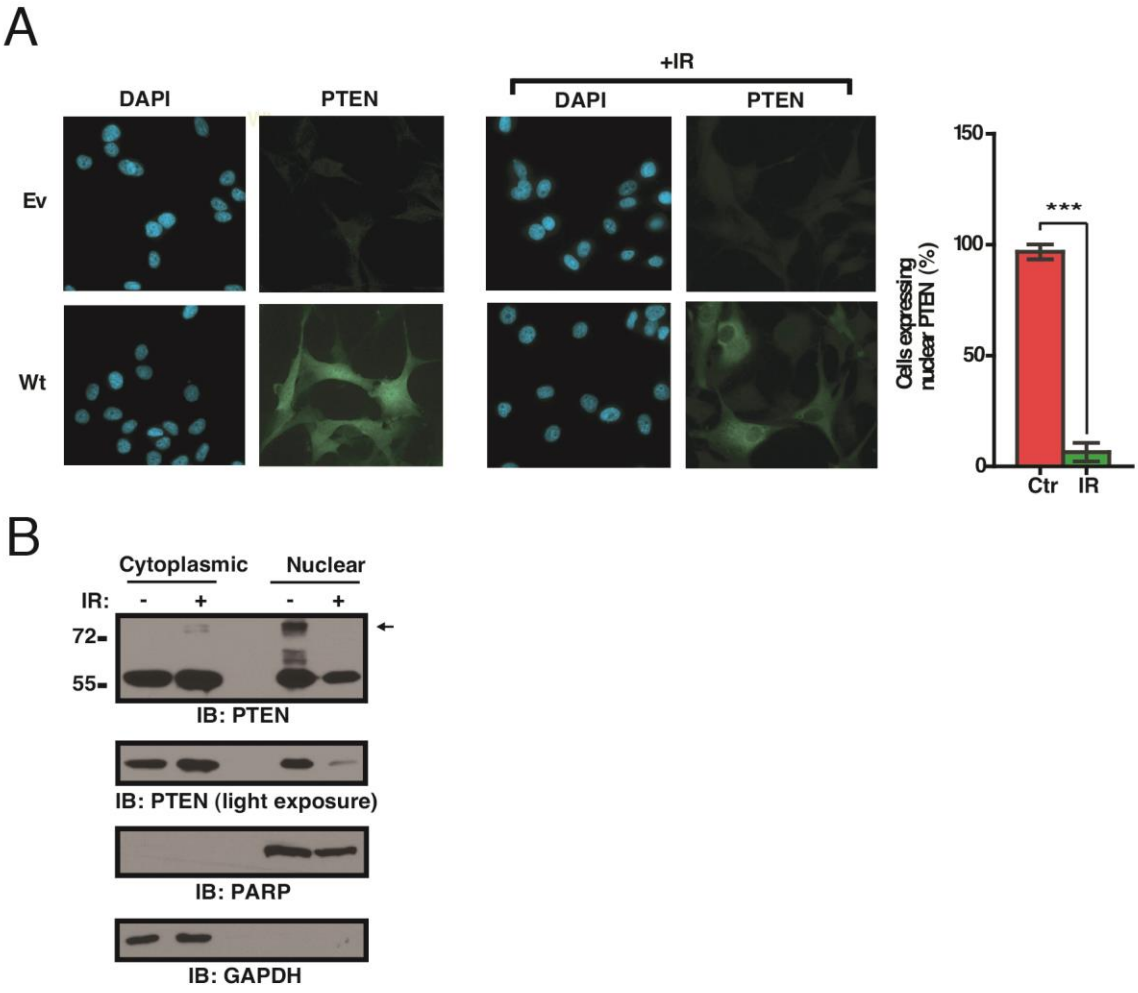

Figure 2.9 PTEN SUMOylation is sensitive to genotoxic stress. (A) Decreased nuclear PTEN localization following genotoxic stress. Immunofluorescence of PTEN-FITC U87MG cells transfected asindicated, 4 hours post-IR. Bar graph represents percentage of cells with nuclear PTEN in controlor IR-treated cells ($p<0.001$, t test, n=5, bars indicate SEM). (B) Decreased nuclear PTEN following genotoxic stress. HeLa cells were treated as indicated, harvested after 4 hours and separated into cytoplasmic and nuclear fractions followed by immunoblotting for PTEN. Fractionation was monitored by immunoblotting for PARP (nuclear protein) and GAPDH (cytoplasmic protein).Arrow indicates SUMO-PTEN.

G129E) activity, or the empty vector control (see Figure 2.7a for expression levels). We monitored the formation of 53BP1 foci, a reliable measure of exposed DNA ends routinely used to monitor the efficiency of DSB repair, by immunofluorescence at different times following genotoxic stress (Figure 2.10a, b). Within 4 hours following IR exposure, comparable levels of 53BP1 foci were visible in all cell lines, indicating the presence of double strand breaks (DSBs). By 24 hours, however, 53BP1 foci had largely resolved in Wt PTEN-reconstituted cells, whereas they remained visible in PTEN-deficient cells. Remarkably, PTEN K254R-reconstituted cells failed to clear 53BP1 foci within the same period, indicative of deficient DNA repair (Figure 2.10b). Intriguingly, PTEN lipid phosphatase activity was dispensable, whereas PTEN protein phosphatase activity was essential for the DDR (Figure 2.10a, b). The involvement of PTEN in the DDR was also investigated in HCT116 cells, their isogenic variants with their *PTEN* gene disrupted (HCT116 *PTEN*$^{-/-}$) (*247*), or the same cells reconstituted with PTEN, PTEN-K254R, -C124S and -G129E, respectively, expressed at levels comparable to the endogenous PTEN levels in parental HCT116 cells using retroviruses (see Figure 2.10c for expression levels). Again, while the Wt PTEN and PTEN-G129E-expressing cells resolved their 53BP1 foci, PTEN-null cells and those containing PTEN K254R and PTEN C124S were deficient in 53BP1 clearance (Figure 2.10d).

Reflecting their impaired capacity to repair DSBs, both U87MG and HCT116 cells expressing the PTEN SUMOylation mutant (PTEN K254R), or the protein phosphatase activity defective mutant (PTEN C124S) were deficient in resolving γH2AX and BRCA1 foci (Figure 2.11a, b, c and d). γH2AX localization to DSBs

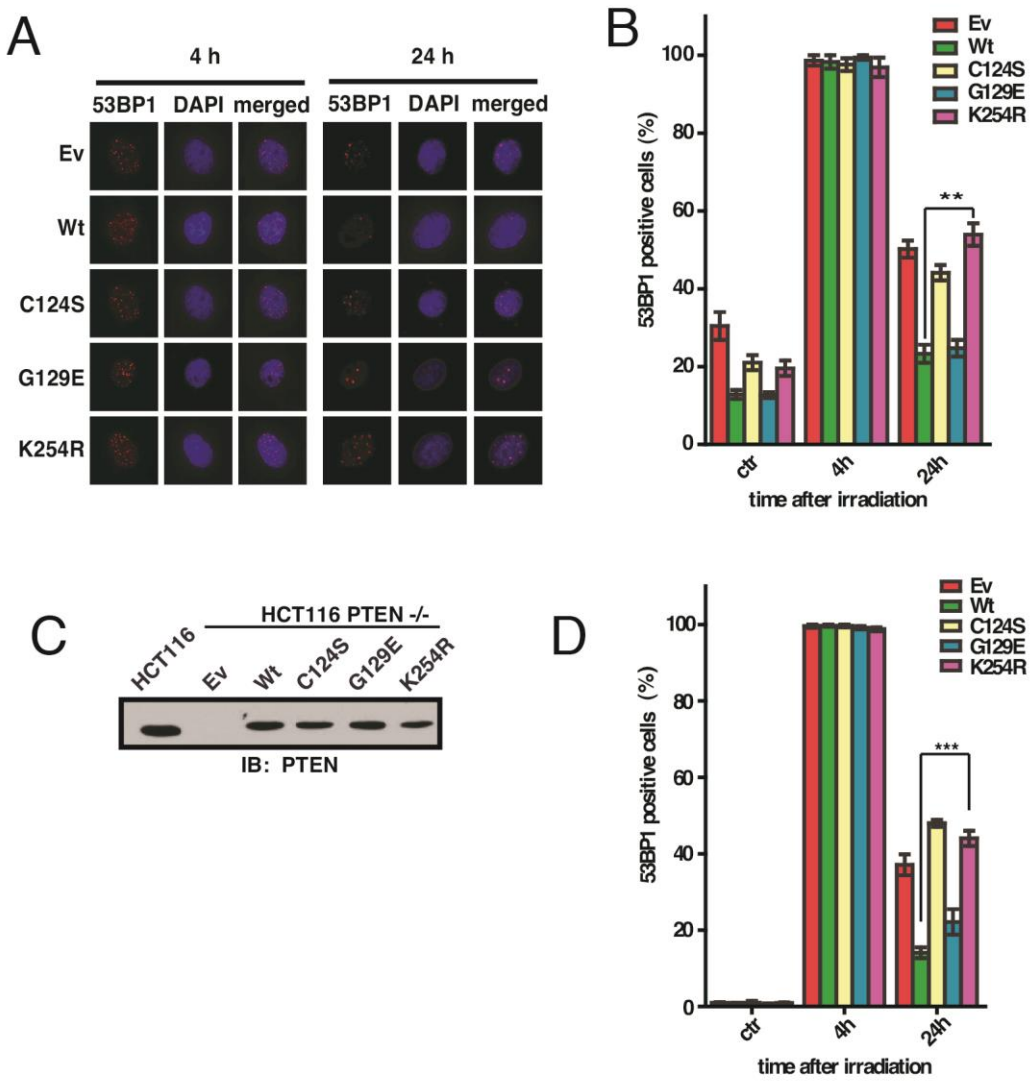

Fig. 2.10. PTEN SUMOylation is required for the resolution of 53BP1 foci. U87MG cells were reconstituted with the indicated proteins. Cells treated with IR (5 Gy) were immunostained at the indicated times with an antibody to 53BP1. (B) Cells containing >5 foci were scored as positive. Bars indicate SEM. (p=0.0014, t test, n=3). (C) HCT116 PTEN-/- cells were transduced with retroviruses carrying empty vector or PTEN-wt, -C124S, -G129E, -K254R or S398A mutants and compared to HCT116 parental cells by immunoblotting with anti-PTEN rabbit monoclonal 138G6 antibody (CST #9559). (B) 53BP1 foci in reconstituted HCT116 cells were analyzed as in B (p=0.002, t test, n=3)

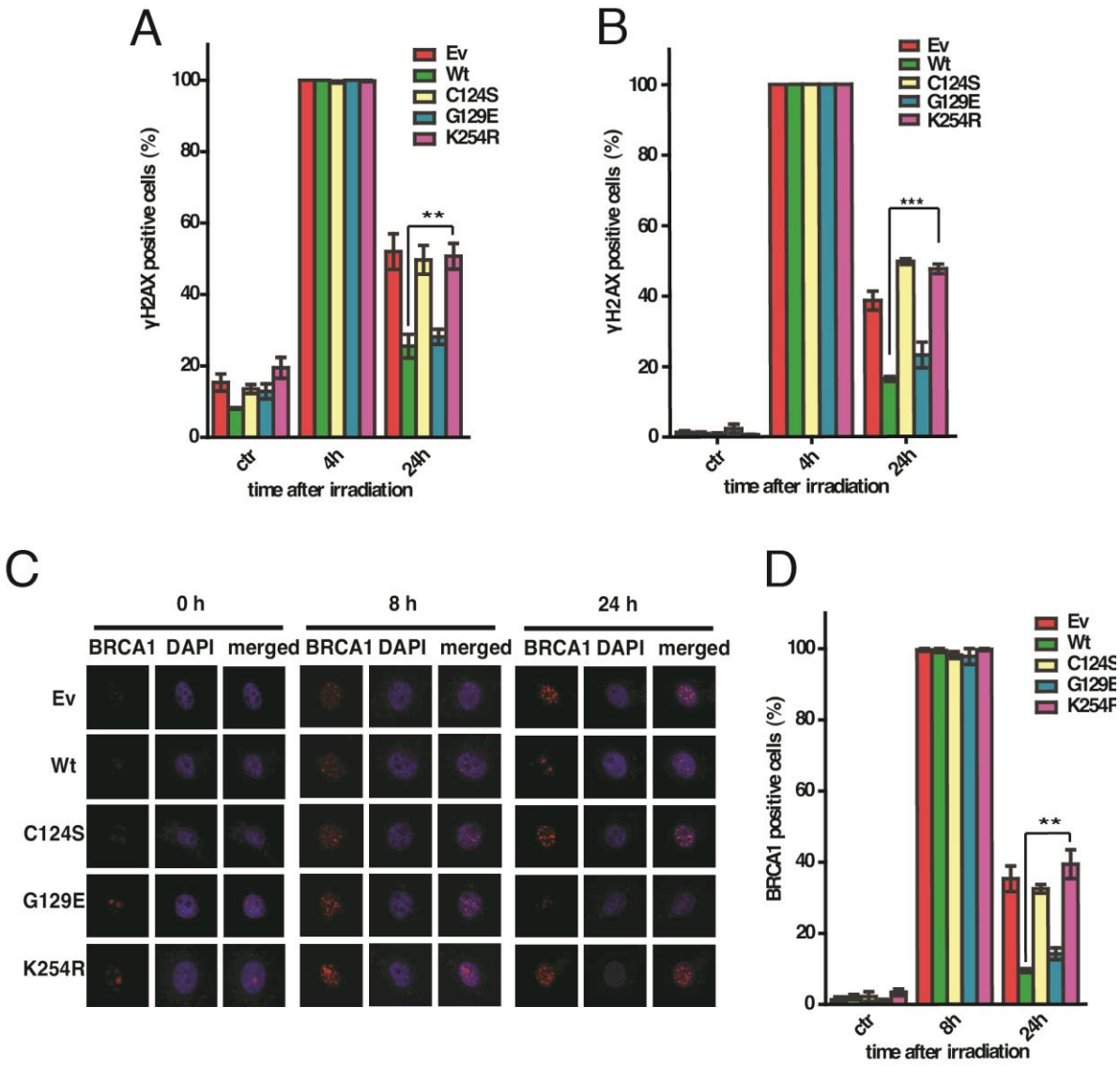

Figure 2.11. PTEN SUMOylation is required for the resolution of γH2AX and BRCA1 foci. (A) (C) (D) U87MG and (B) HCT116 cells were treated with IR (5 Gy) and immunostained at the indicated times with an antibody to γH2AX (A) (B) or BRCA1 (C) (D). Cells containing >5 foci were scored as positive. Bars indicate SEM. γH2AX foci (A) ($p=0.007$, t test, n=3) (B) ($p<0.001$, t test, n=3) and BRCA1 foci (D) ($p=0.0136$, t test, n=3)

precedes 53BP1 recruitment, whereas BRCA1, like 53BP1, plays a role in chromatin remodeling but is recruited independently to sites of damage (*248, 249*). Importantly, equal recruitment of all three markers early following DNA damage (γH2AX and 53BP1 at 4 hours and BRCA1 at 8 hours) indicates intact recognition of DNA breaks by all cell lines, independent of PTEN status (Figure 2.11a, b, c and d).

Following recognition of DSBs, DNA repair pathways are activated, resulting in the assembly of multi-protein complexes at sites of DNA damage (*250*). RAD51 is a key molecule involved in DNA repair by homologous recombination (HR) (*251*). Indicative of HR-based repair deficiency, PTEN-null HCT116 and U87MG cells, as well as their PTEN K254R- and PTEN C124S-expressing counterparts, failed to recruit RAD51 to the sites of DNA damage (Figure 2.12a, b and c). Of note, although PTEN was reported to act as co-factor for the transcription factor E2F1 to regulate the expression of RAD51 (*105*), consistent with a more recent report (*252*), we found no evidence that PTEN status governs *RAD51* mRNA or protein levels in the absence or presence of DNA damage (Figure 2.12d, e and f).

To distinguish the importance of nuclear localization *per se* versus SUMOylation for PTEN function in the response to DSBs, we fused a nuclear localization sequence from SV40 Large T antigen (*253*) to the non-SUMOylatable PTEN mutant (NLS-PTEN K254R). Despite constitutively localizing to the nucleus (Figure 2.13a), this non-SUMOylated PTEN protein (Figure 2.13b) could not rescue the impaired response of U87MG cells to IR, as exemplified by defective recruitment of RAD51 to sites of DNA damage (Figure 2.13c) and an inability to resolve γH2AX foci (Figure

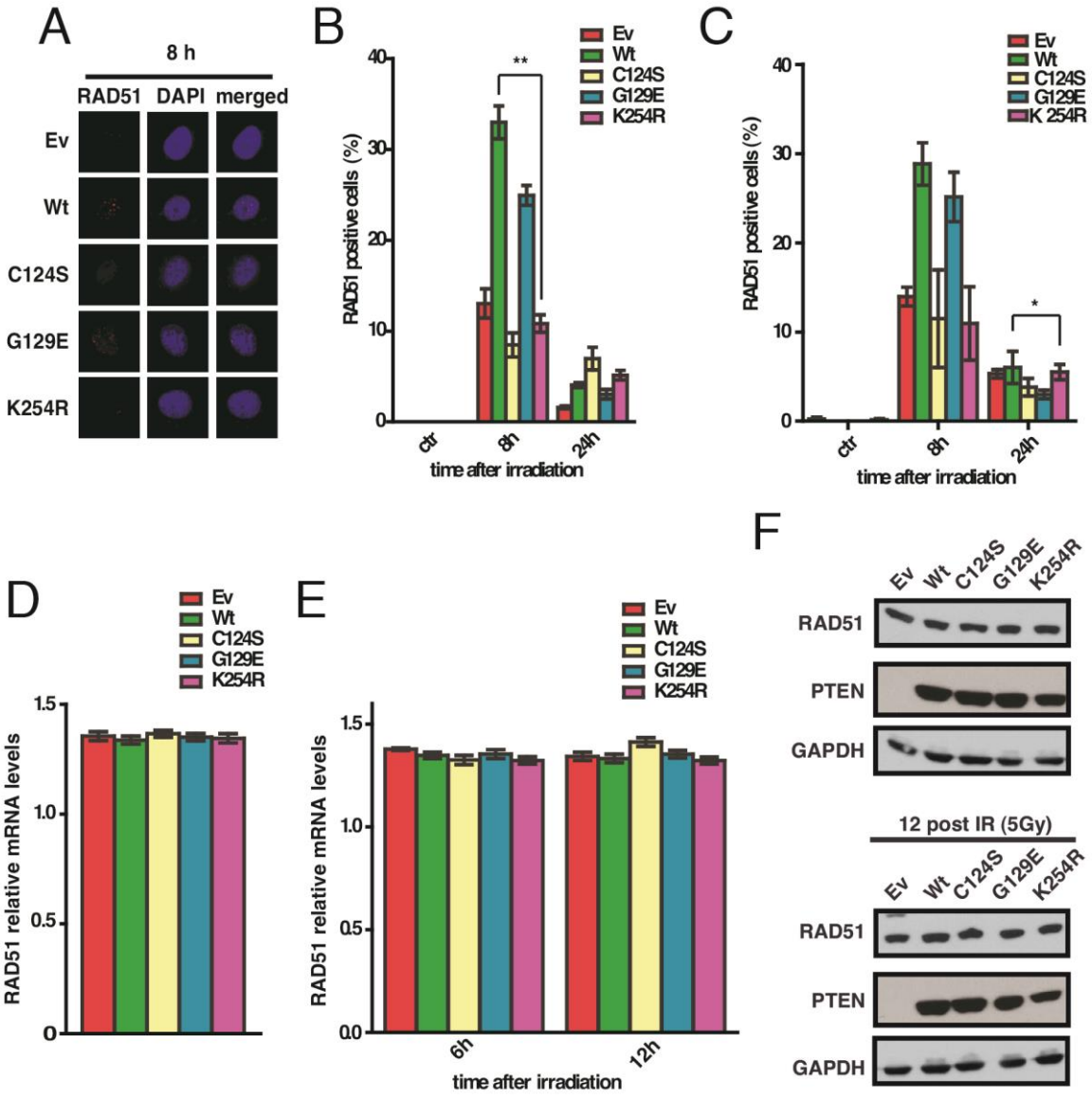

Figure 2.12 Nuclear PTEN is required for RAD51 focus formation but does not affect RAD51 expression. (A) (B) U87MG and (C) HCT116 cells were treated with IR (5 Gy) and immunostained at the indicated times with an antibody to RAD51. Cells containing >5 foci were scored as positive. Bars indicate SEM. (B) (p=0.0016, t test, n=3) (B) (p=0.0196, t test, n=3) (D) qRT-PCR analysis of RAD51 mRNA levels in U87MG cells reconstituted with the indicated FLAG-PTEN proteins. Values are shown relative to human ribosomal protein S9 (housekeeping) (E) qRT-PCR analysis of RAD51 mRNA levels at 6 and 12 hours following 3 Gy IR in U87MG cells reconstituted with the indicated FLAG-PTEN proteins. Data is represented as in (D). (F) Immunoblot of Rad51 in U87MG cells reconstituted with FLAG-PTEN proteins following exposure to 5 Gy IR.

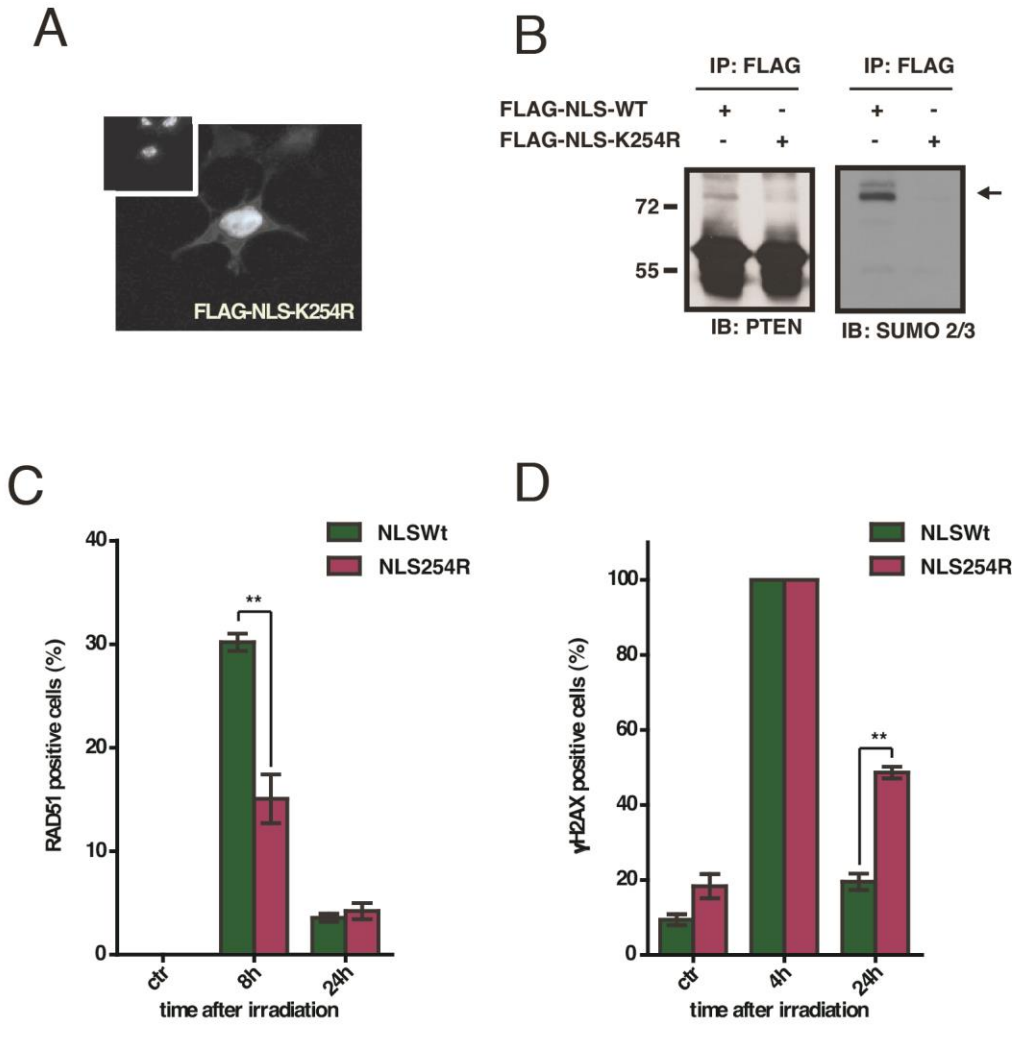

Figure 2.13 NLS-K254R-PTEN cannot rescue the repair defect in PTEN-null cells. (A) NLSK254R-PTEN localizes to the nucleus. Flag-FITC immuno_uorescence images of HEK293 cells transfected with FLAG-NLS-K254R-PTEN. Inset shows DAPI staining. (B) NLS-K254R-PTEN is not SUMOylated. HEK293 cells were transfected with FLAG-NLS-PTEN-WT or FLAG-NLS-K254R, together with His-SUMO2 and myc-His-Ubc9. Immunoprecipitations were performed using M2-Flag agarose, followed by immunoblotting for PTEN (6H2.1). (C and D) U87MG cells were reconstituted with FLAG-NLS-PTEN or FLAG-NLS-K254R-PTEN. Cells were exposed or not (control) to 5 Gy of ionizing radiation, immunostained at the indicated times post-IR for γH2AX or RAD51. DAPI was used to stain DNA. (C) PTEN SUMOylation is required for RAD51 foci formation. Quanti_cation of RAD51-positive cells. (D) SUMOylation of PTEN is required for resolution of γH2AX foci. Cells containing >5 γH2AX foci were scored as positive and counted.

2.13d). Thus, PTEN SUMOylation is required for PTEN's function in the DDR. Of Note, reconstitution of U87MG cells with Wt PTEN or various PTEN mutants did not yield changes in cell cycle distribution (Figure 2.14a) or the engagement of cell cycle checkpoints following IR (Figure 2.14b), indicating that DNA repair deficiency in these cells were not secondary consequences of potential effects of PTEN on the cell cycle.

We also assessed the ability of PTEN mutant-expressing U87MG cells and cells derived from a mouse mammary tumor with a conditional PTEN gene disruption (WAP-Cre *PTEN*$^{-/-}$ MMTC) to repair a stably integrated, DR-GFP plasmid-based reporter, which provides a direct and quantitative measure of HR-mediated DSB repair (*254*). Consistent with their defect in Rad51 focus formation, cells expressing PTEN K254R or PTEN C124S were unable to efficiently repair the reporter (Figure 2.15a and b).

2.3.4. Lack of SUMO-PTEN confers cells sensitivity to genotoxic agents

Next, we monitored the effects of SUMO-defective PTEN on radiosensitivity, by scoring the surviving fraction 5 days after IR exposure, of U87MG cells and *PTEN*$^{-/-}$ MMTC engineered to express PTEN variants (*255*) (*139*). Remarkably, despite their considerable DSB repair defects, in both models, the survival of PTEN-null cells was indistinguishable from that of Wt PTEN-expressing cells (Figure 2.15C and d). This likely reflects the activation of PI3K-mediated cell survival signaling in cells lacking PTEN (see Discussion). In marked contrast, however, PTEN K254R-

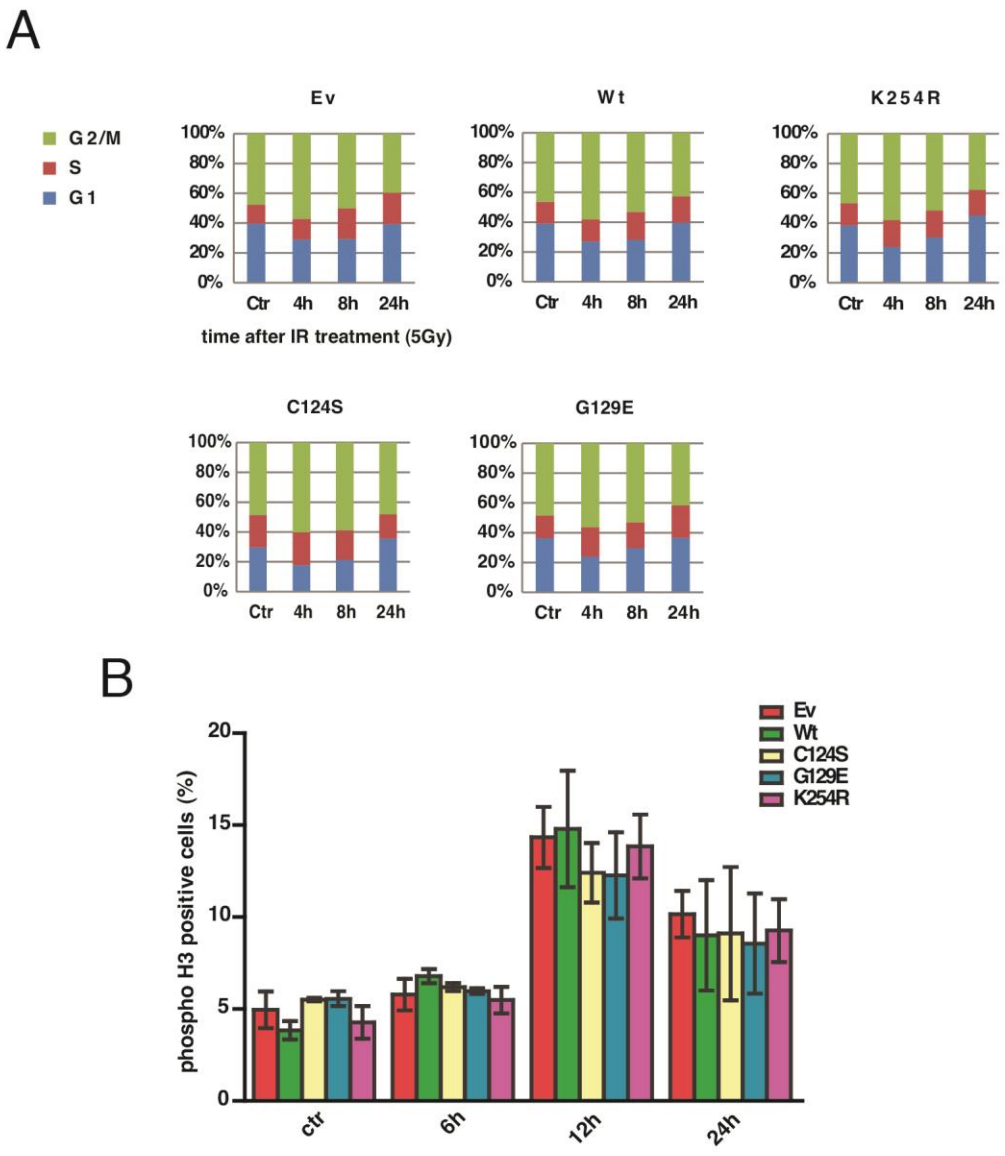

Figure 2.14 PTEN-K254R does not influence the DDR checkpoint. U87MG cells reconstituted with the indicated FLAG-PTEN proteins were exposed to IR and fixed at the indicated time points. (A) Cells were stained with propidium iodide (PI) and analyzed for DNA content. DNA pro_les were analyzed using FLowJo and divided into G1, S and G2/M fractions using the following criteria: 2N = G1, 2N < S < 4N and 4N = G2/M. (B) Cells were stained with an antibody specific against the mitotic marker phosphor-Histone H3 (Ser10) and analyzed by flow cytometry.

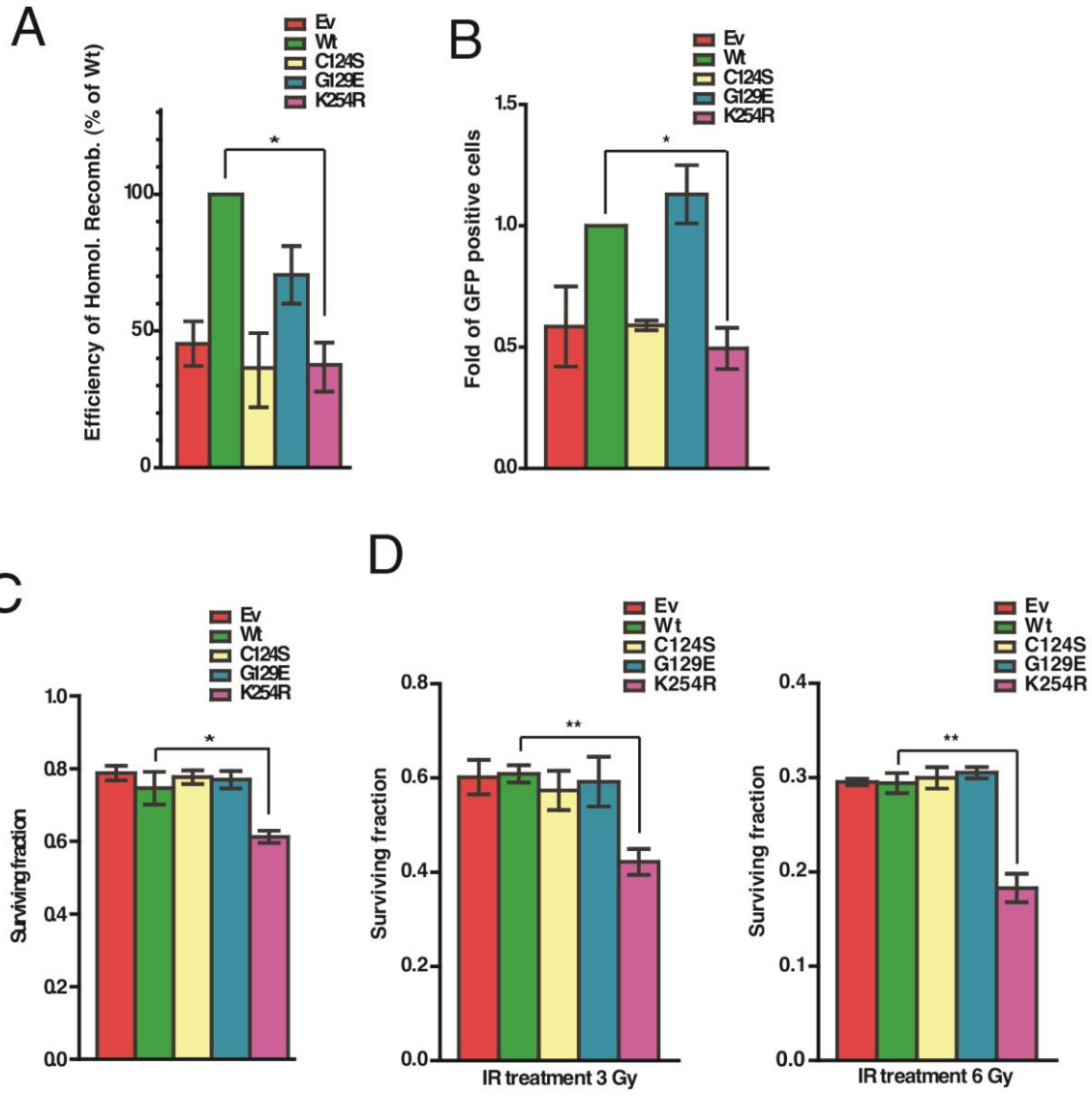

Figure 2.15 Requirement of nuclear SUMO-PTEN for homologous recombination repair of DNA double-strand breaks. (A) Impaired homologous recombination-based DNA repair in PTEN- and SUMO-PTEN-deficient cells. U87MG cells stably expressing a DR-GFP reporter were reconstituted with the indicated PTEN proteins and their HR-mediated repair assessed as previously described (228). HR efficiency is expressed relative to that of wt. (p=0.012, t test, n=2, bars indicate SEM). (B) DR-GFP was stably integrated into MMTC cells. These cells were transduced with retrovirus for empty vector or PTEN-wt, -C124S, -G129E or -K254R mutants, and HR efficiency assesed as in A (C) Lack of SUMO-PTEN increases radiosensitivity. Surviving fraction was determined by Sulforhodamine B staining 6 days following exposure to 3 Gy of IR (p=0.02, t test, n=3, bars indicate SEM). (D) MMTC reconstituted with the indicated PTEN proteins were treated with 3 Gy (p=0.0049, t test, n=3) or 6 Gy (p=0.039, t test, n=3) of ionizing radiation, and the surviving fraction was measured as described in C.

expressing cells exhibited decreased survival following IR exposure (Figure 2.15c and d).

2.3.5. ATM phosphorylates serine 398 of PTEN and regulates SUMO-PTEN abundance and its nuclear localization

To gain further insight into nuclear SUMO-PTEN's involvement in the genotoxic stress response, we monitored its abundance under various conditions. Serum starvation, stimulation with serum or with specific growth factors had no effect on SUMO-PTEN (data not shown). However, IR led to a gradual reduction in SUMO-PTEN levels beginning 1 hour after IR exposure, followed by restoration of steady state levels 8 hours later (Figure 2.16a). Other forms of genotoxic stress, such as treatment with Cisplatin or Doxorubicin, also led to decreased SUMO-PTEN, which reached its lowest level 48 hours after treatment, consistent with a DNA replication-dependent mechanism and the timing of accumulation of DNA damage elicited by these agents (Figure 2.16b).

Upon DNA damage, the protein kinases ATM and ATR phosphorylate multiple targets implicated in the response to this insult (*256*). Inhibition of ATM by treatment with KU55933 significantly impaired SUMO-PTEN turnover in response to ionizing radiation (Figure 2.17a), implicating this kinase in SUMO-PTEN regulation and linking these two major tumor suppressor gene products. To ask if ATM can phosphorylate PTEN directly, the kinase activity of ATM immunoprecipitated from γ-irradiated cells toward PTEN was tested *in vitro* (Figure 2.17b). ATM phosphorylated GST-PTEN to a similar extent as it did p53, a known ATM substrate

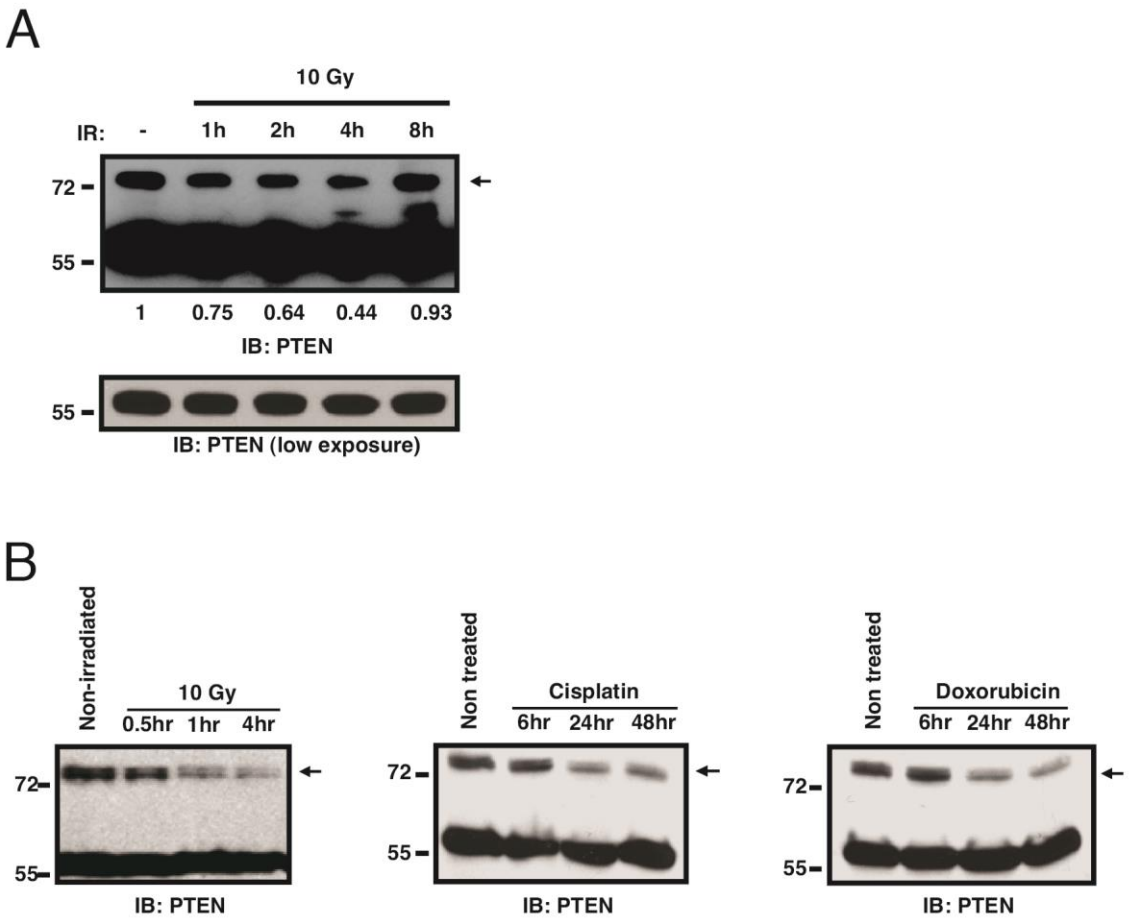

Figure 2.16 Sumo-PTEN turnover following genotoxic stress. (A) Decreased SUMOPTEN after DNA damage. Lysates from HeLa cells treated as indicated were immunoblotted for PTEN. A ratio of SUMO-PTEN to PTEN immunoreactivity relative to the nonirradiated control is indicated. (B) HEK293 cells were treated with ionizing radiation (IR, 10 Gy), Cisplatin (10uM) or Doxorubicin (1uM), and whole cell lysates prepared at indicated times were immunoblotted using PTEN 6H2.1 antibodies.

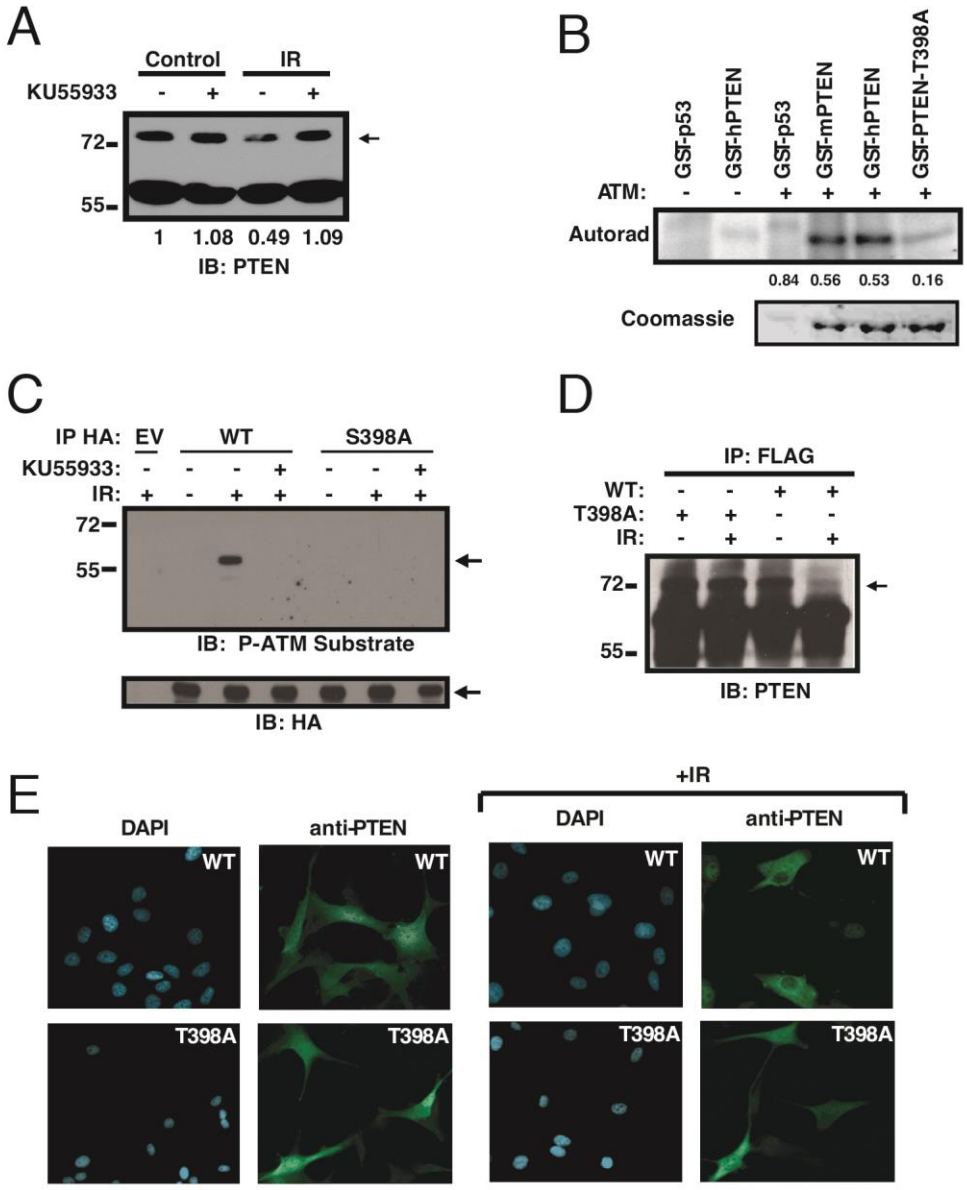

Figure 2.17 PTEN is part of a ATM-regulated signaling cascade. (A) ATM Regulation of SUMO-PTEN abundance by ATM. Lysates from HeLa cells treated as indicated were immunoblotted for PTEN (B) ATM phosphorylates serine/threonine 398 of PTEN in vitro. The indicated GST-fusion proteins were incubated with active ATM in the presence of 32P-ATP. 32P incorporation was quantified by PhosphorImager and normalized to protein loading. (C) Phosphorylation of PTEN S398 in vivo by ATM. HA immunoprecipitates from HEK293 cells transfected and treated as indicated, were immunoblotted with antibody against phospho-ATM substrates (CST #9607). (D) SUMO-PTEN-T398A is not sensitive to IR. Flag immunoprecipitates from HEK293 cells transfected and treated as indicated were immunoblotted for PTEN. (E) PTEN-T398A remains in the nucleus after IR. Cells were treated and imaged as in Fig. 2.8.

(Figure 2.17b). Inspection of the PTEN amino acid sequence for predicted ATM phosphorylation sites identified threonine 398 of human PTEN (serine 398 in mouse PTEN) as a candidate (*257*). Indeed, mutation of this residue to alanine (PTEN T/S398A) largely precluded PTEN phosphorylation by ATM (Figure 2.17c). To examine PTEN S/T398 phosphorylation *in vivo*, epitope tagged Wt mouse PTEN or PTEN S/T398A were immunoprecipitated from transfected HEK293 cells exposed to IR, with or without pretreatment with KU55933 and immunoblotted with an anti-ATM substrate antibody (CST #9607). While Wt PTEN was readily phosphorylated at an ATM site following IR, this was precluded both by the treatment with the ATM inhibitor and the S/T398A substitution (Figure 2.17c) establishing PTEN S/T398 as a *bona fide* ATM phosphorylation site within PTEN. Significantly, unlike Wt SUMO-PTEN, SUMO-PTEN S/T398A was resistant to IR-induced turnover (Figure 2.17d) and was not excluded from the nucleus following IR (Figure 2.17e), consistent with ATM involvement in nuclear SUMO-PTEN regulation.

2.4. PTEN-null cells are sensitive to the combination of DNA damaging agents and PI3K inhibitors *in vitro* and *in vivo*

Taken together, our data establish a nuclear function for PTEN in the DNA damage response, distinct from its regulation of PI3K signaling in the cytoplasm. Considering that the most commonly found PTEN alterations in human tumors lead to a complete loss of PTEN protein and thus both its cytoplasmic and nuclear functions, we compared the sensitivity of U87MG cells reconstituted with either

empty vector control or Wt PTEN, or the isogenic HCT116 and HCT116 *PTEN*$^{-/-}$ cells to BKM-120, a small molecule pan-PI3K inhibitor, IR (2 Gy), or the combination thereof, at doses found to produce minimal toxicity when administered alone (Figure 2.18a, c). Significantly, PTEN-deficient cells displayed increased sensitivity to IR/BKM-120 combination compared to cells expressing Wt PTEN. Consistently, a combination of BKM-120 with the genotoxic agent cisplatin produced the same effect in HCT116 *PTEN*$^{-/-}$ cells (Figure 2.18b, c) raising the possibility that combined action of equivalent clinical agents may have efficacy in treatment of PTEN-deficient tumors. To test this *in vivo*, immunocompromised mice carrying HCT116 and HCT116 *PTEN*$^{-/-}$ xenografts were challenged with a regimen of a single dose of cisplatin, daily BKM-120 for 15 days or the combination thereof (Figure 2.19). While either of the drugs alone had limited effect on the growth of the tumors (<30% tumor growth inhibition (TGI) over the course of the treatment), the combination treatment dramatically reduced the growth of HCT116 *PTEN*$^{-/-}$ xenografts (>90% TGI), but not their PTEN-proficient counterparts (Figure 2.19), further indicative of the synthetic sensitivity of PTEN-deficient cells to the combined action of DNA-damaging agents and PI3K pathway inhibitors.

2.5. Discussion

Accumulating evidence points to a key role for SUMOylation in coordinating the cellular response to DSBs (*231, 232*). Following DNA damage, SUMOs, UBC9, and the E3 ligases PIAS1 and PIAS2 localize to sites flanking DSBs, where they

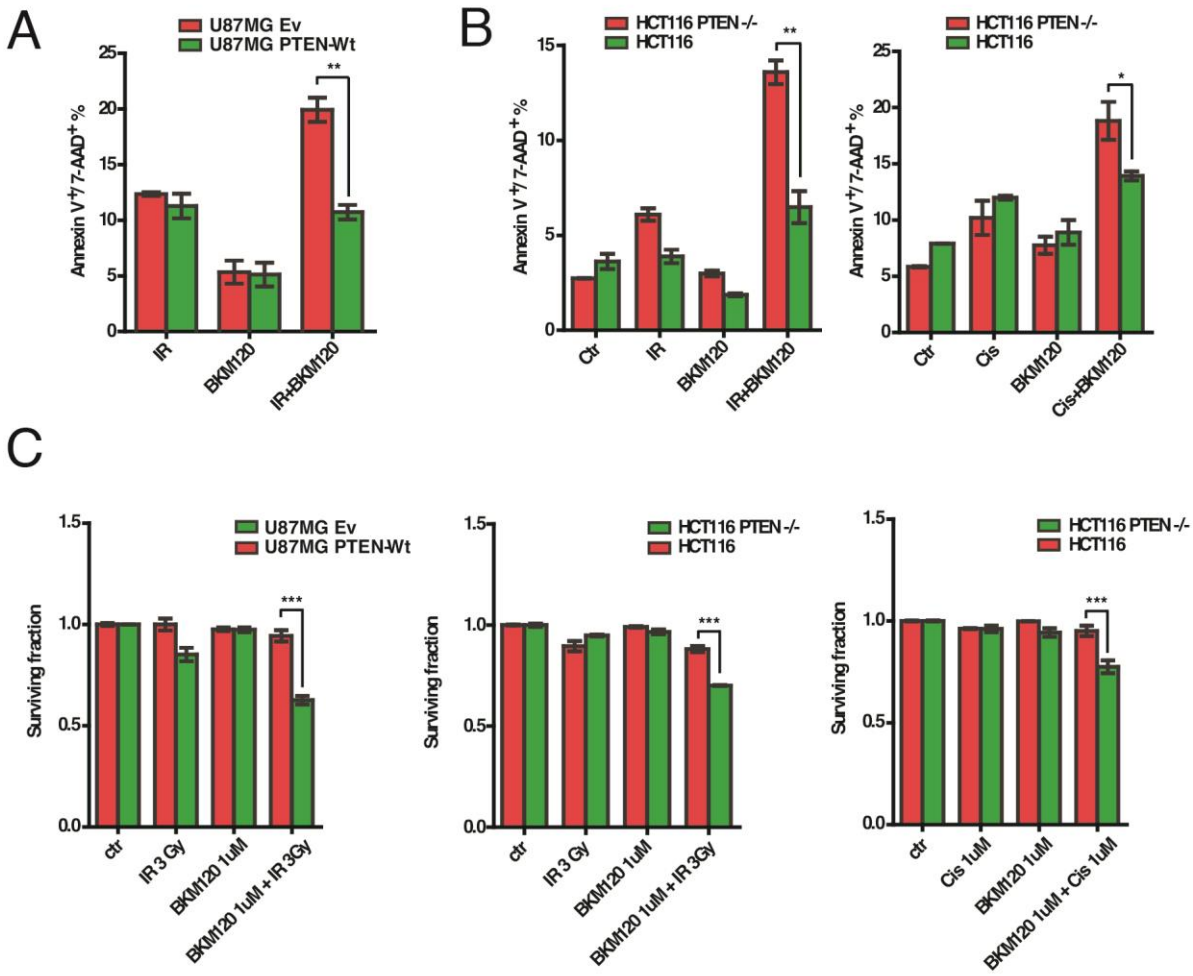

Figure 2.18 PTEN loss sensitizes cells to combination treatment with IR and a pan-PI3K inhibitor. Bars indicate SEM. (A) U87MG cells reconstituted with empty vector or PTEN-wt were treated with either 2 Gy of IR, 500 nM BKM120 or both, and apoptosis was measured by annexin V+/7-AAD+ staining ($p=0.0019$, t test, n=3). (B) HCT116 PTEN-null and parental PTEN-WT cells were treated with either IR (2 Gy) or 1 mM cisplatin alone or in combination with 500 nM BKM120, as indicated. Apoptosis was measured as in (A) (IR+BKM120 $p=0.0025$, t test, n=3) (Cis+BKM120 $p=0.0475$, t test, n=3). (C) Surviving fraction 3 days following indicated treatments. Cells, U87MG reconstituted with empty vector or PTEN-wt and HCT116 PTEN-null and isogenic PTEN-WT cells were treated as indicated, fixed with TCA and stained with Sulforhodamine B. The incorporated dye was solubilized and absorbance measured at 565 nm. Bars indicate SEM. (for all the experiments, $p<0.001$, 2 way Anova, n=3)

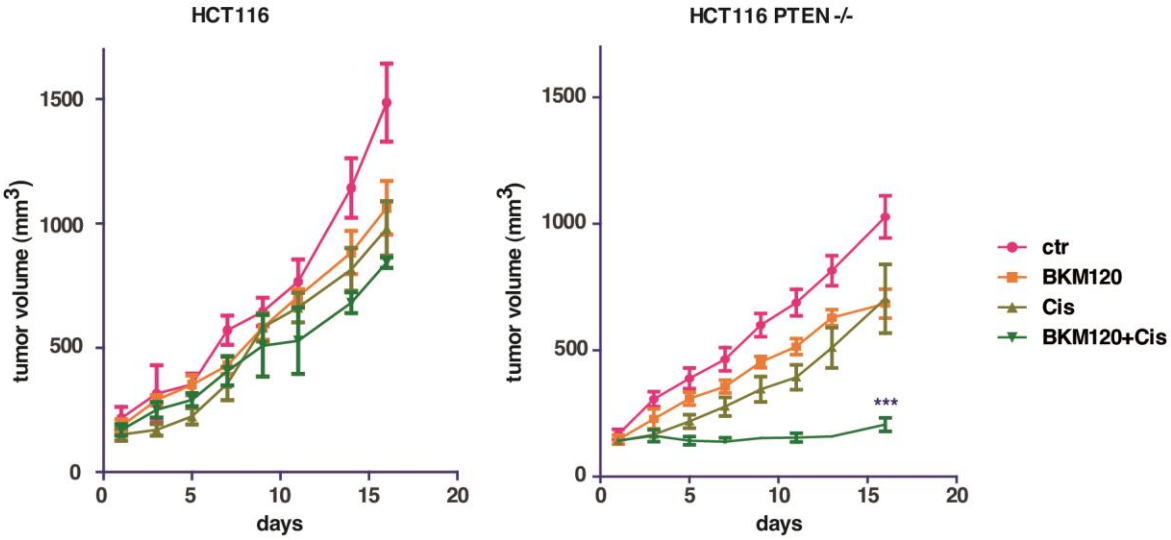

Figure 2.19 PTEN-deficient cells are sensitive to the Cisplatin/PI3K inhibitor combination in vivo. HCT116 parental or HCT116 PTEN-/- cells were injected subcutaneously into NOD/SCID mice (n=10). Mice were treated with cisplatin, BKM120, or both. Data points represent mean tumor volume ± SEM. Tumor growth difference was measured between day 1 and 16 and one way ANOVA was performed ($p=0.0321$ for HCT116 parental and $p<0.0001$ for HCT116 PTEN-/-) followed by Dunnet's multiple comparison test (HCT116 PTEN-/- BKM120 + Cisplatin vs Ctr, $p<0.0001$).

target several proteins involved in the DDR, including BRCA1, 53BP1 and RNF8 (*231, 232, 258*). Several components of the DDR machinery, such as BRCA1, RAD51 and RAD52, also can interact with SUMO moieties on proteins via SUMO-interacting motifs (SIMs) (*259-261*). We discovered that PTEN is SUMOylated at K254 and that mutation of lysine 254 is important for nuclear localization of PTEN. However, SUMOylation appears to be also directly involved in PTEN's function in DNA repair. As shown by the expression of a nucleus-restricted PTEN K254R mutant (NLS-PTEN K254R), forced nuclear localization is not sufficient to induce SUMOylation, nor can NLS-PTEN K254R expression rescue the defect in DNA damage response of PTEN-deficient cells. Moreover, the acute decrease in nuclear SUMO-PTEN upon DNA damage was followed by restoration of nuclear SUMO-PTEN levels within 8 hours, a time consistent with the assembly of the DNA repair machinery (*250*). Nuclear SUMO-PTEN turnover in response to DNA damage was fully ATM-dependent and required PTEN T398, which is phosphorylated by ATM *in vitro* and *in vivo*, establishing a functional link between these two major tumor suppressors. Finally, PTEN protein, but not lipid phosphatase activity was necessary for the DNA damage response.

Considering the requirement of nuclear SUMO-PTEN for efficient HR-mediated DSB repair, further investigations into the PTEN-dependent dynamics of the assembly of HR-mediated DSB repair complexes should lead to the identification of SUMO-PTEN interaction partners and/or targets of its protein phosphatase activity necessary for the efficient DNA damage response.

A recent report identified PTEN SUMOylation at another site, K266, as a means of plasma membrane localization, where PTEN acts to downregulate the PI3K pathway (*67*). Although our mass spectrometry-based assessment did not identify K266 as a PTEN SUMOylation site under the conditions tested, such findings raise the possibility that PTEN SUMOylation on different sites may control its localization to distinct sub-cellular compartments thus governing PTEN's complex functions. Fnls

Our data show that the ATM-nuclear SUMO-PTEN axis impacts cellular sensitivity to genotoxic stress both *in vitro* and *in vivo*. Notably, missense mutations at PTEN K254 or the surrounding residues, which are predicted to abolish SUMOylation, have been identified at low frequency in various human cancers (Cosmic Database, Sanger Institute), providing strong evidence for the importance of this pathway in tumorigenesis. Nevertheless, the influence of PTEN on the cytotoxic effects of DNA damage also likely involves its function in regulating PI3K-mediated cell growth and survival via its cytoplasmic function (*50*). This is best exemplified by the comparable resistance of PTEN-deficient (PTEN-null, or expressing PTEN-C124S) and PTEN-proficient cells to high doses of DNA damaging agents, in contrast to the sensitivity of cells expressing PTEN-K254R, a mutant fully capable of countering PI3K signaling to the same insult. Although PTEN-proficient cells effectively repair their genomes and continue to grow, PTEN-deficient cells fail to execute an apoptotic program due to the constitutive activation of PI3K/Akt survival signaling, in the absence of cytoplasmic PTEN or upon loss of PTEN phosphatase activity, and survive, despite accumulating irreparable DNA

damage (*35*) (Figure 2.20). On the other hand, cells deficient for nuclear, but not cytoplasmic PTEN function (PTEN-K254R), respond to accumulated DNA damage

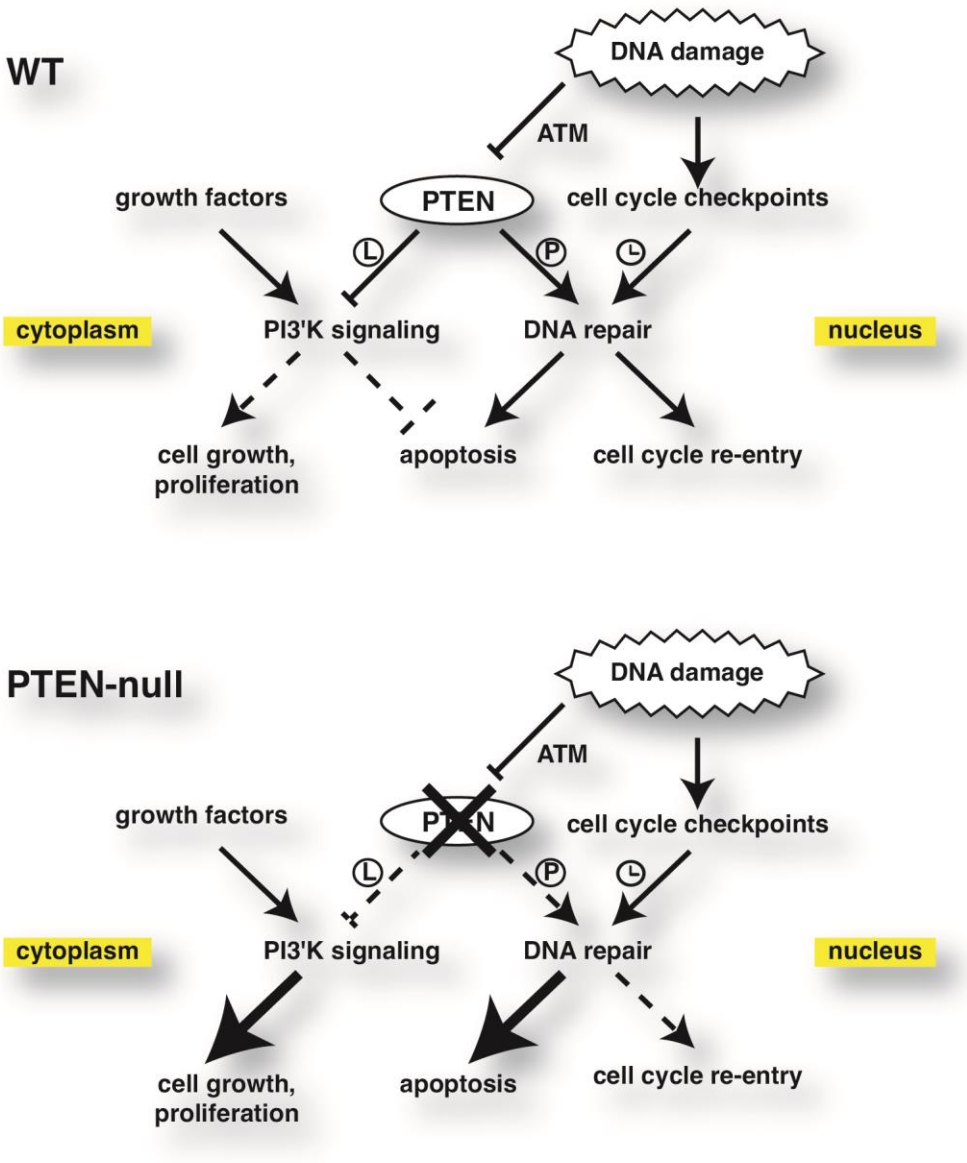

Figure 2.20 Schematic of PTEN effect on cell response to DNA damage.

by physiological activation of apoptosis, contributing to their sensitivity to genotoxic stress.

Importantly, our findings imply that tumors bearing complete loss of PTEN function, which represent the majority of human tumors with PTEN alterations, may be particularly sensitive to the combined action of therapeutics targeting activated PI3K signaling and genomic instability of tumor cells, respectively. Indeed, unlike cells expressing Wt PTEN, PTEN-deficient cells displayed sensitivity to the combined action of IR and a small molecule pan-PI3K inhibitor both *in vitro* and *in vivo*. The selective susceptibility of PTEN-deficient cells to this combination indicates the existence of a therapeutic window for treatment of PTEN-null tumors using such a strategy. By contrast, our results suggest that administering genotoxic agents alone to such tumors could accelerate the acquisition of additional mutations, including those promoting drug resistance or tumor progression. The ongoing clinical development of numerous agents countering activated PI3K signaling in cancer (*262, 263*) and next generation genotoxic agents (*264*) should facilitate testing of these concepts in the clinic.

2.6. Materials and methods

2.6.1. Antibodies

The following antibodies were purchased from Cell Signaling Technology: PTEN (138G6) (#9559), PTEN (#9552), SUMO-2/3 (18H8) (#4971), Ubc9 (#4918), PARP (#9542), Akt (#9272), phospho-Akt (Ser473) (193H12) (#4058), phospho-ATM substrate (#9607) and phospho-GSK-3β (Ser9) (#9336). Histone H3 (ab1971) and phospho-ATM (Ser1981) (ab2888) antibodies were from Abcam. FLAG M2-FITC (F4049), FLAG M2 (F3165) and FLAG M2 agarose (A2220) were from Sigma-Aldrich. GAPDH FL-335 (sc-25778), Rad51 H-92 (sc-8349) and BRCA1 I-20 (sc-646) antibodies were from Santa Cruz Biotechnology. PTEN antibody (6H2.1) (ABM-2052) was from Cascade Bioscience. Phospho-Histone H2A.X (Ser139) (JBW301) (05-636) was from Millipore. Antibody against 53BP1 (A300-272A) was from Bethyl Laboratories. ATM Ab-3 (PC116) was from Calbiochem. Anti-HA was produced in-house from the 12CA5 hybridoma.

2.6.2. Cell culture, transfections, viral infections and reagents

HEK293 cells, U87MG, HCT116 PTEN-null, isogenic PTEN Wt cells and their derivatives were maintained in DMEM, supplemented with 10% FBS (Gibco) and Pen/Strep (100 mg/ml, Hyclone). Puromycin (1 μg/ml) was from Calbiochem. The mouse mammary tumor (MMTC) cell line was cultured in DMEM/F-12 (1:1), supplemented with 10% FBS (Gibco), Pen/Strep (100 mg/ml), insulin (5 μg/ml), hydrocortisone (1 μg/ml) (all from Sigma), and EGF (5 ng/ml, Peprotech).

Transient transfections of HEK293 cells were performed by using Lipofectamine (Invitrogen) or the $CaCl_2$ and Hepes method, as described previously. High efficiency transfection (>90%) of U87MG cells for the homologous recombination assay was achieved using the Amaxa Cell Line Nucleofector Kit T (Lonza, VCA-1002), according to manufacturer's instructions.

All plasmids used were constructed with the exception of plasmids encoding N-terminally His-tagged full-length SUMO1, SUMO2, and SUMO3, generously provided by Dr. Paul E. Fraser (University of Toronto), and pDR-GFP and pISceI (Addgene).

For expression in mammalian cells, *HA-PTEN* was cloned into pcDNA3.1, and *PTEN* was cloned into p3XFLAG-CMV10 and pBabe-Flag. *Ubc9* was cloned into pcDNA3.1-myc-His-A. Full-length *SENP1* and *SENP2* in pGEX4T3 were sub-cloned into pcDNA3.1-myc-His-A.

For expression of recombinant proteins in bacterial cells, full-length and truncation mutants of *PTEN* were cloned into pGEX4T3 and pET32a. *Ubc9* and catalytic fragments of *SENP1* and *SENP2* were cloned into pGEX4T3. *TP53* was cloned into pGEX2TK.

All point mutants were generated by using the QuickChange site-directed mutagenesis kit (Agilent), which also was used to insert the NLS derived from SV40 large T-antigen into p3XFLAG-CMV10-PTEN.

U87MG cells were engineered to express the ecotropic retroviral receptor by selecting a stable clone transfected with pWZL-Neo-EcoR. Viral transduction with retroviruses expressing FLAG-PTEN or mutants was performed in U87MG-EcoR

and MMBC cells by following the procedures described previously (*265*). Cells were subsequently selected with puromycin (1 μg/ml) until negative control cells died (generally 3 days). *Ubc9* siRNA was from Santa Cruz (sc-36773).

2.6.3. Cell lysis, immunoblotting and immunoprecipitations

Unless indicated otherwise, for immunoblotting cells were lysed in Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol), normalized for total protein content, resolved by SDS-PAGE, and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% nonfat milk (Bioshop Canada) and probed with the indicated antibodies.

For immunoprecipitations, cells were lysed for 30 min on ice in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA), supplemented with fresh 20 mM N-ethylmaleimide (NEM), 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate and a protease inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation at 15,000 × g for 15 min at 4 °C. Immunoprecipitations were performed by gentle rotation overnight at 4 °C, and then immune complexes were washed four times in cold RIPA buffer and resuspended in Laemmli buffer.

Immunoprecipitation of endogenous SUMO-PTEN was performed by lysing cells in Laemmli buffer, followed by sonication and centrifugation at 15,000 x *g* for 15 min at 4 °C. Clarified lysates were diluted 10-fold with PBS containing 1% Triton X-100 and the protease inhibitor cocktail, then anti-PTEN antibody was added and the mixture was allowed to rotate for 4 hours at 4 °C. Following the addition of

protein A-Sepharose and rotation for 1 hr, immune complexes were washed 4 times with PBS containing 1% Triton x-100.

2.6.4. Immunofluorescence

Cells cultured on glass cover slips were rinsed in PBS, fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, permeabilized with PBS plus 0.5% Triton X-100 for 5 min, blocked overnight at 4°C with PBS containing 1% bovine serum albumin (Fisher), and then incubated with primary antibodies (PTEN Cell signaling 9559, 1:200; RAD51 1:500; 53BP1 1:500; γH2AX 1:1000; BRCA1 1:100; FLAG-FITC 1:1000). After 3 washes with PBS, 5 min each, at room temperature, samples were incubated for 30 min with a 1:400 dilution (in PBS) of goat anti-rabbit IgG conjugated to the fluorescent Alexa 488 dye or with goat anti-mouse IgG conjugated to the fluorescent Alexa 546 dye (Invitrogen Molecular Probes), washed three times, stained with DAPI and mounted in Mowiol.

2.6.5. Cell proliferation assays

Cell number was assessed indirectly by using the Sulforhodamine B (SRB) assay (*266*).

2.6.6. Phosphorylation reactions

Cells were lysed in ATM buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.2% Tween 20, 1.5 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiothreitol, 50 mM NaF, 500 μM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml aprotinin, 0.1 μg/ml

leupeptin), cleared by centrifugation, and subjected to immunoprecipitations with PC116 ATM antibody (Calbiochem). ATM immunoprecipitates were incubated with recombinant p53 or PTEN in kinase buffer (10 mM HEPES, pH 7.4; 10 mM $MgCl_2$; 50 mM NaCl; 10 mM $MnCl_2$), supplemented with 50 µM ATP and 10 µCi [γ-^{32}P]ATP, for 15 min at 30°C (*267*).

2.6.7. Homologous recombination assays

To assess HR efficiency, U87MG cells were stably transduced with DR-GFP and various PTEN constructs, and subsequently transfected with pCMV3xnlsI-SceI, using Amaxa™ Nucleofector™ Technology (Lonza). At 2 days post-transfection, GFP signals were quantified by using a FACSCalibur flow cytometer (BD Biosciences). Recombination efficiency was calculated as the number of GFP-positive cells in the samples divided by the number of GFP-positive cells in WT-PTEN-reconstituted U87MG cells. For each experiment, 10,000 cells were scored per treatment group.

2.6.8. SUMOylation

Assays were performed with 150 ng of SAEI and SAEII (Boston Biochem), 1 µg of UBC9, 2 µl of 10× SUMOylation reaction buffer (200 mM HEPES, pH 7.5, 50 mM $MgCl_2$ and 20 mM ATP), 1 µg of SUMO1 (Boston Biochem) and 300 ng of recombinant His-THX-PTEN. Reactions were incubated at 37°C for 2 hrs, quenched with SDS–PAGE sample buffer and analysed by SDS–PAGE and immunoblotting with anti-His antibody.

2.6.9. Lipid phosphatase assays

PTEN catalytic activity was determined by using the malachite green PTEN phosphatase assay kit, using PI(3,4,5)P3 diC8 as the substrate (Echelon Bioscence).

2.6.10. In vitro deSUMOylation

FLAG-PTEN immunoprecipitates were incubated in TBS with 1 ug of recombinant SENP catalytic fragments, expressed as His-fusion proteins, for 1 hr at 37 °C. Reactions were terminated by boiling in Laemmli loading buffer, and samples were analysed by SDS-PAGE and immunoblotting with anti-SUMO-2/3 antibodies.

2.6.11. RNA isolation, reverse transcription PCR, and real-time quantitative PCR

Total RNA was isolated by using RNeasy (QIAGEN) and treated with DNase I (Roche Diagnostics). Reverse transcription PCR (RT-PCR) was performed using the TaqMan Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR analysis of *RAD51* was performed using primers specific for human *RAD51*: hRAD51 F1 5'-CGTAAGCCAGGGGCGTTGGG-3'; hRAD51 R1 5'-TGCCATTACTCGGTCCGCAGC-3'

2.6.12. Cell cycle analysis

Cell cycle analysis was carried out by flow cytometry. Briefly, U87MG cells were seeded into 6-well culture plates, treated as indicated, collected, fixed, stained

with propidium iodide (100 µg/mL) and RNAse (20 µg/mL) in PBS for 1 hour, and analyzed on a FACScalibur flow cytometer instrument (BD Biosciences) and CellQuest software (BD Immunocytometry Systems, San Jose, CA). Similarly, indirect immunofluorescence on ethanol-fixed U87 cells (70% in PBS) was performed to quantify mitotic cells using an anti-phospho-H3 (Ser10) specific antibody (CellSignaling) detected by an Alexa488-labelled goat anti-rabbit secondary antibody (Invitrogen). Cells were analysed using a FACScalibur instrument (BD Biosciences) and CellQuest software (BD Immunocytometry Systems, San Jose, CA). Ten thousand events were analyzed for each sample.

2.6.13. Annexin V/7-AAD assay for apoptosis

For Annexin V/7-AAD assays, cells were stained with Annexin V–FITC and 7-AAD, and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD PharMingen, San Diego, CA, USA). Briefly, 1 X 10^5 cells were washed twice with phosphate-buffered saline (PBS), and stained with 5 µl of Annexin V–FITC and 10 µl of &-AAD (5 µg/ml) in 1X binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM $CaCl_2$) for 15 min at room temperature in the dark. The apoptotic cells were determined using a FACSCalibur flow cytometer (BD Biosciences).

2.6.14. In vivo studies

2.6.14.1. Compound preparation

NVP-BKM120 and AZD6244 were formulated in NMP/PEG300 (10/90, v/v). Solutions were freshly prepared for each day of dosing by dissolving the powder,

first in *N*-Methyl-2-pyrrolidone (NMP) with sonication and then by adding the remaining volume of PEG300. The application volume was 10 mL/kg. Cisplatin treatment (6 mg/kg body weight) by intraperitoneal injections was given as a single dose.

2.6.14.2. Xenograft studies

Female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (6–10 weeks of age) were kept under sterile conditions (type III cage, in an Optimal Hygienic Conditions zone) with free access to food and water. Subcutaneous tumors were established by injection of 100 μL of PBS containing 2×10^6 tumor cells (HCT116 PTEN-null and isogenic PTEN-WT), in left flank of each animal. Treatments were initiated when the mean tumor volume in each randomized group (n = 8–10) reached 150 to 200 mm^3. For BKM120, treatments were carried out orally, once per day, using an application volume of 10 mL/kg. Treatments were stopped and animals sacrificed when the tumor size in the vehicle control group reached 1,000 to 1,200 mm^3. Tumor volumes were determined using calipers for measurement of longest (considered as length) and shortest (considered as diameter) dimensions of each tumor and calculated according to the modified ellipsoid formula (length × $diameter^2$) / 2. Data are presented as means ± one SEM. Comparisons between groups and vehicle control group were done using either one-way ANOVA followed by the Dunnett tests. For all tests, the level of significance was set at $P < 0.05$. Calculations were carried out with GraphPad Prism. All experimental procedures strictly adhered to the Canadian Council on Animal Care guidelines.

Chapter 3 Combining DNA damaging agents with PI3K inhibitors: alternatives to mitigate the adverse effects

Conceptually, selective killing of cancer cells is the most sensible approach to treating cancer. Nevertheless, the majority of widely administered cancer treatments (chemotherapy, radiation) do little to spare normal cells and largely rely on the high proliferation of cancer cells to kill them.

The first effective cancer drugs were the cytotoxics. Cancer cells replicate their DNA and divide more frequently than normal cells, thus agents such the DNA-damaging agents, antimetabolites that inhibit DNA synthesis and microtubule inhibitors such as Taxol (paclitaxel) that block the mechanics of cell division, preferentially kill tumor cells. Cytotoxic drugs are highly effective for certain tumor types and still form the backbone of cancer therapeutics. Unfortunately, they don't always work. Some cancers are simply not sensitive, while others might initially respond to the treatment only to recur and become resistant. At the basis of the variability in the therapeutic response lies the inherent genetic, phenotypic and functional heterogeneity that exists within, as well as between, tumors. This means that one cure is likely not going to be effective for all malignancies but rather selection of treatment should be guided by the molecular profile of the cancer. In the past 20 years, much effort has been put forward towards understanding of the complexity of the biology of cancer and ultimately treatments directed against the molecular underpinning of individual cancers. This concept is often called precision or personalized medicine.

Understanding of the genetic origin of cancer, and the eventual identification of oncogenes and tumor suppressor genes in the latter half of the 20th century, set the stage for the development of agents aimed at these cancer changes. Cancer is

a complex disease characterized by numerous genomic alterations but only a subset of these mutations contribute to the tumor's progression. These are classified as "driver" mutations, to distinguish them from the preponderance of neutral, "passenger" mutations that can be found in cancer but do not contribute to disease (*268*). Focusing on proteins and pathways altered as a consequence of driver mutations, "molecularly targeted" agents were rationally designed following the understanding of structure-activity relationships and molecular mechanisms of action, largely revealed by structural biology, and signify the beginning of the "targeted molecular therapeutics era" (*269*). This strategy is best exemplified by the development of the kinase inhibitor class of compounds. Mutated or overexpressed kinases are often involved in the processes of tumor development and progression, and drug discovery has explored numerous approaches to inhibit these targets (*270*).

Given the high incidence of *PI3KCA* mutations in malignancies, pan-PI3K inhibitors were thought to be particularly useful to treat tumors that harbor this specific mutation. Indeed, when tested *in vitro*, cell lines with *PIK3CA* mutation demonstrate increased sensitivity to pan-PI3K inhibitors (*271, 272*). Nonetheless, in early-phase clinical studies pan-PI3K inhibitors proved effective in patients both with and without *PIK3CA* mutation, indicating that these agents may be effective when the PI3K pathway activation is driven by molecular alterations at different levels (*273*).

A number of potent and selective small molecule PI3K inhibitors are currently in clinical trial, evaluating their potential as new anticancer drugs. Early-phase,

single-agent trials with PI3K inhibitors have yet to establish a consistent and distinct association between the most common alterations in the PI3K pathway and response to therapy (*274*). Clinical response might be improved by the use of PI3K inhibitors in combination therapies and the identification of predictive biomarkers that define the patient populations that are more likely to benefit from this treatment.

The major concern in the use of PI3Ki in patients is related to the adverse effects associated with inhibiting this pathway. Preclinical studies in rodents reported metabolic disturbances, in particular increased blood glucose (*274*), and were confirmed by an increase in insulin levels in patients treated with these agents (*275*). Side effects that emerge form inhibiting all isoforms of PI3K for a prolonged period of time to achieve therapeutic effectiveness might prove problematic, especially if these inhibitors are combined with other chemotherapeutics. To overcome such toxicities, it might be useful to administer the agent in alternating sequences of doses while another approach would be to employ isoform-specific PI3K inhibitors.

3.1. Management of PI3K inhibitor administration

3.1.1. Combination of DNA damaging agents and PI3K inhibitor causes severe weight loss

As described in chapter 2, treatment of human cancer cells transplanted into immunodeficient rodents with a combination of cisplatin and BKM120 was effective in reducing the tumor burden brought on by the HCT116 *PTEN* $^{-/-}$ xenografts (Figure

2.19). For HCT116 *PTEN* $^{-/-}$ derived xenografts, bi-weekly treatment with cisplatin and a daily dose of BKM120 as adjuvant lead to tumor growth inhibition (TGI) % of 95.5. This represents a substantial improvement over the effects obtained with cisplatin treatment alone (TGI % 33.5) or BKM120 alone (TGI % 24.7). However, the treated mice suffered from substantial weight losses that, in the most severe cases, lead to interruption of the regimen and euthanasia (fig 3.1). Data in this chapter describe strategies to mitigate the adverse side effects while maintaining the efficacy of treatment.

3.1.2. Discontinuous administration of PI3K inhibitor

In my *in vivo* experiments described in chapter 2, the mice were treated with a dose of 6 mg/ml of cisplatin every fourteen days and a daily dose of 3 mg/kg of BKM120 (protocol A, Figure 3.2). In an effort to mitigate the side effects of the combination treatment, we devised an alternative protocol where the same cisplatin dose was coupled with a discontinuous administration of BKM120 (protocol B, fig 3.2). I hypothesized that the introduction of a “drug holiday” would alleviate the weight loss previously observed with the continuous treatment without losing treatment efficacy. The fourteen day treatment cycle began with a peritoneal injection (IP) of a cisplatin on day 1 (6 mg/kg). Daily BKM120 treatment (3 mg/kg) was performed from day 1 through day 7, while no treatment was given from day 8 to day 14 (holiday). The cycle was repeated for the total of 28 days. Using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunohistochemistry staining, a method for detecting DNA fragmentation that

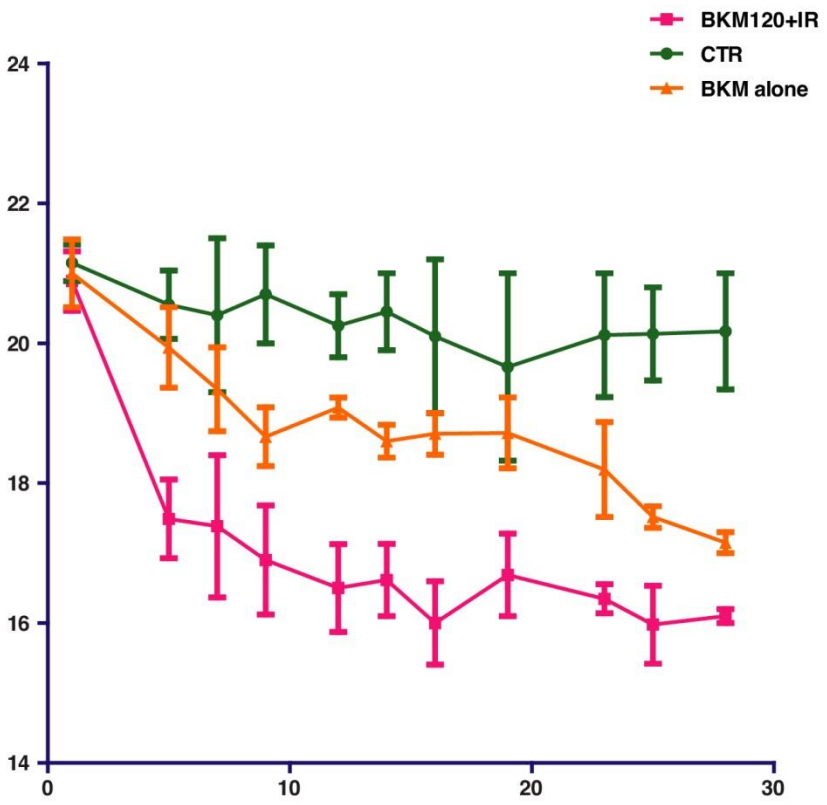

Figure 3.1 BKM120 and Cisplatin treatment cause weight loss in mice. Weight loss was monitored every two days for the duration of the treatment. Error bars indicate the SEM

results from apoptotic signaling cascades, I have previously ascertained that cisplatin rapidly induces apoptosis in HCT116-derived xenografts, reaching a maximum at three days after administration (Figure 3.3). Treatment with BKM120 for seven days following cisplatin injection was chosen to ensure that the adjuvant action of inhibiting the PI3K pathway covers the window of efficacy of the DNA damaging agent.

I also tested another discontinuous protocol, where the two drugs were administered sequentially. This fourteen day cycle featured seven days on (from day one to day seven) and seven days off (from day eight to day fourteen) of BKM120 treatment, with cisplatin administration at day four. We hypothesized that such regimen may give the animal time to adjust to the PI3K inhibitor, mitigating the weight loss. Furthermore, three days of BKM120 pre-treatment may sensitize the cancer cells to the subsequent cisplatin action and improve its efficacy (protocol C, fig 3.2).

3.1.3. Intermittent regimen improves fitness of tumor-bearing mice

NOD/SCID mice were injected with HCT116 *PTEN* $^{-/-}$ cells as described previously and randomized to different treatment groups (5-10 animals per group). Weight and the tumor burden of the animals were monitored and recorded every two-to-three days for the duration of the treatment (28 days).

Mice in protocol B showed an initial weight loss comparable to the ones in protocol A but, while the latter continued to lose weight over the entire treatment period, after seven days, the weight of animals in protocol B begun to recover,

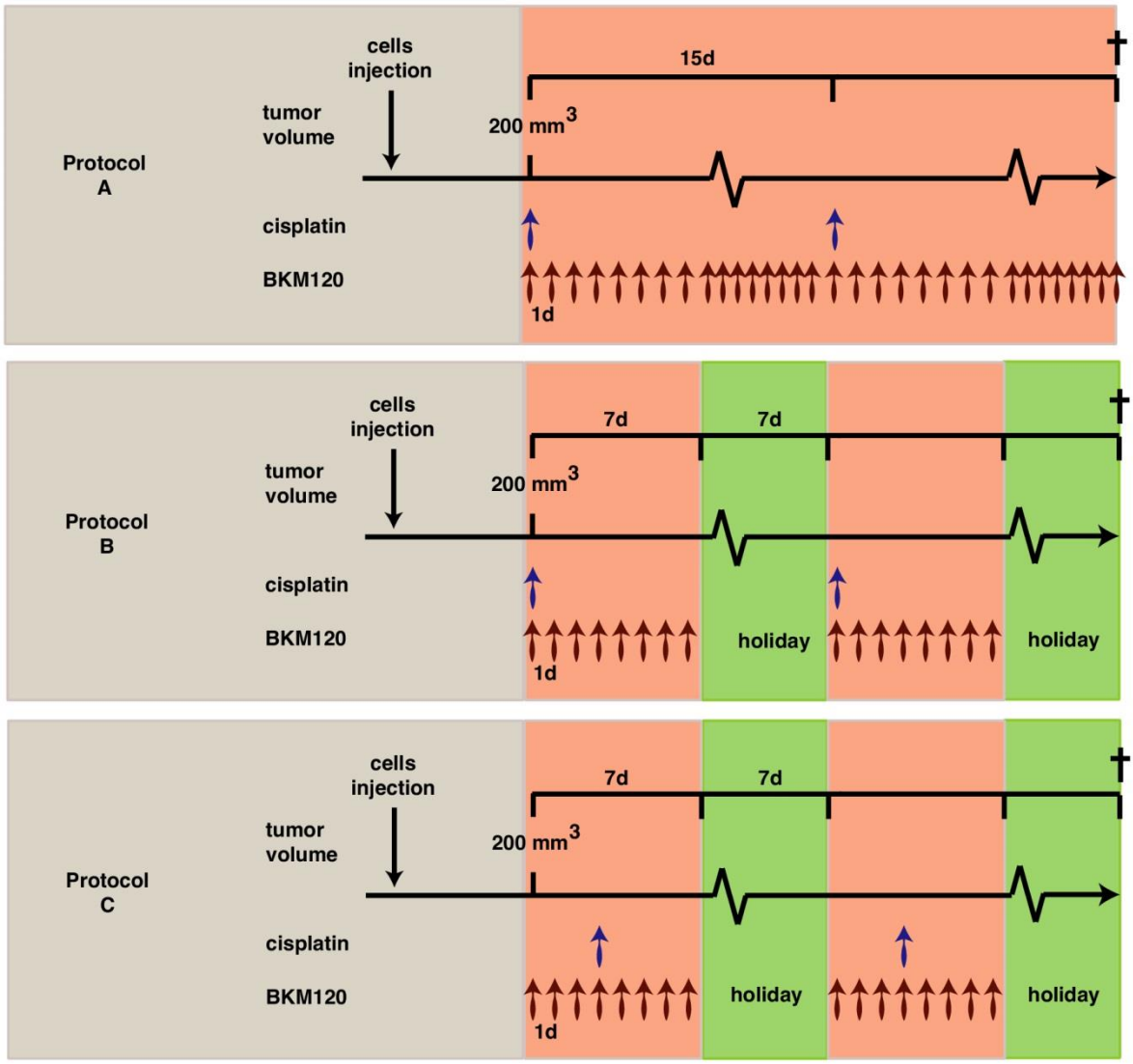

Figure 3.2 Schematic of the different treatment protocols.

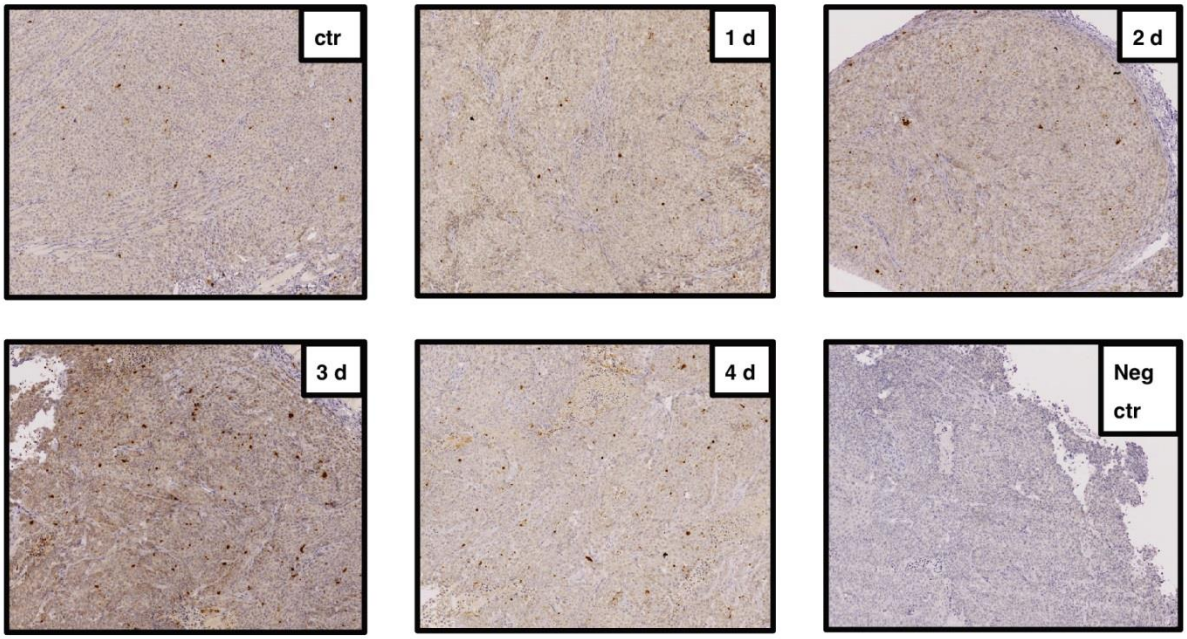

Figure 3.3. Apoptotic cells in xenograft. TUNEL staining was used to study apoptotic cells in transplanted tumors after cisplatin treatment.

trending toward the weight measured at the beginning of the study (fig 3.4). This steady gain in body weight coincided with the period of drug holiday, during which no treatment was administered. The same trend was observed for the second cycle of treatment in the protocol B group. These animals displayed an average of 12% weight loss throughout the study with a weight loss of 11 % of the initial weight at the end of the treatment. In comparison, mice in protocol A lost, respectively, 16% and 23% of their body weight.

Offsetting the administration of cisplatin and BKM120 (protocol C) also shows an improvement in term of fitness over protocol A, with weight loss figures similar to protocol B (average weight loss 12%, final weight loss 15%).

Overall, the intermittent BKM120 regimen had a beneficial impact on the mouse fitness during the treatment.

3.1.4. Intermittent PI3K inhibitor regimen shows comparable performance as the continuous one

Results described in the previous paragraph indicate that there is a marked improvement in terms of fitness of animals following a cycle with discontinuous BKM120 administration. In parallel, we compared the efficacy of protocol B and C to protocol A in inhibition of tumor growth.

To determine the *in vivo* effects of BKM120 on the PI3K pathway, tumor lysates were immunoblotted at the end of the treatment period. Judging by the activation-specific phosphorylation of PKB/Akt, and phosphorylation of ribosomal protein S6 (a downstream target of the mTOR arm of the pathway), protocol A and

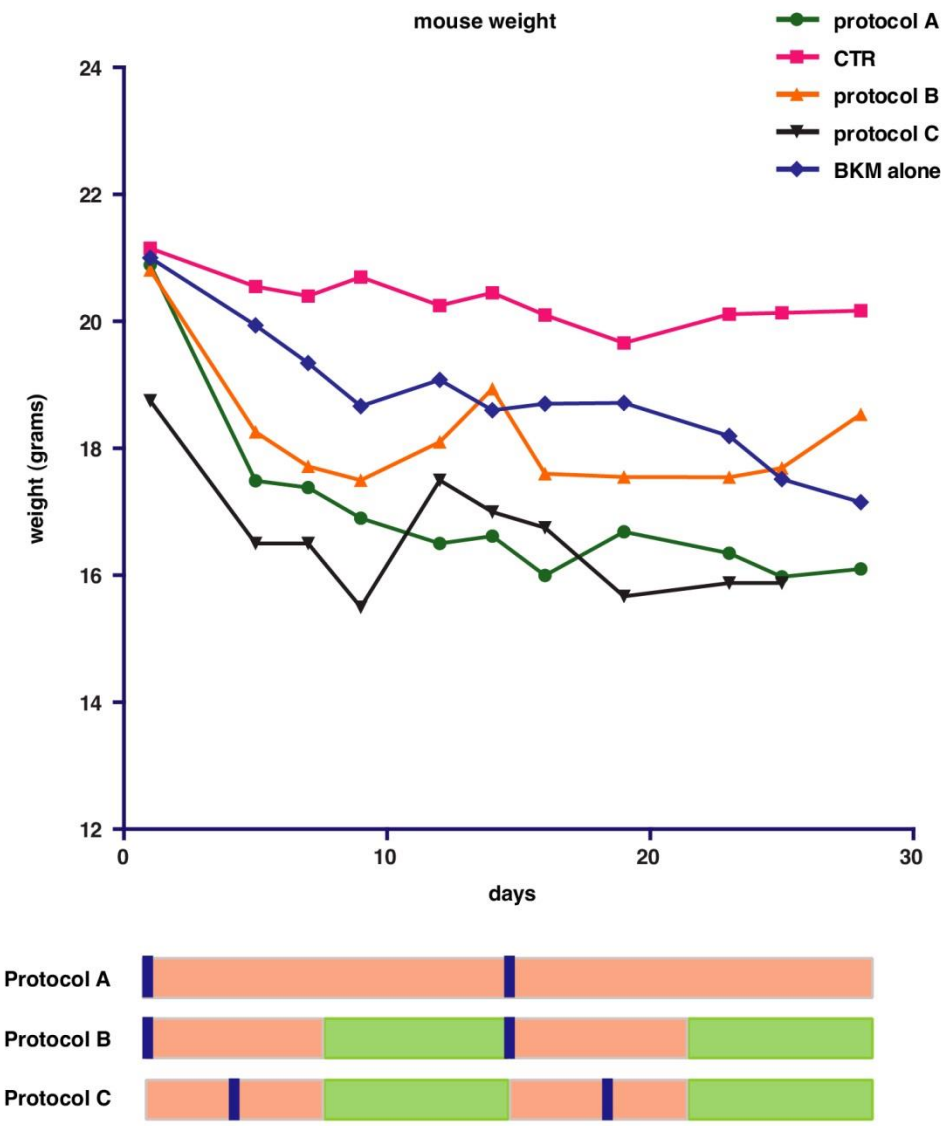

Figure 3.4 Discontinuos treatment alleviates BKM120 and Cisplatin weight loss in mice. Weight loss was monitored every two days for the duration of the treatment. Error bars were omitted to make the graph more interpretable.

protocol B displayed the same efficacy in inhibiting the PI3K signaling (Figure 3.5). This was paralleled by comparable levels of tumor growth inhibition between the regimens. Discontinuous administration of BKM120 in combination with cisplatin for 28 days following protocol B or protocol C resulted in TGI % values of 86.7 and 82.5 respectively, matching tumor growth inhibition obtained with a continuous administration of BKM120 in protocol A (TGI % of 81.9) (fig 3.6).

3.2. PI3K-Isoform specific targeting

The prevalence of PI3K signaling activation in human cancer led to the development of PI3K pathway inhibitors as novel targeted therapeutic agents. The past few years have seen an increasing effort to develop pan- or isoform-selective PI3K inhibitors with diverse selectivity profiles (*276*). Dual pan-class I PI3K–mTOR inhibitors provide the potential advantage of targeting the pathway at two levels (suppressing mTOR in both the mTORC1 and mTORC2 complexes, and PI3K). Their dual activity is based on the structural similarities of the catalytic domain of mTOR and the p110 subunit of PI3K. Nevertheless, inhibition of both PI3K and mTOR has proven an effective therapy strategy in some but not all clinical contexts (*277*). Genetic and biochemical studies have shown that mTORC1 governs a negative feedback loop at the level of RTKs, causing reactivation of the PI3K and the mitogen-activated protein (MAP) pathway upon mTORC1 inhibition (*278*). Depending on the genetic alterations responsible for PI3K pathway activation, it may be desirable to have compounds with better specificity.

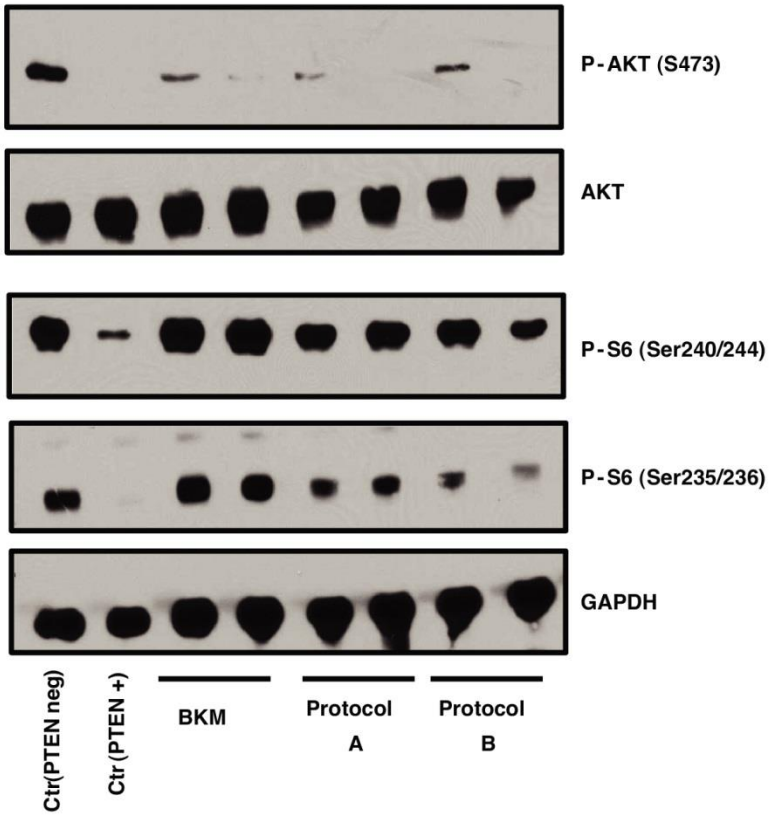

Figure 3.5 PI3K activation is downregulated by BKM120 in vivo. Animals were treated according to the different protocols and the status of the PI3K pathway was estimated by monitoring the activation-specific phosphorylation of PKB/Akt at S473 and of S6 at 235/236 and Ser240/244 by immunoblotting.

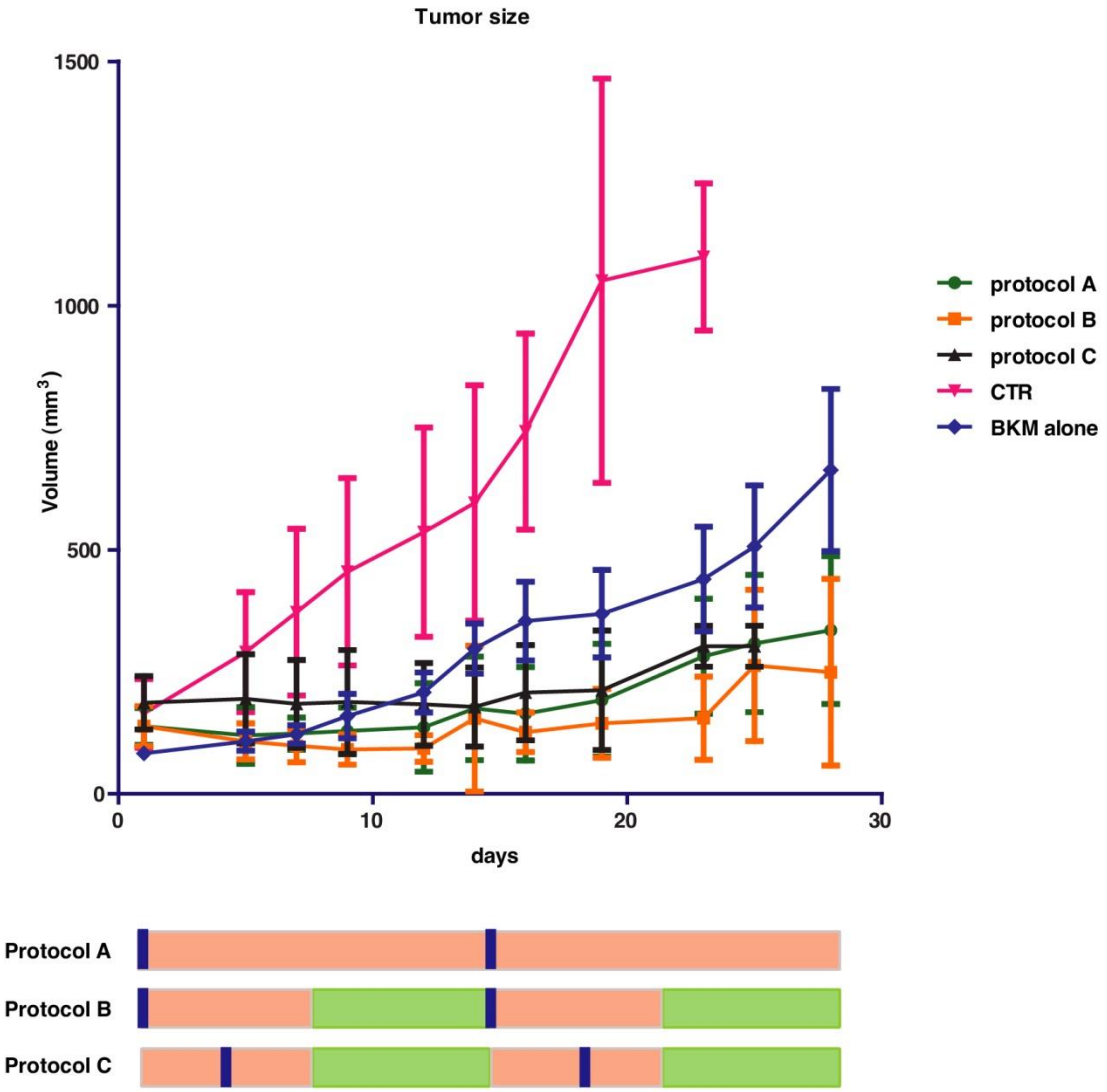

Figure 3.6 Discontinuos treatment is effective in inhibiting tumor growth in mice. HCT116 PTEN-/- cells were injected subcutaneously into NOD/SCID mice (n=10). Mice were treated with cisplatin, BKM120, or both according to the different protocols. Data points represent mean tumor volume ± SEM.

In the previous chapter, the combination of a genotoxic agent with the PI3K inhibitor BKM120 was shown to have synergistic therapeutic activity, specifically in cells that lack PTEN (section 2.4). BKM120 is a specific inhibitor of the pan-class I PI3K family of lipid kinases (in that it targets all four isoforms of class I PI3K α, β, γ, δ) in an ATP-competitive manner.

This broad activity is considered an advantage, considering that most cancer cells express multiple PI3K isoforms with redundant functions in oncogenic signaling. However, the doses needed to fully block all class I PI3Ks for extended periods, pose major tolerability issues in patients. Isoform-selective PI3K inhibitors have the potential to completely block the relevant target, while limiting toxicities associated with broader inhibition profiles.

3.2.1. Specific inhibition of P110α

With the goal of reducing the adverse effects of BKM120, I investigated if a PI3K alpha isoform-specific inhibitor could replicate the synergistic effects of BKM120. I first compared the activity of a PI3Kα-specific inhibitor, BYL719 to that of BKM120. Titration of the drug in U87MG cells confirmed that BYL719 is effective in inhibiting the PI3K pathway even at low concentrations, as shown by the marked reduction of p-AKT levels (Figure 3.7a). Despite being a more potent inhibitor of PI3Kα (BYL719 IC50 = 5 nM) compared to BKM120 (BKM120 IC50 = 52 nM), BYL719 proved less efficient in inhibiting the growth of U87MG cells (Figure 3.7b). Specific PI3Kα inhibition preferentially inhibited the growth of PTEN-null cells (Figure 3.7b), consistent with the data obtained with the pan inhibitor BKM120.

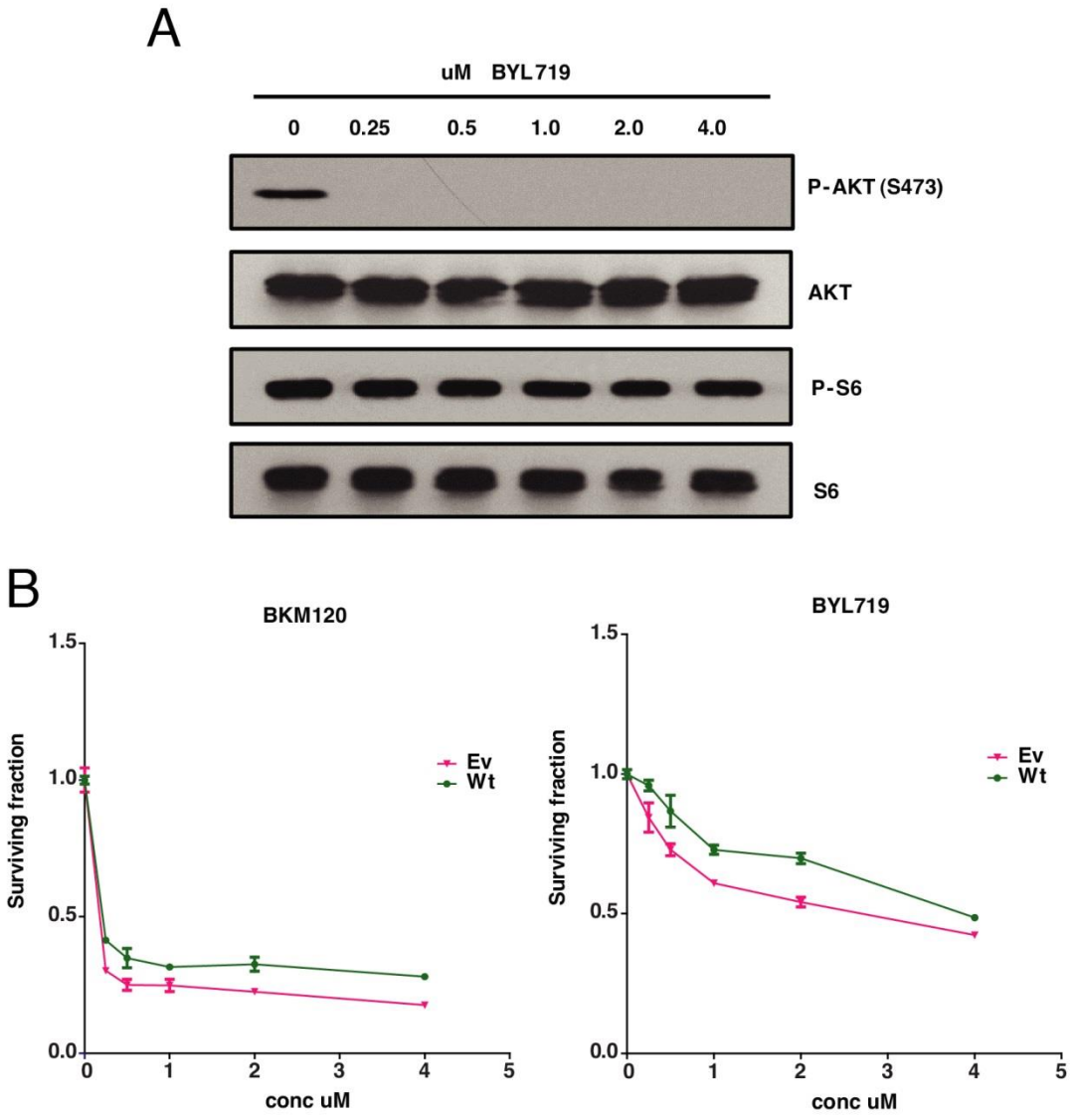

Figure 3.7 U87MG cells are sensitive to BYL719. (A) U87MG cells were treated with BYL719 at the indicated concentrations. The status of the PI3K pathway was estimated by monitoring the activation-specific phosphorylation of PKB/Akt at S473 and of S6 at 235/236 by immunoblotting. (B) U87MG cells were treated with BKM120 and BYL719 at the indicated concentrations. The surviving fraction was measured performing SRB assay after 3 days.

3.2.2. Combination of BYL719 with genotoxic stress

We next tested the activity of the PI3Kα-specific inhibitor in combination with IR, using HCT116 or HCT116 *PTEN*$^{-/-}$ cells (fig 3.8a). Although both cell types were mildly sensitive to the single agent treatments, *PTEN*$^{-/-}$ cells were significantly more sensitive to the treatment combination when compared to parental cells (Figure 3.8a). The same was true when BYL719 was used in combination with another genotoxic agent, cisplatin (Figure 3.8b). Western blot analysis of the samples verified that, in these conditions, BYL719 was effective in inhibiting the PI3K pathway, judged by reduction of p-AKT levels (Figure 3.8c).

To exclude the possibility that the different sensitivity of HCT116 parental and *PTEN*$^{-/-}$ cells to the combination of cisplatin and BYL719 was due to the differences between the two cell lines other than PTEN, we reconstituted HCT116 *PTEN*$^{-/-}$ cells with a construct expressing PTEN or mock DNA (Ev, used as control). Re-expression of PTEN effectively rescued the growth inhibition effects of cisplatin and BYL719 combination on HCT116 *PTEN*$^{-/-}$ cells, further establishing PTEN (or lack thereof) as the factor conferring augmented sensitivity (fig 3.9a).

DNA damage leads to the initiation of a coordinate network of signal transduction pathways involved in cell cycle arrest, apoptosis, stress response and activation of DNA repair processes. It is generally accepted that DNA damage and subsequent induction of apoptosis may be the primary cytotoxic mechanism of cell killing by cisplatin, as well as other DNA-damaging agents (*279, 280*). Indeed, when tested on HCT116 cells, cisplatin and IR induced apoptosis (Figure 3.9b), whereas specific inhibition of PI3Kα with BYL719 had no detectable effects. Consistent with

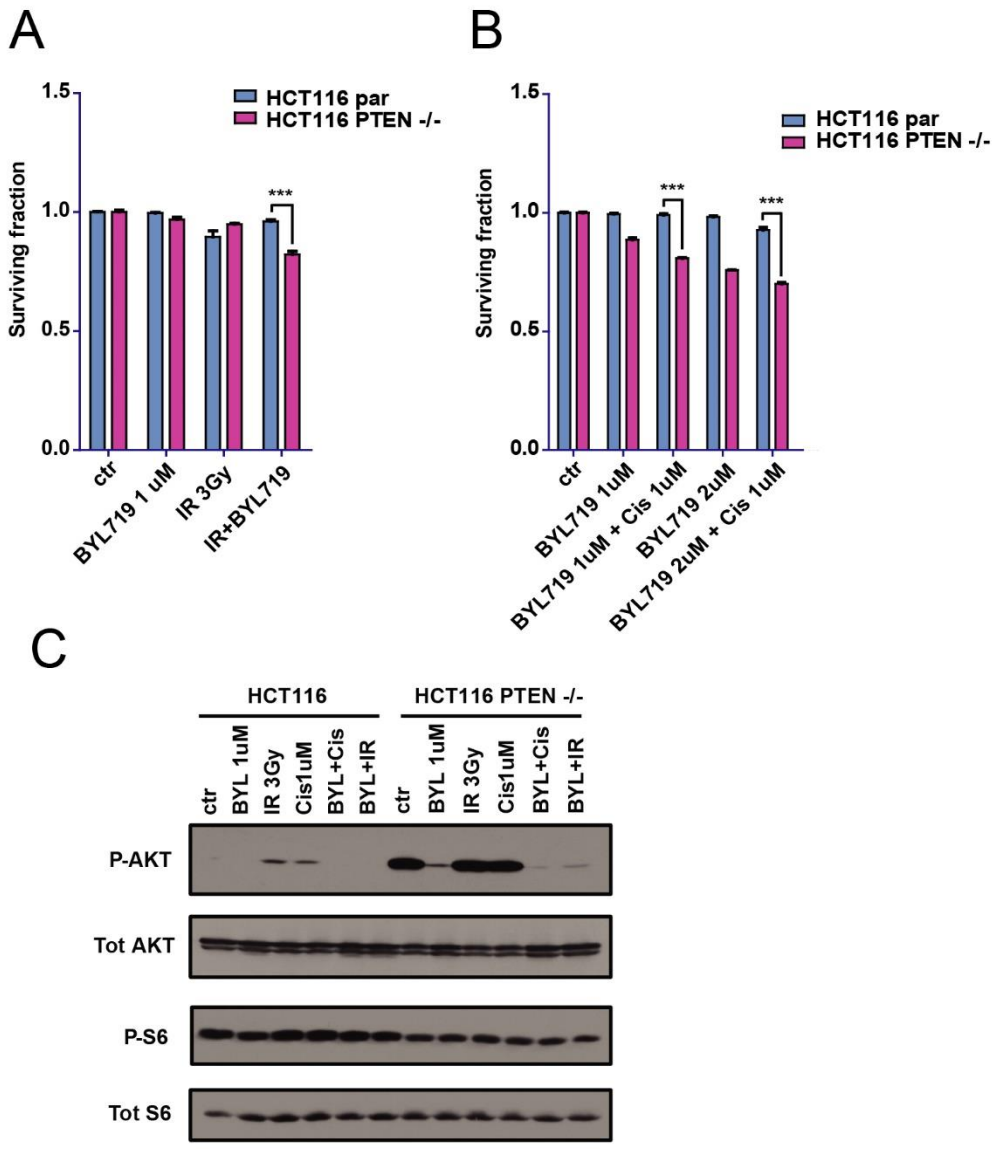

Figure 3.8. PTEN loss sensitizes HCT116 cells to combination treatment with IR and a p110α specific inhibitor. (A,B) Surviving fraction 3 days following indicated treatments. PTEN-wt and HCT116 PTEN-null cells were treated as indicated, fixed with TCA and stained with Sulforhodamine B. The incorporated dye was solubilized and absorbance measured at 565 nm. Bars indicate SEM. Two way analysys of variance followed by Bonferroni test $p<0.0001$. (C) Cells as in A and B were treated as indicated and the status of the PI3K pathway was estimated by monitoring the activation-specific phosphorylation of PKB/Akt at S473 and of S6 at 235/236 by immunoblotting.

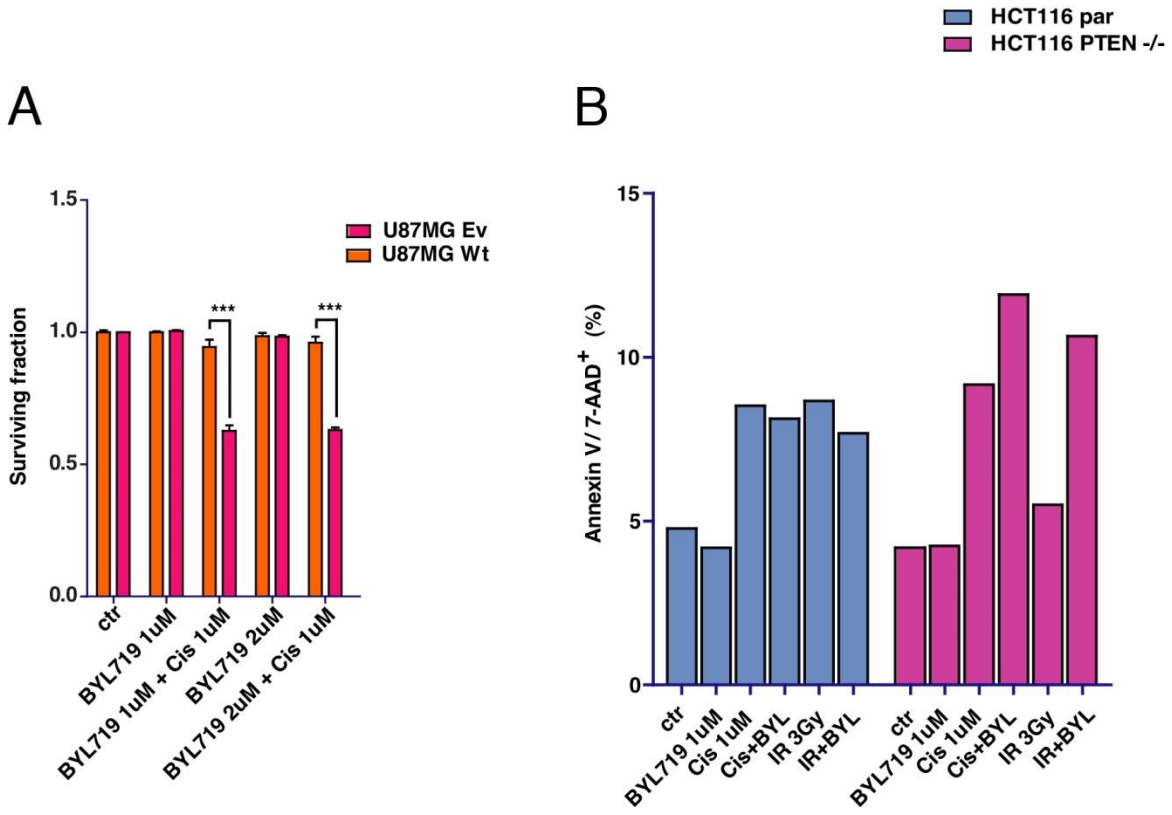

Figure 3.9. IR and a p110α specific inhibitor combination synergistically induce apoptosis in PTEN-null cells. (A) HCT116 PTEN-null cells reconstituted with Ev or WT-PTEN were treated as indicated, fixed with TCA and stained with Sulforhodamine B. The incorporated dye was solubilized and absorbance measured at 565 nm. Bars indicate SEM. (B) PTEN-wt and HCT116 PTEN-null cells were treated was indicate and apoptosis was measured by annexin V+/7-AAD+ staining.

the growth inhibition data, in HCT116 *PTEN*$^{-/-}$ cells, co-treatment with BYL719 potentiated the cisplatin and IR pro-apoptotic effect, while this increase was not found in HCT116 parental cells that express PTEN. Even though this conceptually replicated the results obtained with BKM120, the BYL719 combination lead to a less effective inhibition of cell growth.

3.2.3. Specific inhibition of P110β

Following a similar experimental scheme described in the previous paragraph, I tested the effects of specific p110β inhibition using a small molecule inhibitor, TGX221. TGX-221 is a potent, selective and cell permeable inhibitor of PI3K p110β (IC50 = 9 nM), but not p110 α (IC50 > 1000 nM). When tested in U87MG cells, TGX221 did affect P-Akt levels and the phosphorylation of the downstream target of PI3K pathway, S6 (fig 3.10a). I next compared TGX221 efficacy in inhibiting the growth of U87MG cells in combination with IR. The p110β-specific inhibitor was tested, side by side with BYL719, in a panel that also included BKM120 as positive control, as well as a combination of TGX221 and BYL719 (fig 3.10b). Inhibition of p110β with TGX221 led to partial sensitivity of PTEN-negative cells, similarly to what was noted with p110α inhibition. However, only when the specific p110 α inhibitor BYL719 and p110 β inhibitor TGX221 were used in combination, the growth inhibition in cells that lack PTEN reached levels

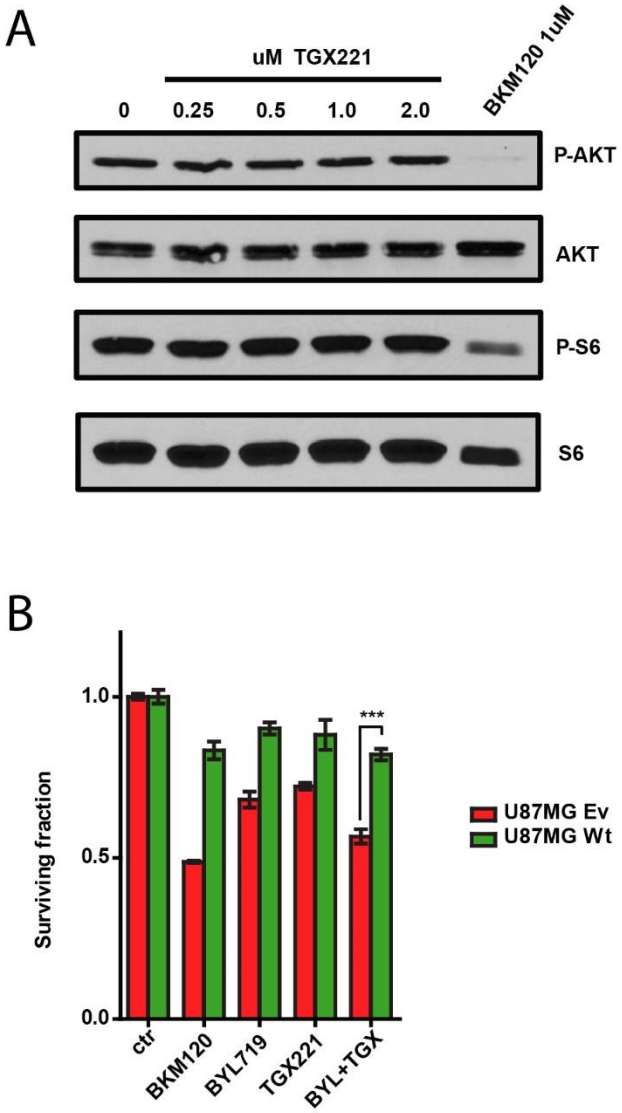

Figure 3.10. p110α and p110β inhibition sensitizes cells to IR reatment. (A) Cells were treated as indicated and the status of the PI3K pathway was estimated by monitoring the activation-specific phosphorylation of PKB/Akt at S473 and of S6 at 235/236 by immunoblotting. (B) U87 cells were treated as indicated, fixed with TCA and stained with Sulforhodamine B. The incorporated dye was solubilized and absorbance measured at 565 nm. Bars indicate SEM. Two way analysys of variance followed by Bonferroni test $p<0.0001$.

comparable to the ones obtained with the pan-inhibitor BKM120 (fig 3.10b).

3.2.4. PI3K targeting with small interfering RNA (siRNA)

To investigate the effect of specific inhibition of PI3Kα or PI3Kβ, I used an alternative experimental approach to block PI3K activity. Instead of small molecules, we used siRNA to specifically target PI3K α (siPI3KCA) or β (siPI3KCB) and evaluated the effects in combination with IR in U87MG cells. Immunoblot (Figure. 3.11a) and RTqPCR (Figure. 3.11b) analysis revealed that siPI3KCA and siPI3KCB where effective in reducing the levels of p110α and p110β, respectively. Reflecting the results obtained with the chemical inhibitors, siPI3KCA as well as siPI3KCB demonstrated a limited degree of synergism with the DNA damaging agents in inhibiting PTEN-deficient cells. Nonetheless, the highest degree of growth inhibition is obtained when both siPI3KCA and siPI3KCB are simultaneously combined with IR (figure 3.12). Collectively, blocking of PI3K signaling with specific siRNAs recapitulates the outcomes of chemical inhibition with small molecules underlying the importance of dual p110 α/β targeting to achieve sensitivity of PTEN-null cells to the combination treatment.

3.3. Discussion

3.3.1. Cisplatin combined with alternating doses of PI3Ki is effective in inhibiting tumor growth

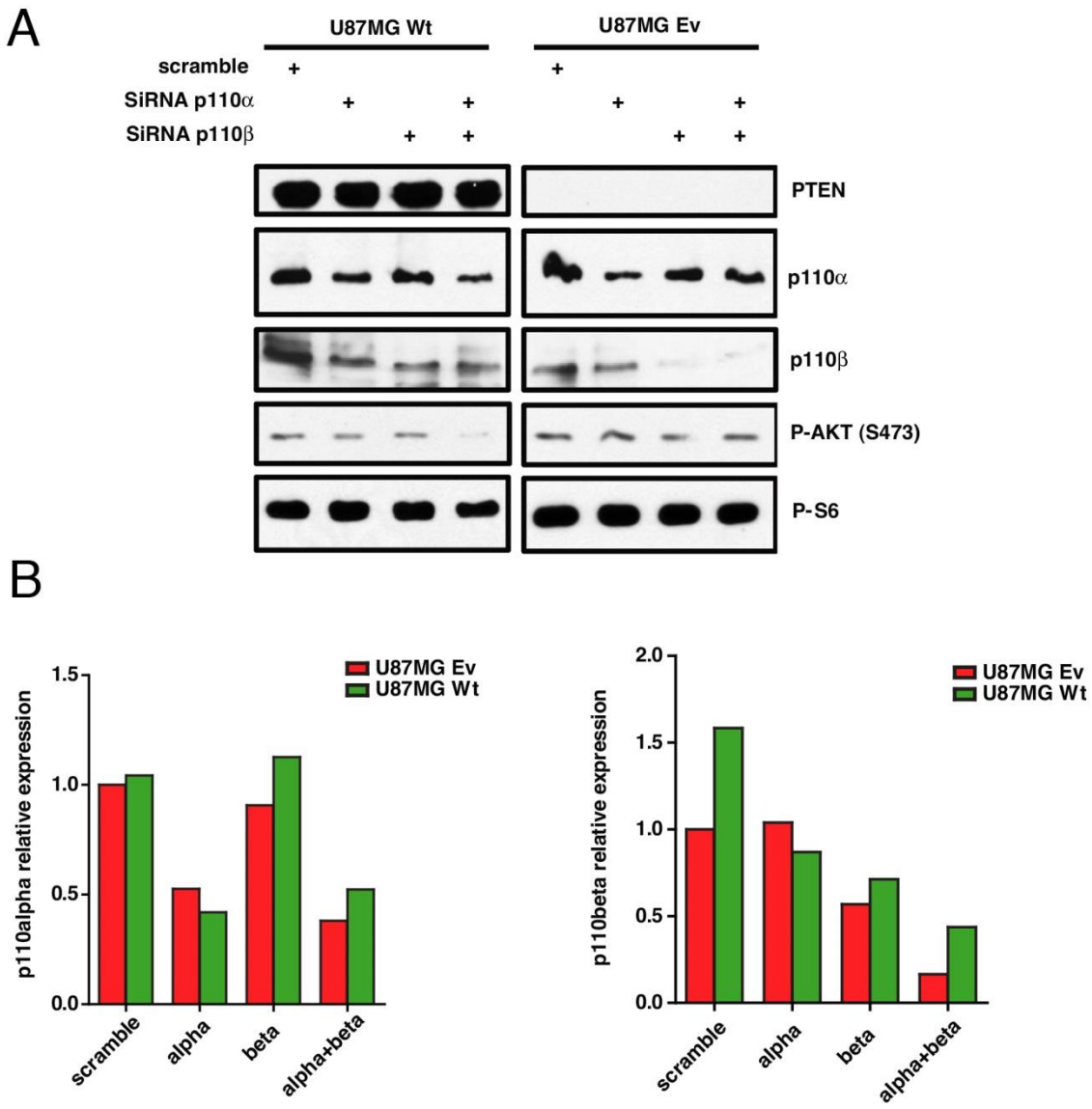

Figure 3.11 SiRNA targeting of p110α and p110β. (A) U87MG cells were transfected with SiRNAs against p110α and p110β and the status of the PI3K pathway was estimated by monitoring the activation-specific phosphorylation of PKB/Akt at S473 and of S6 at 235/236 by immunoblotting. (B) mRNA levels of p110α and p110β in U87MG cells that were transfected with SiRNAs p110a and p110b.

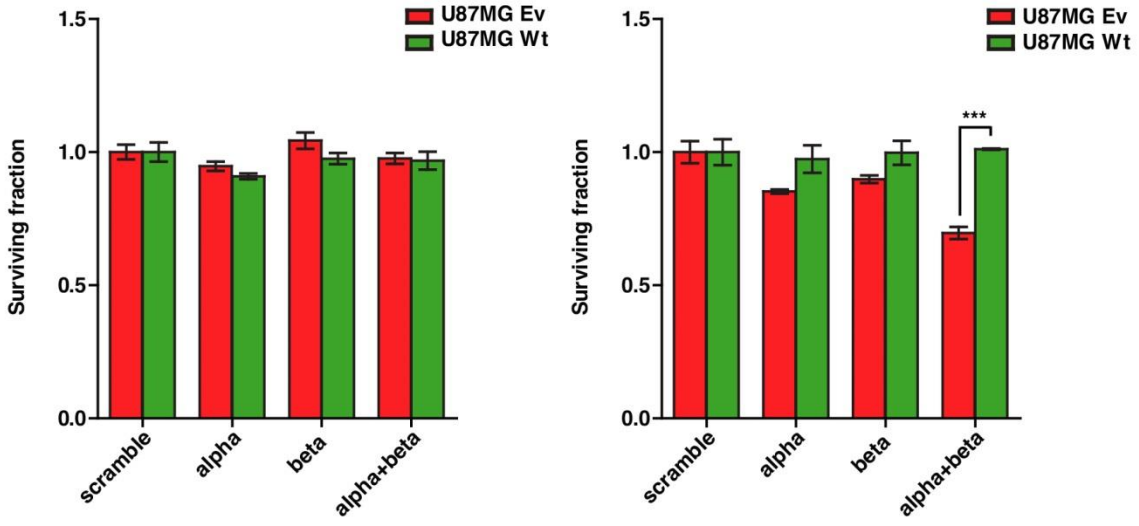

Figure 3.12 p110α and p110β silencing sensitizes cells to IR reatment. U87MG cells were transfected with SiRNAs against p110α and p110β, fixed with TCA and stained with Sulforhodamine B. The incorporated dye was solubilized and absorbance measured at 565 nm. Bars indicate SEM.

An involuntary decrease in weight due to fat and muscle loss, clinically referred to as cachexia, is a common condition associated with cancer and occurs in up to 80% of patients at the later stages of the disease. Cachexia is often associated with decreased response to therapy, quality of life and survival (*281, 282*). It has been suggested that chemotherapy itself may be a major contributor to the development and sustainment of cancer cachexia. Cisplatin, which is part of the standard of care for the treatment of cancers such as lung, head and neck, ovary, testicular, and bladder cancer, is known to have side effects such as nausea, diarrhea, and anorexia that exacerbate the effects of cachexia.

When tested *in vivo*, we observed that continuous treatment with BKM120 also causes weight loss in the animals, which could not be attributed to the tumor burden or the oral gavage procedure (Figure 3.5). Expectedly, addition of BKM120 treatment to the cisplatin regiment caused even more severe weight loss with a body mass decrease greater than 10 %.

To try to reduce the weight loss associated with this protocol we tested an intermittent cycle where the administration of the PI3K inhibitor BKM120 is suspended for a short period of time. The introduction of a period of “holiday” is primarily intended to allow the animals to recover from the weight loss caused by the combination treatment.

There is increasing interest in whether introducing breaks in courses of chemotherapy can relieve side effects without decreasing efficacy. A large Phase III trial was set up in the UK by the Medical Research Council (MRC) to test the effect of intermittent treatment compared to continuous standard chemotherapy in patients

with advanced colorectal cancer (*283*). While survival rates were unaffected by the break in treatment, the researchers found there were benefits in quality of life, including less time spent in chemotherapy, fewer hospital visits and reduced side-effects.

In our model, the introduction of a “holiday” period in the treatment had a similar beneficial effect on the fitness of the animals without impinging on the efficacy. Indeed, we determined that the intermittent regimen produced tumor growth inhibition values equivalent to the continuous protocol. Interestingly, examination of tumor samples at the end of the two cycles of treatment revealed that the inhibitory effect on PI3K pathway of BKM120 was comparable between intermittent and continuous regimen. It is noteworthy that, in tumor samples from protocol B, the levels of P-Akt remain low even though the animals have not received BKM120 for seven days at the time of analysis. This is in agreement with the notion that animals in the intermittent protocol do not show a spurt in tumor growth during the breaks in BKM120 regimen. It appears then, that the combination of DNA damaging agent and PI3K inhibition has lasting effects on tumor cells compatible with a model where the combination of PI3K inhibitor and genotoxic stress causes an increase in apoptosis and a decrease in clonogenic survival. Experiments have shown that inhibition of PI3K/Akt pathway leads to radiosensitization of cells derived from glioblastoma and various carcinomas, including colon, bladder, prostate, head and neck, and cervix (*284-288*). Loss of PTEN and the presence of active Akt counter apoptosis induced by radiation. Taken together, these observations suggest that inhibition of the PI3K pathway should be

concurrent with the genotoxic stress to prevent Akt-induced resistance to chemotherapy. Moreover, PI3K inhibition can be relieved between chemotherapy cycles to help reduce adverse effects without consequences on the efficacy of treatment.

3.3.2. Proliferation of PTEN-null cells is effectively repressed by p110α and p110β inhibition

The development of specific PI3K inhibitors was thought to provide an effective weapon for the treatment of tumor that harbors mutation in the PI3K pathway. In particular, tumors with mutations in the *PIK3CA* gene or *PTEN,* and therefore 'addicted' to PI3K signaling, are thought to be particularly sensitive to inhibitors of this pathway. Several pan-class I PI3K inhibitors in clinical trials target all four class I PI3K isoforms with similar potencies. However, in an effort to minimize side effects and in the prospective that these inhibitors must be given for prolonged periods to be effective, the focus is shifting toward the generation of p110 isoform-specific agents. Since p110α and p110β seem to have independent roles in insulin responses and energy metabolism it is possible that a drug aimed at either one would have fewer side effects than a compound that inhibits both. As an additional bonus, these potential drugs might avoid toxicity to the immune system, which is largely dependent on p110δ and p110γ for function (*289*).

Availability of inhibitors with specific activity toward p110α or p110β could prove of specific effectiveness in certain tumor types. For example, targeting p110α may work well in tumors encompassing oncogenic forms of RTKs and Ras since

p110α plays a central role in cellular transformation and tumorigenesis induced by these lesions (*247, 290*).

Despite promising preliminary results in the clinic, concerns with the cumulative toxicity of led to the development of isoform-specific PI3K inhibitors. In particular, agents that spare p110δ, an isoform predominantly expressed in the immune system, may circumvent immune-related side effects. Experimental characterization of the p110α inhibitors BYL719 on a large panel of cancer cell lines revealed that *PIK3CA* alterations and *HER2* amplification are associated with increased sensitivity (*291*). Conversely, *PTEN* and *BRAF* mutation, as well as concurrent *PIK3CA* and *KRAS* mutations were associated with resistance (*291*). In a separate study, the selective p110α inhibitor INK1402 was found to be more effective in *PIK3CA*-mutated cell lines, compared with those with mutated or absent PTEN (*292*). These findings concur with *in vitro* and *in vivo* studies suggesting that tumors with PTEN loss may be better suited to treatment with p110β-specific inhibitors (*293*).

My data suggests that the synergistic effect of BKM120 with genotoxic agents such as cisplatin or IR is dependent on the ability of this small molecule to inhibit both α and β isoforms of PI3K. The use of specific inhibitors (or specific siRNAs) to target p110 α or β alone only leads to partial growth inhibition in cells that do not express PTEN. These results are further corroborated by a recent report which shows that in primary and immortalized mouse fibroblast cell lines, both p110 α and p110 β controlled steady-state PtdIns(3,4,5)P-3 levels and Akt signaling induced by heterozygous *PTEN* loss (*294*).

Taken together, the data suggests that that both α and beta isoforms of p110 are contributing to the resistance to DNA damaging agents in a PTEN null context and, in order to achieve a clinical response, inhibition of both isoforms is desirable.

3.4. Materials and methods

3.4.1. Antibodies

The following antibodies were purchased from Cell Signaling Technology: PTEN (138G6) (#9559), Akt (#9272), phospho-Akt (Ser473) (193H12) (#4058), phospho-S6 (Ser235/236) (#2211), phospho-S6 (Ser240/244) (#2215), PI3 kinase p110α (#4255). GAPDH FL-335 (sc-25778) and PI3 kinase p110β (sc-602) antibodies were from Santa Cruz Biotechnology.

3.4.2. Cell culture, transfections, viral infections and reagents

U87MG, HCT116 PTEN-null, isogenic PTEN Wt cells and their derivatives were maintained in DMEM, supplemented with 10% FBS (Gibco) and Pen/Strep (100 mg/ml, Hyclone). Puromycin (1 μg/ml) was from Calbiochem. The mouse mammary tumor (MMTC) cell line was cultured in DMEM/F-12 (1:1), supplemented with 10% FBS (Gibco), Pen/Strep (100 mg/ml), insulin (5 μg/ml), hydrocortisone (1 ug/ml) (all from Sigma), and EGF (5 ng/ml, Peprotech).

Transient transfection of cells with SiRNA was performed by using Lipofectamine (Invitrogen) as described previously. *PI3KCA* and *PI3KCB* siRNA were from Dharmacon (*PI3KCA* L-003018-00-0005, *PI3KCB* L-003019-00-0005).

U87MG cells were engineered to express the ecotropic retroviral receptor by selecting a stable clone transfected with pWZL-Neo-EcoR. Viral transduction with retroviruses expressing FLAG-PTEN or Ev was performed in U87MG-EcoR cells by following the procedures described previously (*265*). Cells were subsequently

selected with puromycin (1 μg/ml) until negative control cells died (generally 3 days).

3.4.3. Cell lysis, immunoblotting and immunoprecipitations

Unless indicated otherwise, for immunoblotting cells were lysed in Laemmli sample buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol), normalized for total protein content, resolved by SDS-PAGE, and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% nonfat milk (Bioshop Canada) and probed with the indicated antibodies.

3.4.4. Cell proliferation assays

Cell number was assessed indirectly by using the Sulforhodamine B (SRB) assay (*266*).

3.4.5. RNA isolation and real-time quantitative PCR

Total RNA was isolated by using RNeasy (QIAGEN) and treated with DNase I (Roche Diagnostics). Reverse transcription PCR (RT-PCR) was performed using the TaqMan Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR analysis of PI3 kinase p110α and PI3 kinase p110β expression was performed using primers specific for human PI3KCA (F1 5'-CGTAAGCCAGGGGCGTTGGG-3'; R1 5'-TGCCATTACTCGGTCCGCAGC-3') and PI3KCB: hRAD51 (F1 5'-CGTAAGCCAGGGGCGTTGGG-3'; R1 5'-TGCCATTACTCGGTCCGCAGC-3')

3.4.6. TUNEL assays

TUNEL assays were performed using the In Situ Cell Death Detection Kit, POD from Roche. Sections were deparaffinized with xylene and rehydrated through graded alcohol washes. Antigen retrieval was performed in sodium citrate buffer (10 mM) by applying microwave irradiation for 1 min. The slides were then incubated for 5 min in 3% hydrogen peroxide and immersed for 30 min at room temperature in 0.1 M Tris-HCl, pH 7.5 containing 3% BSA and 20% normal bovine serum. TUNEL reaction mixture containing a 1:20 dilution of TdT enzyme was added to the slides at 37°C for 1.5 h in a humidified atmosphere chamber. Fifty microliters of Converter-POD was then added to each slide and incubated at 37°C for 45 min in a humidified atmosphere chamber. DAB substrate was applied for 1 min followed by counterstaining in hematoxylin.

3.4.7. Annexin V/7-AAD assay for apoptosis

For Annexin V/7-AAD assays, cells were stained with Annexin V–FITC and 7-AAD, and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD PharMingen, San Diego, CA, USA). Briefly, 1 X 10^5 cells were washed twice with phosphate-buffered saline (PBS), and stained with 5 μl of Annexin V–FITC and 10 μl of &-AAD (5 μg/ml) in 1X binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM $CaCl_2$) for 15 min at room temperature in the dark. The apoptotic cells were determined using a FACSCalibur flow cytometer (BD Biosciences).

3.4.8. In vivo studies

3.4.8.1. Compound preparation

NVP-BKM120 was formulated in NMP/PEG300 (10/90, v/v). Solutions were freshly prepared for each day of dosing by dissolving the powder, first in *N*-Methyl-2-pyrrolidone (NMP) with sonication and then by adding the remaining volume of PEG300. The application volume was 10 mL/kg. Cisplatin treatment (6 mg/kg body weight) by intraperitoneal injections was given as a single dose.

3.4.8.2. Xenograft studies

Female nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (6–10 weeks of age) were kept under sterile conditions (type III cage, in an Optimal Hygienic Conditions zone) with free access to food and water. Subcutaneous tumors were established by injection of 100 μL of PBS containing 2×10^6 tumor cells (HCT116 PTEN-null and isogenic PTEN-WT), in left flank of each animal. Treatments were initiated when the mean tumor volume in each randomized group (n = 8–10) reached 150 to 200 mm^3. For BKM120, treatments were carried out orally, once per day, using an application volume of 10 mL/kg. Treatments were stopped and animals sacrificed when the tumor size in the vehicle control group reached 1,000 to 1,200 mm^3. Mouse weight was measured using a mouse weighting scale. Data are presented as means ± one SEM. Tumor volumes were determined using calipers for measurement of longest (considered as length) and shortest (considered as diameter) dimensions of each tumor and calculated according to the modified ellipsoid formula (length × $diameter^2$) / 2. Data are presented as means ± one SEM. Comparisons between groups and vehicle control group were done using either one-way ANOVA followed by the Dunnett tests. For all tests, the level of significance was set at $P < 0.05$. Calculations were carried out

with GraphPad Prism. All experimental procedures strictly adhered to the Canadian Council on Animal Care guidelines.

Chapter 4 Future directions

Invariably found activated in many human cancers, the PI3K pathway has been a major drug development target. Several of its components, including the PI3K catalytic subunit p110α (PIK3CA) and the members of the AKT family, can drive malignant transformation when they are mutationally activated or overexpressed, as does functional inactivation of the tumor suppressor PTEN. Considering the pleiotropic impact of PI3K pathway activation on control of proliferation, cell migration, metabolism, resistance to apoptosis and tumor-associated angiogenesis, specific inhibitors of this pathway are thought of as potential therapeutics for many human cancers.

The development of targeted anti-cancer therapeutics has been historically focused on oncogenes as tumor promoters. Designing anti-cancer therapeutics directed at loss of tumor suppressors has proven to be more difficult, as it is easier to devise a strategy to inhibit an overexpressed or hyperactivated protein than to replenish the function of a silenced or inactivated one. Hence, most of the strategies against PTEN-deficient cancers utilize inhibitors of kinases that lie at the level of PI3K or downstream of it. While there has been some success with these strategies in early clinical trials, the value of PTEN as a clinical biomarker remains controversial. In a retrospective non-randomized study that included various cancer types treated with mTOR targeted therapeutics, PTEN status, along with *PIK3CA* mutations, were found to be predictive of response (*277*). In colorectal adenocarcinomas, lack of *PTEN* gene was associated with greater response to cetuximab, an anti-EGFR monoclonal antibody (*295*). On the other hand, in HER2-positive metastatic breast cancers, PTEN status was not significantly associated

with response to trastuzumab, a monoclonal antibody that interferes with the HER2/neu receptor (*296*).

4.1. PTEN-deficient tumors and combination therapy: testing sensitivity in patient derived xenograft

Patient-Derived Xenografts (or PDXs) models are considered superior to traditional cell line-xenograft models of cancer because they maintain more similarities to the parental tumors (*297-299*). In the PDX model, the primary tumors, obtained fresh from patient surgery, are transferred into an immunodeficient mouse, generally a NOD/SCID mouse. The result is a tumor that more closely resemble the original cancer in the morphology, cellular heterogeneity, and molecular profile (*297-299*) and are proving useful for preclinical evaluation of experimental therapeutics. The main advantage of PDXs is that they are derived directly from patient tissue without prior *in vitro* culture, maintaining the heterogeneity of the clinical disease. Because the whole tumor fragments are directly implanted *in vivo*, the tumor material is not processed using dissociation protocols. Even though engraftment frequency and growth rate of implanted tumors can be variable, PDX models of different tumor types have been successfully established (*300*). Many of these tumors, in particular breast, ovarian, and brain, are characterized by high frequency of PTEN loss making them ideal for further testing of concepts developed in this thesis. Particularly relevant would be the comparison of the susceptibility of PTEN wild type or PTEN null PDXs to the combination of cisplatin and PI3Ki.

Cohorts can be generated from multiple donors and used to test for differential response. A homogeneous response to drug treatments amongst the different cohorts will identify loss of nuclear PTEN as the determinant of increased sensitivity to these agents. Conversely, high variability in the response will suggest the existence of cooperating alterations or other factors beyond PTEN loss (and the consequent genomic instability). This will provide an ideal opportunity to undertake additional molecular analyses to identify these mediators of sensitivity, which could reveal potential biomarkers and suggest opportunities for drug combinations.

The identification of tumor biological markers can be useful in categorizing patients who will actually benefit from a specific treatment. The differential expression of molecules likely to be involved in the sensitivity of the tumor cells to therapy might explain the varying responses of tumor patients to a therapy, and might provide important tools for exploiting this aspect in the clinic. Genome-scale gene expression analyses of PDX tumors performed prior to treatment can help identify subsets that may benefit from the therapeutic combination of cisplatin and PI3Ki. Pattern of expression can be used to generate a gene expression signature associated with the response. This gene expression signature can subsequently be validated in cohorts of tumors from additional donors.

4.2. PTEN-K254R and PTEN-T398A knock-in mice

Knockout of the *PTEN* gene in mice leads to early embryonic lethality, whereas heterozygous deletion results in a fully penetrant cancer susceptibility

phenotype (*35*). In an effort to understand the role of loss of PTEN induced genomic instability in these phenotypes, it will prove useful to genetically engineer mice to carry mutations in key residues implicated in PTEN posttranslational modifications relevant to its nuclear function, lysine 254 and threonine 398. The mutations can be introduced using the knock-in strategy by homologous recombination in mouse embryonic stem (ES) cells. Mice heterozygous or homozygous for PTEN-K254R and PTEN-T398A would be initially monitored for viability and gross phenotypic defects. In case of embryonic lethality, mice will be staged to determine time of death and possible causes based on morphological analysis.

The evidence that either of the PTEN mutants, K254R and T398A, retain full lipid phosphatase activity implies that the homozygous mutant strains are likely to be viable. These mice, alongside the heterozygotes, should be monitored for spontaneous tumorigenesis, with particular emphasis on the endometrium and the mammary tissue where deletion of one *PTEN* allele causes a fully penetrant tumor phenotype (*125*). Considering the role of PTEN in the maintenance of genomic integrity, these mice should be subjected to genotoxic challenges, such as sub-lethal doses of whole body ionizing radiation (3 Gy) and monitored for the appearance and timing of thymic lymphomas, the most likely tumors resulting from such treatment (*301*).

To evaluate the specific consequences of loss of nuclear PTEN function-induced genomic instability on tumorigenesis, mice bearing PTEN-K254R or PTEN-T398A mutation should be crossed with other transgenic mice predisposed to cancer. A good strategy for this study would be the use of mice expressing the neu

(ErbB2) oncogene under the transcriptional control of the mouse mammary tumor virus promoter/enhancer (MMTVneu). MMTVneu mice develop focal mammary tumors that first appear at 4 months, with a median onset of 205 days (*302*). This model has been shown to recapitulate the gene expression profile of human Luminal B breast cancers, (*303*), and has been used test clinically-relevant therapies and identify potential biomarkers of drug sensitivity and resistance (*304*). Previous studies in this mouse model reported a tendency for the developing tumors to accumulate p53 mutations (*305*). Indeed, in bi-transgenic mice carrying MMTV-ErbB2 and WAP-p53–172H, tumor latency was shortened to 154 days, indicative of strong cooperativity between the MMTV-ErbB2 model and genomic instability brought on by p53 impairment (*305*). Moreover, the relatively prolonged rate of tumor development in the MMTV/Neu mice compared to other models (such as MMTV-PyVT) offers an opportunity to better appreciate the expected acceleration caused by the PTEN K254R mutation. Finally, because K254R mutation of PTEN replicates the genomic instability observed in PTEN-null scenarios without compromising its lipid phosphatase activity and ability to regulate PI3K signaling, the contribution of nuclear and cytoplasmic PTEN to tumorigenesis can be uncoupled.

A similar experimental design can be applied to knock-in mouse expressing a PTEN T398A mutation. Mutation of PTEN threonine 398 abolishes ATM phosphorylation at this position and prevents its nuclear exclusion following genotoxic stress. Cells expressing PTEN T398A show milder DNA repair defects compared to cells expressing PTEN K254R, thus offering insight into the

contribution of partial loss of PTEN nuclear function in DNA repair. Combined, future experiments should establish how the disruption of the crosstalk between these two major tumor suppressors such as ATM and PTEN affects the formation of tumors. We expect that the knock-in of PTEN K254R and T398A will accelerate tumorigenesis in MMTVneu mice by both reducing tumor latency and increasing tumor numbers and growth.

4.3. Identification of SUMO-PTEN protein-protein interactions

SUMOylation has been shown to have a central role in the assembly and function of proteins at the site of DNA damage (*232, 233, 236, 238, 241, 259*). Likewise, SUMO-PTEN plays a specific role in the nucleus and cells that express the non-SUMOylable mutant PTEN-K254R have defective HR and display genomic instability. However the detailed mechanism by which PTEN achieves this function remains unknown. The identification of SUMO-PTEN interactors would represent a considerable step toward discovery of understanding the molecular mechanism of PTEN action in DDR.

Mass-spectrometry provides an unbiased and powerful tool for the study of protein-protein interaction (*306*). Mass spectrometry is able to resolve complex mixtures of proteins using a technique in which gas phase peptides are ionized and their mass-to-charge ratio is measured. A spectrum is acquired to give the mass-to-charge ratio of all compounds in the sample and, thanks to the use of bioinformatics, identify the components of the mixture. In the case of co-

immunoprecipitated proteins, mass-spectrometry can be used for the identification of functional interactions. However, in the case of SUMOylated proteins, low steady-state levels of endogenous SUMOylation and the difficulty in separating the SUMOylated form from the non-modified protein species are obstacles in the use of conventional methods for identifying protein-protein interactions.

Nevertheless, certain improvements to the general approach may facilitate the detection of SUMO-specific PTEN interactions. Restricting affinity purification of PTEN to the nuclear fraction will lead to the enrichment for the SUMOylated form while excluding PTEN cytoplasmic interactions. To distinguish the SUMO-specific nuclear PTEN interactions, a “subtraction list” should be generated by determining the interactome of PTEN-K254R under the same conditions. To ensure that the interactions are physiologically relevant, the expression levels of PTEN and PTEN K254R should be comparable to those of the endogenous PTEN. This can be achieved by retroviral infection with PTEN constructs carried by the pBabe-FLAG backbone. Alternatively, MEFs isolated from wild type and PTEN-K254R knock-in mice could be used.

SUMO-PTEN is involved in the cellular DNA repair and governs the correct assembly of repair proteins, such RAD51, at the DNA damage foci. To facilitate the capture of PTEN interactions towards this function, nuclear-PTEN pull-downs should be performed following cell exposure to IR. Considering the dynamics of SUMO-PTEN turnover following DNA damage and the time of assembly of DNA repair proteins, 4 to 6 hours after the damage would be a good optimization starting point (*307*). Potential interactors would then be verified by co-immunoprecipitation

and immunofluorescence microscopy. In particular, *in situ* proximity ligation assay (PLA) (*308*), for its specificity and sensitivity in detecting and quantifying protein-protein interactions, would be the method of choice for validating experiments.

Each candidate binding partner should also be further explored for the regions of interaction by truncation and co-immunoprecipitation analysis. Functional relationships should be pursued by overexpression/knockdown of candidate interactors, monitoring for rescue of DDR phenotypes elicited by nuclear PTEN loss, as well as regulation of nuclear PTEN turnover and 398 phosphorylation. Ultimately, verified relationships should be examined genetically in model organisms.

4.4. Concluding remarks

PTEN mutations are detected in at least 30 different tumor types (Catalogue of Somatic Mutations in Cancer, COSMIC). Loss of PTEN expression accounts for many more human cancers (*309*), making *PTEN* one of the most commonly lost or mutated tumor suppressor gene.

Loss of PTEN is associated with high-grade malignancies, poor outcome and poor response to therapy in many human cancers (*310-312*). Screening PTEN loss by immunohistochemistry is being implemented in a number of cancer clinical trials. Despite numerous preclinical studies, it is unclear if PTEN loss of function represents an actionable feature in the clinical setting and, to date, no unified personalized therapy exists for these tumors.

The drugs tested in the clinical setting include almost exclusively RTK/PI3K/mTOR inhibitors. Data on drug sensitivity associated with PTEN deficiency collected from preclinical studies has only been partially validated in the clinic. In particular, early clinical data from patients receiving single-agent PI3K pathway inhibitors therapies produced mixed results. A phase I study with the pan-PI3K inhibitors Buparlisib (BKM120) showed no association between PTEN status and clinical response (*313*).

Equally PTEN loss only with the activation of the PI3K pathway, fails to take into account a prominent role for PTEN in the maintenance of genomic instability (*158, 164, 314-316*), possibly explaining failure of anti-PI3K therapeutics in PTEN-deficient cancers. Importantly, PI3KCA mutations and PTEN loss are not mutually exclusive events in tumors (*317*). Loss of PTEN is likely associated with a distinctive set of molecular and metabolic changes within the cancer cells, possibly creating vulnerabilities and opportunities for synthetic lethal interactions.

My work revealed sensitivity of PTEN deficient cells to the combination of genotoxic stress and PI3K inhibition both *in vitro* and *in vivo*. Importantly, such combination was found to spare cells wild type for PTEN. While the mechanism of action of cisplatin leverages on their intrinsic genomic instability of PTEN-deficient cells, the concomitant inhibition of PI3K works to blunt their survival advantage. Consistent with such notion, one of the mechanisms of resistance to cisplatin is the activation of the PI3K/Akt pathway (*318*), which suppresses cisplatin induced apoptosis. Co-treatment with BKM120 effectively reduces PI3K pathway activation and ensures the normal functioning of the apoptotic response.

Clinical testing of these observations is underway. Targeted screening for PTEN loss in several ongoing clinical trials evaluating the effectiveness of the combination of PI3K inhibitors and conventional chemotherapy drugs will provide more insight into the value of PTEN as a biomarker.

Parallel to these efforts, molecular biology and biochemistry efforts are shedding light on the molecular mechanisms of PTEN regulation relevant to tumorigenesis. Further work in these areas will lead towards development of new therapeutic strategies and refinement of the existing ones, with the ultimate goal of treatment of PTEN-deficient cancers.

References

1. T. Maehama, J. E. Dixon, The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *The Journal of biological chemistry* **273**, 13375-13378 (1998); published online EpubMay 29 (
2. D. Hanahan, R. A. Weinberg, The hallmarks of cancer. *Cell* **100**, 57-70 (2000); published online EpubJan 7 (
3. R. J. T. Joy, The Theory and Practice of Oncology - Historical Evolution and Present Principles - Raven,Rw. *Isis* **83**, 639-640 (1992); published online EpubDec (Doi 10.1086/356301).
4. R. J. Huebner, G. J. Todaro, Oncogenes of RNA tumor viruses as determinants of cancer. *Proceedings of the National Academy of Sciences of the United States of America* **64**, 1087-1094 (1969); published online EpubNov (
5. S. H. Bigner, J. Mark, M. S. Mahaley, D. D. Bigner, Patterns of the early, gross chromosomal changes in malignant human gliomas. *Hereditas* **101**, 103-113 (1984).
6. G. J. Kitange, K. L. Templeton, R. B. Jenkins, Recent advances in the molecular genetics of primary gliomas. *Curr Opin Oncol* **15**, 197-203 (2003); published online EpubMay (
7. M. Yoshimoto, J. C. Cutz, P. A. Nuin, A. M. Joshua, J. Bayani, A. J. Evans, M. Zielenska, J. A. Squire, Interphase FISH analysis of PTEN in histologic sections shows genomic deletions in 68% of primary prostate cancer and 23% of high-grade prostatic intra-epithelial neoplasias. *Cancer Genet Cytogenet* **169**, 128-137 (2006); published online EpubSep (S0165-4608(06)00252-4 [pii]
10.1016/j.cancergencyto.2006.04.003).
8. P. A. Steck, M. A. Pershouse, S. A. Jasser, W. K. Yung, H. Lin, A. H. Ligon, L. A. Langford, M. L. Baumgard, T. Hattier, T. Davis, C. Frye, R. Hu, B. Swedlund, D. H. Teng, S. V. Tavtigian, Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* **15**, 356-362 (1997); published online EpubApr (10.1038/ng0497-356).
9. J. Li, C. Yen, D. Liaw, K. Podsypanina, S. Bose, S. I. Wang, J. Puc, C. Miliaresis, L. Rodgers, R. McCombie, S. H. Bigner, B. C. Giovanella, M. Ittmann, B. Tycko, H. Hibshoosh, M. H. Wigler, R. Parsons, PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**, 1943-1947 (1997); published online EpubMar 28 (
10. D. M. Li, H. Sun, TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res* **57**, 2124-2129 (1997); published online EpubJun 1 (

11. D. J. Marsh, V. Coulon, K. L. Lunetta, P. Rocca-Serra, P. L. Dahia, Z. Zheng, D. Liaw, S. Caron, B. Duboue, A. Y. Lin, A. L. Richardson, J. M. Bonnetblanc, J. M. Bressieux, A. Cabarrot-Moreau, A. Chompret, L. Demange, R. A. Eeles, A. M. Yahanda, E. R. Fearon, J. P. Fricker, R. J. Gorlin, S. V. Hodgson, S. Huson, D. Lacombe, C. Eng, et al., Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation. *Hum Mol Genet* **7**, 507-515 (1998); published online EpubMar (ddb041 [pii]).
12. X. Zhou, H. Hampel, H. Thiele, R. J. Gorlin, R. C. Hennekam, M. Parisi, R. M. Winter, C. Eng, Association of germline mutation in the PTEN tumour suppressor gene and Proteus and Proteus-like syndromes. *Lancet* **358**, 210-211 (2001); published online EpubJul 21 (S0140673601054125 [pii]).
13. J. A. Hobert, C. Eng, PTEN hamartoma tumor syndrome: an overview. *Genet Med* **11**, 687-694 (2009); published online EpubOct (10.1097/GIM.0b013e3181ac9aea).
14. R. J. Gorlin, M. M. Cohen, Jr., L. M. Condon, B. A. Burke, Bannayan-Riley-Ruvalcaba syndrome. *Am J Med Genet* **44**, 307-314 (1992); published online EpubOct 1 (10.1002/ajmg.1320440309).
15. M. M. Cohen, Jr., Overgrowth syndromes: an update. *Adv Pediatr* **46**, 441-491 (1999).
16. N. Chalhoub, S. J. Baker, PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol* **4**, 127-150 (2009)10.1146/annurev.pathol.4.110807.092311).
17. D. J. Marsh, J. B. Kum, K. L. Lunetta, M. J. Bennett, R. J. Gorlin, S. F. Ahmed, J. Bodurtha, C. Crowe, M. A. Curtis, M. Dasouki, T. Dunn, H. Feit, M. T. Geraghty, J. M. Graham, Jr., S. V. Hodgson, A. Hunter, B. R. Korf, D. Manchester, S. Miesfeldt, V. A. Murday, K. L. Nathanson, M. Parisi, B. Pober, C. Romano, C. Eng, et al., PTEN mutation spectrum and genotype-phenotype correlations in Bannayan-Riley-Ruvalcaba syndrome suggest a single entity with Cowden syndrome. *Human molecular genetics* **8**, 1461-1472 (1999); published online EpubAug (
18. A. M. Martelli, C. Evangelisti, W. Chappell, S. L. Abrams, J. Basecke, F. Stivala, M. Donia, P. Fagone, F. Nicoletti, M. Libra, V. Ruvolo, P. Ruvolo, C. R. Kempf, L. S. Steelman, J. A. McCubrey, Targeting the translational apparatus to improve leukemia therapy: roles of the PI3K/PTEN/Akt/mTOR pathway. *Leukemia* **25**, 1064-1079 (2011); published online EpubJul (10.1038/leu.2011.46
leu201146 [pii]).
19. V. Stambolic, D. MacPherson, D. Sas, Y. Lin, B. Snow, Y. Jang, S. Benchimol, T. W. Mak, Regulation of PTEN transcription by p53. *Mol Cell* **8**, 317-325 (2001); published online EpubAug (S1097-2765(01)00323-9 [pii]).
20. M. T. Lau, C. Klausen, P. C. Leung, E-cadherin inhibits tumor cell growth by suppressing PI3K/Akt signaling via beta-catenin-Egr1-mediated PTEN expression. *Oncogene* **30**, 2753-2766 (2011); published online EpubJun 16 (10.1038/onc.2011.6
onc20116 [pii]).

21. L. B. Song, J. Li, W. T. Liao, Y. Feng, C. P. Yu, L. J. Hu, Q. L. Kong, L. H. Xu, X. Zhang, W. L. Liu, M. Z. Li, L. Zhang, T. B. Kang, L. W. Fu, W. L. Huang, Y. F. Xia, S. W. Tsao, M. Li, V. Band, H. Band, Q. H. Shi, Y. X. Zeng, M. S. Zeng, The polycomb group protein Bmi-1 represses the tumor suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells. *J Clin Invest* **119**, 3626-3636 (2009); published online EpubDec (10.1172/JCI39374
39374 [pii]).
22. W. H. Chappell, T. D. Green, J. D. Spengeman, J. A. McCubrey, S. M. Akula, F. E. Bertrand, Increased protein expression of the PTEN tumor suppressor in the presence of constitutively active Notch-1. *Cell Cycle* **4**, 1389-1395 (2005); published online EpubOct (2028 [pii]).
23. J. T. Whelan, S. L. Forbes, F. E. Bertrand, CBF-1 (RBP-J kappa) binds to the PTEN promoter and regulates PTEN gene expression. *Cell Cycle* **6**, 80-84 (2007); published online EpubJan 1 (3648 [pii]).
24. T. Palomero, M. L. Sulis, M. Cortina, P. J. Real, K. Barnes, M. Ciofani, E. Caparros, J. Buteau, K. Brown, S. L. Perkins, G. Bhagat, A. M. Agarwal, G. Basso, M. Castillo, S. Nagase, C. Cordon-Cardo, R. Parsons, J. C. Zuniga-Pflucker, M. Dominguez, A. A. Ferrando, Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med* **13**, 1203-1210 (2007); published online EpubOct (nm1636 [pii]
10.1038/nm1636).
25. A. Mirmohammadsadegh, A. Marini, S. Nambiar, M. Hassan, A. Tannapfel, T. Ruzicka, U. R. Hengge, Epigenetic silencing of the PTEN gene in melanoma. *Cancer Res* **66**, 6546-6552 (2006); published online EpubJul 1 (66/13/6546 [pii]
10.1158/0008-5472.CAN-06-0384).
26. T. Frisk, T. Foukakis, T. Dwight, J. Lundberg, A. Hoog, G. Wallin, C. Eng, J. Zedenius, C. Larsson, Silencing of the PTEN tumor-suppressor gene in anaplastic thyroid cancer. *Genes Chromosomes Cancer* **35**, 74-80 (2002); published online EpubSep (10.1002/gcc.10098).
27. Y. H. Kang, H. S. Lee, W. H. Kim, Promoter methylation and silencing of PTEN in gastric carcinoma. *Lab Invest* **82**, 285-291 (2002); published online EpubMar (
28. Y. E. Whang, X. Wu, H. Suzuki, R. E. Reiter, C. Tran, R. L. Vessella, J. W. Said, W. B. Isaacs, C. L. Sawyers, Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proc Natl Acad Sci U S A* **95**, 5246-5250 (1998); published online EpubApr 28 (
29. P. L. Dahia, R. C. Aguiar, J. Alberta, J. B. Kum, S. Caron, H. Sill, D. J. Marsh, J. Ritz, A. Freedman, C. Stiles, C. Eng, PTEN is inversely correlated with the cell survival factor Akt/PKB and is inactivated via multiple mechanismsin haematological malignancies. *Hum Mol Genet* **8**, 185-193 (1999); published online EpubFeb (ddc034 [pii]).

30. P. J. Shetty, N. Pasupuleti, S. Chava, K. Nasaruddin, Q. Hasan, Altered transcription and expression of PTEN in breast tumors: is it regulated by hypermethylation? *Breast Dis* **33**, 27-33 (2011)10.3233/BD-2010-0312 B617630393462442 [pii]).
31. N. Bar, R. Dikstein, miR-22 forms a regulatory loop in PTEN/AKT pathway and modulates signaling kinetics. *PLoS One* **5**, e10859 (2010)10.1371/journal.pone.0010859).
32. J. T. Huse, C. Brennan, D. Hambardzumyan, B. Wee, J. Pena, S. H. Rouhanifard, C. Sohn-Lee, C. le Sage, R. Agami, T. Tuschl, E. C. Holland, The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. *Genes Dev* **23**, 1327-1337 (2009); published online EpubJun 1 (10.1101/gad.1777409 23/11/1327 [pii]).
33. F. Meng, R. Henson, H. Wehbe-Janek, K. Ghoshal, S. T. Jacob, T. Patel, MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* **133**, 647-658 (2007); published online EpubAug (S0016-5085(07)01002-5 [pii] 10.1053/j.gastro.2007.05.022).
34. L. Poliseno, L. Salmena, J. Zhang, B. Carver, W. J. Haveman, P. P. Pandolfi, A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* **465**, 1033-1038 (2010); published online EpubJun 24 (10.1038/nature09144 nature09144 [pii]).
35. V. Stambolic, A. Suzuki, J. L. de la Pompa, G. M. Brothers, C. Mirtsos, T. Sasaki, J. Ruland, J. M. Penninger, D. P. Siderovski, T. W. Mak, Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39 (1998).
36. J. A. Engelman, J. Luo, L. C. Cantley, The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* **7**, 606-619 (2006); published online EpubAug (nrg1879 [pii] 10.1038/nrg1879).
37. R. J. Haslam, H. B. Koide, B. A. Hemmings, Pleckstrin domain homology. *Nature* **363**, 309-310 (1993); published online EpubMay 27 (10.1038/363309b0).
38. M. J. Macias, A. Musacchio, H. Ponstingl, M. Nilges, M. Saraste, H. Oschkinat, Structure of the pleckstrin homology domain from beta-spectrin. *Nature* **369**, 675-677 (1994); published online EpubJun 23 (10.1038/369675a0).
39. K. M. Ferguson, M. A. Lemmon, J. Schlessinger, P. B. Sigler, Crystal structure at 2.2 A resolution of the pleckstrin homology domain from human dynamin. *Cell* **79**, 199-209 (1994); published online EpubOct 21 (
40. K. M. Ferguson, M. A. Lemmon, J. Schlessinger, P. B. Sigler, Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* **83**, 1037-1046 (1995); published online EpubDec 15 (

41. S. Koshiba, T. Kigawa, J. H. Kim, M. Shirouzu, D. Bowtell, S. Yokoyama, The solution structure of the pleckstrin homology domain of mouse Son-of-sevenless 1 (mSos1). *Journal of molecular biology* **269**, 579-591 (1997); published online EpubJun 20 (10.1006/jmbi.1997.1041).
42. M. Hyvonen, M. Saraste, Structure of the PH domain and Btk motif from Bruton's tyrosine kinase: molecular explanations for X-linked agammaglobulinaemia. *The EMBO journal* **16**, 3396-3404 (1997); published online EpubJun 16 (10.1093/emboj/16.12.3396).
43. D. R. Alessi, M. Deak, A. Casamayor, F. B. Caudwell, N. Morrice, D. G. Norman, P. Gaffney, C. B. Reese, C. N. MacDougall, D. Harbison, A. Ashworth, M. Bownes, 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol* **7**, 776-789 (1997); published online EpubOct 1 (S0960-9822(06)00336-8 [pii]).
44. D. D. Sarbassov, D. A. Guertin, S. M. Ali, D. M. Sabatini, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098-1101 (2005); published online EpubFeb 18 (307/5712/1098 [pii]
10.1126/science.1106148).
45. J. Feng, J. Park, P. Cron, D. Hess, B. A. Hemmings, Identification of a PKB/Akt hydrophobic motif Ser-473 kinase as DNA-dependent protein kinase. *The Journal of biological chemistry* **279**, 41189-41196 (2004); published online EpubSep 24 (10.1074/jbc.M406731200
M406731200 [pii]).
46. N. Hay, N. Sonenberg, Upstream and downstream of mTOR. *Genes Dev* **18**, 1926-1945 (2004); published online EpubAug 15 (10.1101/gad.1212704
18/16/1926 [pii]).
47. D. H. Kim, D. D. Sarbassov, S. M. Ali, J. E. King, R. R. Latek, H. Erdjument-Bromage, P. Tempst, D. M. Sabatini, mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**, 163-175 (2002); published online EpubJul 26 (S0092867402008085 [pii]).
48. J. R. Testa, P. N. Tsichlis, AKT signaling in normal and malignant cells. *Oncogene* **24**, 7391-7393 (2005); published online EpubNov 14 (10.1038/sj.onc.1209100).
49. N. Fujita, S. Sato, K. Katayama, T. Tsuruo, Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization. *The Journal of biological chemistry* **277**, 28706-28713 (2002); published online EpubAug 9 (10.1074/jbc.M203668200
M203668200 [pii]).
50. I. Vivanco, C. L. Sawyers, The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nature reviews* **2**, 489-501 (2002); published online EpubJul (
51. M. Tamura, J. Gu, E. H. Danen, T. Takino, S. Miyamoto, K. M. Yamada, PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. *The Journal of biological chemistry* **274**, 20693-20703 (1999); published online EpubJul 16 (

52. K. Okumura, M. Zhao, R. A. Depinho, F. B. Furnari, W. K. Cavenee, Cellular transformation by the MSP58 oncogene is inhibited by its physical interaction with the PTEN tumor suppressor. *Proc Natl Acad Sci U S A* **102**, 2703-2706 (2005); published online EpubFeb 22 (0409370102 [pii]
10.1073/pnas.0409370102).
53. Z. Mounir, J. L. Krishnamoorthy, G. P. Robertson, D. Scheuner, R. J. Kaufman, M. M. Georgescu, A. E. Koromilas, Tumor suppression by PTEN requires the activation of the PKR-eIF2alpha phosphorylation pathway. *Sci Signal* **2**, ra85 (2009)10.1126/scisignal.2000389
2/102/ra85 [pii]).
54. X. S. Liu, B. Song, B. D. Elzey, T. L. Ratliff, S. F. Konieczny, L. Cheng, N. Ahmad, X. Liu, Polo-like kinase 1 facilitates loss of Pten tumor suppressor-induced prostate cancer formation. *The Journal of biological chemistry* **286**, 35795-35800 (2011); published online EpubOct 14 (10.1074/jbc.C111.269050
C111.269050 [pii]).
55. M. S. Song, A. Carracedo, L. Salmena, S. J. Song, A. Egia, M. Malumbres, P. P. Pandolfi, Nuclear PTEN regulates the APC-CDH1 tumor-suppressive complex in a phosphatase-independent manner. *Cell* **144**, 187-199 (2011); published online EpubJan 21 (10.1016/j.cell.2010.12.020
S0092-8674(10)01473-X [pii]).
56. J. O. Lee, H. Yang, M. M. Georgescu, A. Di Cristofano, T. Maehama, Y. Shi, J. E. Dixon, P. Pandolfi, N. P. Pavletich, Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell* **99**, 323-334 (1999); published online EpubOct 29 (
57. B. Z. Harris, W. A. Lim, Mechanism and role of PDZ domains in signaling complex assembly. *Journal of cell science* **114**, 3219-3231 (2001); published online EpubSep (
58. S. S. Shenoy, H. Nanda, M. Losche, Membrane association of the PTEN tumor suppressor: electrostatic interaction with phosphatidylserine-containing bilayers and regulatory role of the C-terminal tail. *J Struct Biol* **180**, 394-408 (2012); published online EpubDec (10.1016/j.jsb.2012.10.003
S1047-8477(12)00271-7 [pii]).
59. J. Yuvaniyama, J. M. Denu, J. E. Dixon, M. A. Saper, Crystal structure of the dual specificity protein phosphatase VHR. *Science* **272**, 1328-1331 (1996); published online EpubMay 31 (
60. G. McConnachie, I. Pass, S. M. Walker, C. P. Downes, Interfacial kinetic analysis of the tumour suppressor phosphatase, PTEN: evidence for activation by anionic phospholipids. *Biochem J* **371**, 947-955 (2003); published online EpubMay 1 (10.1042/BJ20021848
BJ20021848 [pii]).
61. X. He, N. Arrotta, D. Radhakrishnan, Y. Wang, T. Romigh, C. Eng, Cowden syndrome-related mutations in PTEN associate with enhanced proteasome activity. *Cancer Res* **73**, 3029-3040 (2013); published online EpubMay 15 (10.1158/0008-5472.CAN-12-3811

0008-5472.CAN-12-3811 [pii]).
62. L. Davidson, H. Maccario, N. M. Perera, X. Yang, L. Spinelli, P. Tibarewal, B. Glancy, A. Gray, C. J. Weijer, C. P. Downes, N. R. Leslie, Suppression of cellular proliferation and invasion by the concerted lipid and protein phosphatase activities of PTEN. *Oncogene* **29**, 687-697 (2010); published online EpubFeb 4 (10.1038/onc.2009.384
onc2009384 [pii]).
63. M. Tamura, J. Gu, K. Matsumoto, S. Aota, R. Parsons, K. M. Yamada, Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science* **280**, 1614-1617 (1998); published online EpubJun 5 (
64. M. P. Myers, I. Pass, I. H. Batty, J. Van der Kaay, J. P. Stolarov, B. A. Hemmings, M. H. Wigler, C. P. Downes, N. K. Tonks, The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc Natl Acad Sci U S A* **95**, 13513-13518 (1998); published online EpubNov 10 (
65. W. Cho, Membrane targeting by C1 and C2 domains. *The Journal of biological chemistry* **276**, 32407-32410 (2001); published online EpubAug 31 (10.1074/jbc.R100007200
R100007200 [pii]).
66. E. A. Nalefski, J. J. Falke, The C2 domain calcium-binding motif: structural and functional diversity. *Protein Sci* **5**, 2375-2390 (1996); published online EpubDec (10.1002/pro.5560051201).
67. J. Huang, J. Yan, J. Zhang, S. Zhu, Y. Wang, T. Shi, C. Zhu, C. Chen, X. Liu, J. Cheng, T. Mustelin, G. S. Feng, G. Chen, J. Yu, SUMO1 modification of PTEN regulates tumorigenesis by controlling its association with the plasma membrane. *Nat Commun* **3**, 911 (2012)10.1038/ncomms1919
ncomms1919 [pii]).
68. F. Vazquez, S. Ramaswamy, N. Nakamura, W. R. Sellers, Phosphorylation of the PTEN Tail Regulates Protein Stability and Function. *Molecular and cellular biology* **20**, 5010-5018 (2000).
69. M. Valiente, A. Andres-Pons, B. Gomar, J. Torres, A. Gil, C. Tapparel, S. E. Antonarakis, R. Pulido, Binding of PTEN to specific PDZ domains contributes to PTEN protein stability and phosphorylation by microtubule-associated serine/threonine kinases. *The Journal of biological chemistry* **280**, 28936-28943 (2005); published online EpubAug 12 (10.1074/jbc.M504761200).
70. T. Ikenoue, K. Inoki, B. Zhao, K. L. Guan, PTEN acetylation modulates its interaction with PDZ domain. *Cancer Res* **68**, 6908-6912 (2008); published online EpubSep 1 (10.1158/0008-5472.CAN-08-1107).
71. A. M. Al-Khouri, Y. Ma, S. H. Togo, S. Williams, T. Mustelin, Cooperative phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) by casein kinases and glycogen synthase kinase 3beta. *The Journal of biological chemistry* **280**, 35195-35202 (2005); published online EpubOct 21 (
72. H. Maccario, N. M. Perera, L. Davidson, C. P. Downes, N. R. Leslie, PTEN is destabilized by phosphorylation on Thr366. *Biochem J* **405**, 439-444 (2007); published online EpubAug 1 (10.1042/BJ20061837).

73. N. Patsoukis, L. Li, D. Sari, V. Petkova, V. A. Boussiotis, PD-1 Increases PTEN Phosphatase Activity While Decreasing PTEN Protein Stability by Inhibiting Casein Kinase 2. *Molecular and cellular biology* **33**, 3091-3098 (2013); published online EpubAug (10.1128/MCB.00319-13).
74. F. Vazquez, S. R. Grossman, Y. Takahashi, M. V. Rokas, N. Nakamura, W. R. Sellers, Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. *The Journal of biological chemistry* **276**, 48627-48630 (2001); published online EpubDec 28 (10.1074/jbc.C100556200).
75. M. Raftopoulou, S. Etienne-Manneville, A. Self, S. Nicholls, A. Hall, Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. *Science* **303**, 1179-1181 (2004); published online EpubFeb 20 (10.1126/science.1092089
303/5661/1179 [pii]).
76. Z. Li, X. Dong, Z. Wang, W. Liu, N. Deng, Y. Ding, L. Tang, T. Hla, R. Zeng, L. Li, D. Wu, Regulation of PTEN by Rho small GTPases. *Nat Cell Biol* **7**, 399-404 (2005); published online EpubApr (
77. X. Wang, L. C. Trotman, T. Koppie, A. Alimonti, Z. Chen, Z. Gao, J. Wang, H. Erdjument-Bromage, P. Tempst, C. Cordon-Cardo, P. P. Pandolfi, X. Jiang, NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. *Cell* **128**, 129-139 (2007); published online EpubJan 12 (S0092-8674(06)01548-0 [pii]
10.1016/j.cell.2006.11.039).
78. E. K. Yim, G. Peng, H. Dai, R. Hu, K. Li, Y. Lu, G. B. Mills, F. Meric-Bernstam, B. T. Hennessy, R. J. Craven, S. Y. Lin, Rak functions as a tumor suppressor by regulating PTEN protein stability and function. *Cancer cell* **15**, 304-314 (2009); published online EpubApr 7 (10.1016/j.ccr.2009.02.012).
79. K. Okumura, M. Mendoza, R. M. Bachoo, R. A. DePinho, W. K. Cavenee, F. B. Furnari, PCAF modulates PTEN activity. *The Journal of biological chemistry* **281**, 26562-26568 (2006); published online EpubSep 8 (
80. Y. Qu, J. Zhang, S. Wu, B. Li, S. Liu, J. Cheng, SIRT1 promotes proliferation and inhibits apoptosis of human malignant glioma cell lines. *Neuroscience letters* **525**, 168-172 (2012); published online EpubSep 13 (10.1016/j.neulet.2012.07.025).
81. L. C. Trotman, X. Wang, A. Alimonti, Z. Chen, J. Teruya-Feldstein, H. Yang, N. P. Pavletich, B. S. Carver, C. Cordon-Cardo, H. Erdjument-Bromage, P. Tempst, S. G. Chi, H. J. Kim, T. Misteli, X. Jiang, P. P. Pandolfi, Ubiquitination regulates PTEN nuclear import and tumor suppression. *Cell* **128**, 141-156 (2007); published online EpubJan 12 (10.1016/j.cell.2006.11.040).
82. F. Fouladkou, T. Landry, H. Kawabe, A. Neeb, C. Lu, N. Brose, V. Stambolic, D. Rotin, The ubiquitin ligase Nedd4-1 is dispensable for the regulation of PTEN stability and localization. *Proc Natl Acad Sci U S A* **105**, 8585-8590 (2008); published online EpubJun 24 (
83. S. Maddika, S. Kavela, N. Rani, V. R. Palicharla, J. L. Pokorny, J. N. Sarkaria, J. Chen, WWP2 is an E3 ubiquitin ligase for PTEN. *Nat Cell Biol* **13**, 728-733 (2011); published online EpubJun (10.1038/ncb2240).

84. J. H. Seo, Y. Ahn, S. R. Lee, C. Yeol Yeo, K. Chung Hur, The major target of the endogenously generated reactive oxygen species in response to insulin stimulation is phosphatase and tensin homolog and not phosphoinositide-3 kinase (PI-3 kinase) in the PI-3 kinase/Akt pathway. *Molecular biology of the cell* **16**, 348-357 (2005); published online EpubJan (10.1091/mbc.E04-05-0369).
85. J. Kwon, S. R. Lee, K. S. Yang, Y. Ahn, Y. J. Kim, E. R. Stadtman, S. G. Rhee, Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. *Proc Natl Acad Sci U S A* **101**, 16419-16424 (2004); published online EpubNov 23 (0407396101 [pii]
10.1073/pnas.0407396101).
86. J. Cao, J. Schulte, A. Knight, N. R. Leslie, A. Zagozdzon, R. Bronson, Y. Manevich, C. Beeson, C. A. Neumann, Prdx1 inhibits tumorigenesis via regulating PTEN/AKT activity. *The EMBO journal* **28**, 1505-1517 (2009); published online EpubMay 20 (10.1038/emboj.2009.101).
87. D. S. Pei, Y. F. Sun, Y. J. Song, S-nitrosylation of PTEN Invovled in ischemic brain injury in rat hippocampal CA1 region. *Neurochem Res* **34**, 1507-1512 (2009); published online EpubAug (10.1007/s11064-009-9938-3).
88. N. Numajiri, K. Takasawa, T. Nishiya, H. Tanaka, K. Ohno, W. Hayakawa, M. Asada, H. Matsuda, K. Azumi, H. Kamata, T. Nakamura, H. Hara, M. Minami, S. A. Lipton, T. Uehara, On-off system for PI3-kinase-Akt signaling through S-nitrosylation of phosphatase with sequence homology to tensin (PTEN). *Proc Natl Acad Sci U S A* **108**, 10349-10354 (2011); published online EpubJun 21 (10.1073/pnas.1103503108).
89. S. M. Planchon, K. A. Waite, C. Eng, The nuclear affairs of PTEN. *Journal of cell science* **121**, 249-253 (2008); published online EpubFeb 1 (10.1242/jcs.022459).
90. M. M. Georgescu, K. H. Kirsch, P. Kaloudis, H. Yang, N. P. Pavletich, H. Hanafusa, Stabilization and productive positioning roles of the C2 domain of PTEN tumor suppressor. *Cancer Res* **60**, 7033-7038 (2000); published online EpubDec 15 (
91. E. Terrien, A. Chaffotte, M. Lafage, Z. Khan, C. Prehaud, F. Cordier, C. Simenel, M. Delepierre, H. Buc, M. Lafon, N. Wolff, Interference with the PTEN-MAST2 interaction by a viral protein leads to cellular relocalization of PTEN. *Sci Signal* **5**, ra58 (2012); published online EpubAug 14 (10.1126/scisignal.2002941).
92. N. B. Adey, L. Huang, P. A. Ormonde, M. L. Baumgard, R. Pero, D. V. Byreddy, S. V. Tavtigian, P. L. Bartel, Threonine phosphorylation of the MMAC1/PTEN PDZ binding domain both inhibits and stimulates PDZ binding. *Cancer Res* **60**, 35-37 (2000); published online EpubJan 1 (
93. A. Perren, P. Komminoth, P. Saremaslani, C. Matter, S. Feurer, J. A. Lees, P. U. Heitz, C. Eng, Mutation and expression analyses reveal differential subcellular compartmentalization of PTEN in endocrine pancreatic tumors compared to normal islet cells. *Am J Pathol* **157**, 1097-1103 (2000); published online EpubOct (

94. G. L. Mutter, M. C. Lin, J. T. Fitzgerald, J. B. Kum, C. Eng, Changes in endometrial PTEN expression throughout the human menstrual cycle. *J Clin Endocrinol Metab* **85**, 2334-2338 (2000).
95. P. Deleris, D. Bacqueville, S. Gayral, L. Carrez, J. P. Salles, B. Perret, M. Breton-Douillon, SHIP-2 and PTEN are expressed and active in vascular smooth muscle cell nuclei, but only SHIP-2 is associated with nuclear speckles. *The Journal of biological chemistry* **278**, 38884-38891 (2003); published online EpubOct 3 (
96. O. Gimm, A. Perren, L. P. Weng, D. J. Marsh, J. J. Yeh, U. Ziebold, E. Gil, R. Hinze, L. Delbridge, J. A. Lees, G. L. Mutter, B. G. Robinson, P. Komminoth, H. Dralle, C. Eng, Differential nuclear and cytoplasmic expression of PTEN in normal thyroid tissue, and benign and malignant epithelial thyroid tumors. *Am J Pathol* **156**, 1693-1700 (2000).
97. M. Tachibana, M. Shibakita, S. Ohno, S. Kinugasa, H. Yoshimura, S. Ueda, T. Fujii, M. A. Rahman, D. K. Dhar, N. Nagasue, Expression and prognostic significance of PTEN product protein in patients with esophageal squamous cell carcinoma. *Cancer* **94**, 1955-1960 (2002); published online EpubApr 1 (
98. D. C. Whiteman, X. P. Zhou, M. C. Cummings, S. Pavey, N. K. Hayward, C. Eng, Nuclear PTEN expression and clinicopathologic features in a population-based series of primary cutaneous melanoma. *Int J Cancer* **99**, 63-67 (2002); published online EpubMay 1 (
99. Y. Lindsay, D. McCoull, L. Davidson, N. R. Leslie, A. Fairservice, A. Gray, J. Lucocq, C. P. Downes, Localization of agonist-sensitive PtdIns(3,4,5)P3 reveals a nuclear pool that is insensitive to PTEN expression. *Journal of cell science* **119**, 5160-5168 (2006); published online EpubDec 15 (
100. J. H. Chung, C. Eng, Nuclear-cytoplasmic partitioning of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) differentially regulates the cell cycle and apoptosis. *Cancer Res* **65**, 8096-8100 (2005); published online EpubSep 15 (
101. T. Minaguchi, K. A. Waite, C. Eng, Nuclear localization of PTEN is regulated by Ca(2+) through a tyrosil phosphorylation-independent conformational modification in major vault protein. *Cancer Res* **66**, 11677-11682 (2006); published online EpubDec 15 (
102. A. Gil, A. Andres-Pons, E. Fernandez, M. Valiente, J. Torres, J. Cervera, R. Pulido, Nuclear localization of PTEN by a Ran-dependent mechanism enhances apoptosis: Involvement of an N-terminal nuclear localization domain and multiple nuclear exclusion motifs. *Molecular biology of the cell* **17**, 4002-4013 (2006); published online EpubSep (
103. J. L. Liu, Z. Mao, T. A. LaFortune, M. M. Alonso, G. E. Gallick, J. Fueyo, W. K. Yung, Cell cycle-dependent nuclear export of phosphatase and tensin homologue tumor suppressor is regulated by the phosphoinositide-3-kinase signaling cascade. *Cancer Res* **67**, 11054-11063 (2007); published online EpubNov 15 (
104. G. Denning, B. Jean-Joseph, C. Prince, D. L. Durden, P. K. Vogt, A short N-terminal sequence of PTEN controls cytoplasmic localization and is required

for suppression of cell growth. *Oncogene* **26**, 3930-3940 (2007); published online EpubJun 7 (1210175 [pii]
10.1038/sj.onc.1210175).
105. W. H. Shen, A. S. Balajee, J. Wang, H. Wu, C. Eng, P. P. Pandolfi, Y. Yin, Essential role for nuclear PTEN in maintaining chromosomal integrity. *Cell* **128**, 157-170 (2007); published online EpubJan 12 (
106. F. Bernassola, M. Karin, A. Ciechanover, G. Melino, The HECT family of E3 ubiquitin ligases: multiple players in cancer development. *Cancer cell* **14**, 10-21 (2008); published online EpubJul 8 (
107. A. Bononi, M. Bonora, S. Marchi, S. Missiroli, F. Poletti, C. Giorgi, P. P. Pandolfi, P. Pinton, Identification of PTEN at the ER and MAMs and its regulation of Ca signaling and apoptosis in a protein phosphatase-dependent manner. *Cell Death Differ*, (2013); published online EpubJun 28 (10.1038/cdd.2013.77
cdd201377 [pii]).
108. B. D. Hopkins, B. Fine, N. Steinbach, M. Dendy, Z. Rapp, J. Shaw, K. Pappas, J. S. Yu, C. Hodakoski, S. Mense, J. Klein, S. Pegno, M. L. Sulis, H. Goldstein, B. Amendolara, L. Lei, M. Maurer, J. Bruce, P. Canoll, H. Hibshoosh, R. Parsons, A Secreted PTEN Phosphatase that Enters Cells to Alter Signaling and Survival. *Science*, (2013); published online EpubJun 6 (science.1234907 [pii]
10.1126/science.1234907).
109. C. L. Bonifant, J. S. Kim, T. Waldman, NHERFs, NEP, MAGUKs, and more: interactions that regulate PTEN. *J Cell Biochem* **102**, 878-885 (2007); published online EpubNov 1 (
110. Y. Takahashi, F. C. Morales, E. L. Kreimann, M. M. Georgescu, PTEN tumor suppressor associates with NHERF proteins to attenuate PDGF receptor signaling. *The EMBO journal* **25**, 910-920 (2006); published online EpubFeb 22 (10.1038/sj.emboj.7600979).
111. J. R. Molina, N. K. Agarwal, F. C. Morales, Y. Hayashi, K. D. Aldape, G. Cote, M. M. Georgescu, PTEN, NHERF1 and PHLPP form a tumor suppressor network that is disabled in glioblastoma. *Oncogene* **31**, 1264-1274 (2012); published online EpubMar 8 (10.1038/onc.2011.324).
112. M. C. Subauste, P. Nalbant, E. D. Adamson, K. M. Hahn, Vinculin controls PTEN protein level by maintaining the interaction of the adherens junction protein beta-catenin with the scaffolding protein MAGI-2. *The Journal of biological chemistry* **280**, 5676-5681 (2005); published online EpubFeb 18 (10.1074/jbc.M405561200).
113. L. Kotelevets, J. van Hengel, E. Bruyneel, M. Mareel, F. van Roy, E. Chastre, The lipid phosphatase activity of PTEN is critical for stabilizing intercellular junctions and reverting invasiveness. *The Journal of cell biology* **155**, 1129-1135 (2001); published online EpubDec 24 (10.1083/jcb.200105109).
114. L. Cotter, M. Ozcelik, C. Jacob, J. A. Pereira, V. Locher, R. Baumann, J. B. Relvas, U. Suter, N. Tricaud, Dlg1-PTEN interaction regulates myelin thickness to prevent damaging peripheral nerve overmyelination. *Science*

328, 1415-1418 (2010); published online EpubJun 11 (10.1126/science.1187735).
115. P. Kreis, M. T. van Diepen, B. J. Eickholt, Regulation of PTEN in neurons by myosin-based transport mechanisms. *Advances in enzyme regulation* **50**, 119-124 (2010)10.1016/j.advenzreg.2009.10.014).
116. N. R. Leslie, L. Spinelli, P. Tibarewal, G. Zilidis, N. Weerasinghe, J. C. Lim, H. Maccario, C. P. Downes, Indirect mechanisms of carcinogenesis via downregulation of PTEN function. *Advances in enzyme regulation* **50**, 112-118 (2010)10.1016/j.advenzreg.2009.10.015).
117. M. T. van Diepen, M. Parsons, C. P. Downes, N. R. Leslie, R. Hindges, B. J. Eickholt, MyosinV controls PTEN function and neuronal cell size. *Nat Cell Biol* **11**, 1191-1196 (2009); published online EpubOct (10.1038/ncb1961).
118. J. Zhou, L. F. Parada, A motor driving PTEN. *Nat Cell Biol* **11**, 1177-1179 (2009); published online EpubOct (10.1038/ncb1009-1177).
119. C. L. Carpenter, L. C. Cantley, Phosphoinositide kinases. *Biochemistry* **29**, 11147-11156 (1990); published online EpubDec 25 (
120. R. B. Chagpar, P. H. Links, M. C. Pastor, L. A. Furber, A. D. Hawrysh, M. D. Chamberlain, D. H. Anderson, Direct positive regulation of PTEN by the p85 subunit of phosphatidylinositol 3-kinase. *Proc Natl Acad Sci U S A* **107**, 5471-5476 (2010); published online EpubMar 23 (10.1073/pnas.0908899107).
121. B. Fine, C. Hodakoski, S. Koujak, T. Su, L. H. Saal, M. Maurer, B. Hopkins, M. Keniry, M. L. Sulis, S. Mense, H. Hibshoosh, R. Parsons, Activation of the PI3K pathway in cancer through inhibition of PTEN by exchange factor P-REX2a. *Science* **325**, 1261-1265 (2009); published online EpubSep 4 (10.1126/science.1173569).
122. L. He, C. Fan, A. Kapoor, A. J. Ingram, A. P. Rybak, R. C. Austin, J. Dickhout, J. C. Cutz, J. Scholey, D. Tang, alpha-Mannosidase 2C1 attenuates PTEN function in prostate cancer cells. *Nat Commun* **2**, 307 (2011)10.1038/ncomms1309).
123. L. He, A. Ingram, A. P. Rybak, D. Tang, Shank-interacting protein-like 1 promotes tumorigenesis via PTEN inhibition in human tumor cells. *J Clin Invest* **120**, 2094-2108 (2010); published online EpubJun (10.1172/JCI40778).
124. Y. C. Kim, H. Kitaura, T. Taira, S. M. Iguchi-Ariga, H. Ariga, Oxidation of DJ-1-dependent cell transformation through direct binding of DJ-1 to PTEN. *International journal of oncology* **35**, 1331-1341 (2009); published online EpubDec (
125. A. Suzuki, J. L. de la Pompa, V. Stambolic, A. J. Elia, T. Sasaki, I. del Barco Barrantes, A. Ho, A. Wakeham, A. Itie, W. Khoo, M. Fukumoto, T. W. Mak, High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Curr Biol* **8**, 1169-1178 (1998); published online EpubOct 22 (S0960-9822(07)00488-5 [pii]).
126. H. Kishimoto, K. Hamada, M. Saunders, S. Backman, T. Sasaki, T. Nakano, T. W. Mak, A. Suzuki, Physiological functions of Pten in mouse tissues. *Cell Struct Funct* **28**, 11-21 (2003); published online EpubFeb (

127. A. Di Cristofano, B. Pesce, C. Cordon-Cardo, P. P. Pandolfi, Pten is essential for embryonic development and tumour suppression. *Nat Genet* **19**, 348-355 (1998); published online EpubAug (10.1038/1235).
128. K. Podsypanina, L. H. Ellenson, A. Nemes, J. Gu, M. Tamura, K. M. Yamada, C. Cordon-Cardo, G. Catoretti, P. E. Fisher, R. Parsons, Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc Natl Acad Sci U S A* **96**, 1563-1568 (1999); published online EpubFeb 16 (
129. M. C. Hollander, A. R. Balogh, J. Liwanag, W. Han, R. I. Linnoila, M. R. Anver, P. A. Dennis, Strain-specific spontaneous and NNK-mediated tumorigenesis in Pten+/- mice. *Neoplasia* **10**, 866-872 (2008); published online EpubAug (
130. V. Stambolic, M. S. Tsao, D. Macpherson, A. Suzuki, W. B. Chapman, T. W. Mak, High incidence of breast and endometrial neoplasia resembling human Cowden syndrome in pten+/- mice. *Cancer Res* **60**, 3605-3611 (2000); published online EpubJul 1 (
131. Y. Li, K. Podsypanina, X. Liu, A. Crane, L. K. Tan, R. Parsons, H. E. Varmus, Deficiency of Pten accelerates mammary oncogenesis in MMTV-Wnt-1 transgenic mice. *BMC Mol Biol* **2**, 2 (2001).
132. L. C. Trotman, M. Niki, Z. A. Dotan, J. A. Koutcher, A. Di Cristofano, A. Xiao, A. S. Khoo, P. Roy-Burman, N. M. Greenberg, T. Van Dyke, C. Cordon-Cardo, P. P. Pandolfi, Pten dose dictates cancer progression in the prostate. *PLoS Biol* **1**, E59 (2003); published online EpubDec (10.1371/journal.pbio.0000059).
133. K. Rajewsky, H. Gu, R. Kuhn, U. A. Betz, W. Muller, J. Roes, F. Schwenk, Conditional gene targeting. *J Clin Invest* **98**, 600-603 (1996); published online EpubAug 1 (10.1172/JCI118828).
134. G. Li, G. W. Robinson, R. Lesche, H. Martinez-Diaz, Z. Jiang, N. Rozengurt, K. U. Wagner, D. C. Wu, T. F. Lane, X. Liu, L. Hennighausen, H. Wu, Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland. *Development* **129**, 4159-4170 (2002); published online EpubSep (
135. S. Wang, J. Gao, Q. Lei, N. Rozengurt, C. Pritchard, J. Jiao, G. V. Thomas, G. Li, P. Roy-Burman, P. S. Nelson, X. Liu, H. Wu, Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer cell* **4**, 209-221 (2003); published online EpubSep (S1535610803002150 [pii]).
136. S. A. Backman, D. Ghazarian, K. So, O. Sanchez, K. U. Wagner, L. Hennighausen, A. Suzuki, M. S. Tsao, W. B. Chapman, V. Stambolic, T. W. Mak, Early onset of neoplasia in the prostate and skin of mice with tissue-specific deletion of Pten. *Proc Natl Acad Sci U S A* **101**, 1725-1730 (2004); published online EpubFeb 10 (10.1073/pnas.0308217100
0308217100 [pii]).
137. X. Ma, A. C. Ziel-van der Made, B. Autar, H. A. van der Korput, M. Vermeij, P. van Duijn, K. B. Cleutjens, R. de Krijger, P. Krimpenfort, A. Berns, T. H. van der Kwast, J. Trapman, Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased

epithelial cell proliferation but not by reduced apoptosis. *Cancer Res* **65**, 5730-5739 (2005); published online EpubJul 1 (65/13/5730 [pii]
10.1158/0008-5472.CAN-04-4519).
138. C. K. Ratnacaram, M. Teletin, M. Jiang, X. Meng, P. Chambon, D. Metzger, Temporally controlled ablation of PTEN in adult mouse prostate epithelium generates a model of invasive prostatic adenocarcinoma. *Proc Natl Acad Sci U S A* **105**, 2521-2526 (2008); published online EpubFeb 19 (10.1073/pnas.0712021105
0712021105 [pii]).
139. S. A. Backman, V. Stambolic, A. Suzuki, J. Haight, A. Elia, J. Pretorius, M. S. Tsao, P. Shannon, B. Bolon, G. O. Ivy, T. W. Mak, Deletion of Pten in mouse brain causes seizures, ataxia and defects in soma size resembling Lhermitte-Duclos disease. *Nat Genet* **29**, 396-403 (2001); published online EpubDec (10.1038/ng782
ng782 [pii]).
140. M. Groszer, R. Erickson, D. D. Scripture-Adams, R. Lesche, A. Trumpp, J. A. Zack, H. I. Kornblum, X. Liu, H. Wu, Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. *Science* **294**, 2186-2189 (2001); published online EpubDec 7 (10.1126/science.1065518
1065518 [pii]).
141. C. H. Kwon, X. Zhu, J. Zhang, L. L. Knoop, R. Tharp, R. J. Smeyne, C. G. Eberhart, P. C. Burger, S. J. Baker, Pten regulates neuronal soma size: a mouse model of Lhermitte-Duclos disease. *Nat Genet* **29**, 404-411 (2001); published online EpubDec (10.1038/ng781
ng781 [pii]).
142. J. Murata, M. Tada, Y. Sawamura, K. Mitsumori, H. Abe, K. Nagashima, Dysplastic gangliocytoma (Lhermitte-Duclos disease) associated with Cowden disease: report of a case and review of the literature for the genetic relationship between the two diseases. *J Neurooncol* **41**, 129-136 (1999); published online EpubJan (
143. C. H. Kwon, B. W. Luikart, C. M. Powell, J. Zhou, S. A. Matheny, W. Zhang, Y. Li, S. J. Baker, L. F. Parada, Pten regulates neuronal arborization and social interaction in mice. *Neuron* **50**, 377-388 (2006); published online EpubMay 4 (S0896-6273(06)00220-0 [pii]
10.1016/j.neuron.2006.03.023).
144. D. Haas-Kogan, D. Stokoe, PTEN in brain tumors. *Expert Rev Neurother* **8**, 599-610 (2008); published online EpubApr (10.1586/14737175.8.4.599).
145. Q. Wei, L. Clarke, D. K. Scheidenhelm, B. Qian, A. Tong, N. Sabha, Z. Karim, N. A. Bock, R. Reti, R. Swoboda, E. Purev, J. F. Lavoie, M. L. Bajenaru, P. Shannon, D. Herlyn, D. Kaplan, R. M. Henkelman, D. H. Gutmann, A. Guha, High-grade glioma formation results from postnatal pten loss or mutant epidermal growth factor receptor expression in a transgenic mouse glioma model. *Cancer Res* **66**, 7429-7437 (2006); published online EpubAug 1 (66/15/7429 [pii]
10.1158/0008-5472.CAN-06-0712).

146. A. Suzuki, M. T. Yamaguchi, T. Ohteki, T. Sasaki, T. Kaisho, Y. Kimura, R. Yoshida, A. Wakeham, T. Higuchi, M. Fukumoto, T. Tsubata, P. S. Ohashi, S. Koyasu, J. M. Penninger, T. Nakano, T. W. Mak, T cell-specific loss of Pten leads to defects in central and peripheral tolerance. *Immunity* **14**, 523-534 (2001); published online EpubMay (S1074-7613(01)00134-0 [pii]).
147. A. Suzuki, T. Kaisho, M. Ohishi, M. Tsukio-Yamaguchi, T. Tsubata, P. A. Koni, T. Sasaki, T. W. Mak, T. Nakano, Critical roles of Pten in B cell homeostasis and immunoglobulin class switch recombination. *J Exp Med* **197**, 657-667 (2003); published online EpubMar 3 (
148. L. Pasqualucci, G. Bhagat, M. Jankovic, M. Compagno, P. Smith, M. Muramatsu, T. Honjo, H. C. Morse, 3rd, M. C. Nussenzweig, R. Dalla-Favera, AID is required for germinal center-derived lymphomagenesis. *Nat Genet* **40**, 108-112 (2008); published online EpubJan (ng.2007.35 [pii]
10.1038/ng.2007.35).
149. J. Zhang, J. C. Grindley, T. Yin, S. Jayasinghe, X. C. He, J. T. Ross, J. S. Haug, D. Rupp, K. S. Porter-Westpfahl, L. M. Wiedemann, H. Wu, L. Li, PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature* **441**, 518-522 (2006); published online EpubMay 25 (nature04747 [pii]
10.1038/nature04747).
150. O. H. Yilmaz, R. Valdez, B. K. Theisen, W. Guo, D. O. Ferguson, H. Wu, S. J. Morrison, Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* **441**, 475-482 (2006); published online EpubMay 25 (nature04703 [pii]
10.1038/nature04703).
151. Y. Horie, A. Suzuki, E. Kataoka, T. Sasaki, K. Hamada, J. Sasaki, K. Mizuno, G. Hasegawa, H. Kishimoto, M. Iizuka, M. Naito, K. Enomoto, S. Watanabe, T. W. Mak, T. Nakano, Hepatocyte-specific Pten deficiency results in steatohepatitis and hepatocellular carcinomas. *J Clin Invest* **113**, 1774-1783 (2004); published online EpubJun (10.1172/JCI20513).
152. C. Kurlawalla-Martinez, B. Stiles, Y. Wang, S. U. Devaskar, B. B. Kahn, H. Wu, Insulin hypersensitivity and resistance to streptozotocin-induced diabetes in mice lacking PTEN in adipose tissue. *Molecular and cellular biology* **25**, 2498-2510 (2005); published online EpubMar (25/6/2498 [pii]
10.1128/MCB.25.6.2498-2510.2005).
153. K. T. Nguyen, P. Tajmir, C. H. Lin, N. Liadis, X. D. Zhu, M. Eweida, G. Tolasa-Karaman, F. Cai, R. Wang, T. Kitamura, D. D. Belsham, M. B. Wheeler, A. Suzuki, T. W. Mak, M. Woo, Essential role of Pten in body size determination and pancreatic beta-cell homeostasis in vivo. *Molecular and cellular biology* **26**, 4511-4518 (2006); published online EpubJun (26/12/4511 [pii]
10.1128/MCB.00238-06).
154. L. Plum, X. Ma, B. Hampel, N. Balthasar, R. Coppari, H. Munzberg, M. Shanabrough, D. Burdakov, E. Rother, R. Janoschek, J. Alber, B. F. Belgardt, L. Koch, J. Seibler, F. Schwenk, C. Fekete, A. Suzuki, T. W. Mak, W. Krone, T. L. Horvath, F. M. Ashcroft, J. C. Bruning, Enhanced PIP3

signaling in POMC neurons causes KATP channel activation and leads to diet-sensitive obesity. *J Clin Invest* **116**, 1886-1901 (2006); published online EpubJul (10.1172/JCI27123).
155. B. Stiles, Y. Wang, A. Stahl, S. Bassilian, W. P. Lee, Y. J. Kim, R. Sherwin, S. Devaskar, R. Lesche, M. A. Magnuson, H. Wu, Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity [corrected]. *Proc Natl Acad Sci U S A* **101**, 2082-2087 (2004); published online EpubFeb 17 (10.1073/pnas.0308617100
0308617100 [pii]).
156. L. C. Cantley, B. G. Neel, New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A* **96**, 4240-4245 (1999); published online EpubApr 13 (
157. L. Simpson, R. Parsons, PTEN: life as a tumor suppressor. *Exp Cell Res* **264**, 29-41 (2001); published online EpubMar 10 (10.1006/excr.2000.5130
S0014-4827(00)95130-9 [pii]).
158. J. Puc, R. Parsons, PTEN loss inhibits CHK1 to cause double stranded-DNA breaks in cells. *Cell Cycle* **4**, 927-929 (2005); published online EpubJul (1795 [pii]).
159. A. Gupta, Q. Yang, R. K. Pandita, C. R. Hunt, T. Xiang, S. Misri, S. Zeng, J. Pagan, J. Jeffery, J. Puc, R. Kumar, Z. Feng, S. N. Powell, A. Bhat, T. Yaguchi, R. Wadhwa, S. C. Kaul, R. Parsons, K. K. Khanna, T. K. Pandita, Cell cycle checkpoint defects contribute to genomic instability in PTEN deficient cells independent of DNA DSB repair. *Cell cycle* **8**, 2198-2210 (2009); published online EpubJul 15 (
160. M. Masson, V. Rolli, F. Dantzer, C. Trucco, V. Schreiber, S. Fribourg, M. Molinete, A. Ruf, E. A. Miranda, C. Niedergang, et al., Poly(ADP-ribose) polymerase: structure-function relationship. *Biochimie* **77**, 456-461 (1995).
161. K. Sugimura, S. Takebayashi, H. Taguchi, S. Takeda, K. Okumura, PARP-1 ensures regulation of replication fork progression by homologous recombination on damaged DNA. *The Journal of cell biology* **183**, 1203-1212 (2008); published online EpubDec 29 (10.1083/jcb.200806068
jcb.200806068 [pii]).
162. L. Virag, Structure and function of poly(ADP-ribose) polymerase-1: role in oxidative stress-related pathologies. *Current vascular pharmacology* **3**, 209-214 (2005); published online EpubJul (
163. A. M. Mendes-Pereira, S. A. Martin, R. Brough, A. McCarthy, J. R. Taylor, J. S. Kim, T. Waldman, C. J. Lord, A. Ashworth, Synthetic lethal targeting of PTEN mutant cells with PARP inhibitors. *EMBO Mol Med* **1**, 315-322 (2009); published online EpubSep (10.1002/emmm.200900041).
164. K. J. Dedes, D. Wetterskog, A. M. Mendes-Pereira, R. Natrajan, M. B. Lambros, F. C. Geyer, R. Vatcheva, K. Savage, A. Mackay, C. J. Lord, A. Ashworth, J. S. Reis-Filho, PTEN deficiency in endometrioid endometrial adenocarcinomas predicts sensitivity to PARP inhibitors. *Sci Transl Med* **2**, 53ra75 (2010); published online EpubOct 13 (10.1126/scitranslmed.3001538
2/53/53ra75 [pii]).

165. M. D. Forster, K. J. Dedes, S. Sandhu, S. Frentzas, R. Kristeleit, A. Ashworth, C. J. Poole, B. Weigelt, S. B. Kaye, L. R. Molife, Treatment with olaparib in a patient with PTEN-deficient endometrioid endometrial cancer. *Nature reviews. Clinical oncology* **8**, 302-306 (2011); published online EpubMay (10.1038/nrclinonc.2011.42).
166. L. A. Loeb, R. J. Monnat, Jr., DNA polymerases and human disease. *Nat Rev Genet* **9**, 594-604 (2008); published online EpubAug (10.1038/nrg2345
nrg2345 [pii]).
167. J. H. Hoeijmakers, Genome maintenance mechanisms for preventing cancer. *Nature* **411**, 366-374 (2001); published online EpubMay 17 (10.1038/35077232).
168. J. Bartek, J. Lukas, DNA damage checkpoints: from initiation to recovery or adaptation. *Current opinion in cell biology* **19**, 238-245 (2007); published online EpubApr (10.1016/j.ceb.2007.02.009).
169. Y. Shiloh, ATM and related protein kinases: safeguarding genome integrity. *Nature reviews* **3**, 155-168 (2003); published online EpubMar (10.1038/nrc1011).
170. J. Lukas, C. Lukas, J. Bartek, Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time. *DNA repair* **3**, 997-1007 (2004); published online EpubAug-Sep (10.1016/j.dnarep.2004.03.006).
171. J. Bartek, C. Lukas, J. Lukas, Checking on DNA damage in S phase. *Nature reviews. Molecular cell biology* **5**, 792-804 (2004); published online EpubOct (10.1038/nrm1493).
172. M. B. Kastan, J. Bartek, Cell-cycle checkpoints and cancer. *Nature* **432**, 316-323 (2004); published online EpubNov 18 (10.1038/nature03097).
173. J. Smith, L. M. Tho, N. Xu, D. A. Gillespie, The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv Cancer Res* **108**, 73-112 (2010)10.1016/B978-0-12-380888-2.00003-0
B978-0-12-380888-2.00003-0 [pii]).
174. J. Campisi, F. d'Adda di Fagagna, Cellular senescence: when bad things happen to good cells. *Nature reviews. Molecular cell biology* **8**, 729-740 (2007); published online EpubSep (10.1038/nrm2233).
175. T. D. Halazonetis, V. G. Gorgoulis, J. Bartek, An oncogene-induced DNA damage model for cancer development. *Science* **319**, 1352-1355 (2008); published online EpubMar 7 (10.1126/science.1140735).
176. J. Jiricny, The multifaceted mismatch-repair system. *Nature reviews. Molecular cell biology* **7**, 335-346 (2006); published online EpubMay (nrm1907 [pii]
10.1038/nrm1907).
177. S. S. David, V. L. O'Shea, S. Kundu, Base-excision repair of oxidative DNA damage. *Nature* **447**, 941-950 (2007); published online EpubJun 21 (nature05978 [pii]
10.1038/nature05978).
178. K. K. Khanna, S. P. Jackson, DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet* **27**, 247-254 (2001); published online EpubMar (10.1038/85798).

179. M. R. Lieber, The mechanism of human nonhomologous DNA end joining. *The Journal of biological chemistry* **283**, 1-5 (2008); published online EpubJan 4 (R700039200 [pii]
10.1074/jbc.R700039200).
180. J. San Filippo, P. Sung, H. Klein, Mechanism of eukaryotic homologous recombination. *Annu Rev Biochem* **77**, 229-257 (2008)10.1146/annurev.biochem.77.061306.125255).
181. E. Weterings, D. J. Chen, The endless tale of non-homologous end-joining. *Cell Res* **18**, 114-124 (2008); published online EpubJan (10.1038/cr.2008.3
cr20083 [pii]).
182. J. M. Bradbury, S. P. Jackson, The complex matter of DNA double-strand break detection. *Biochem Soc Trans* **31**, 40-44 (2003); published online EpubFeb (10.1042/).
183. M. Podhorecka, A. Skladanowski, P. Bozko, H2AX Phosphorylation: Its Role in DNA Damage Response and Cancer Therapy. *J Nucleic Acids* **2010**, (2010)10.4061/2010/920161
920161 [pii]).
184. A. Shibata, S. Conrad, J. Birraux, V. Geuting, O. Barton, A. Ismail, A. Kakarougkas, K. Meek, G. Taucher-Scholz, M. Lobrich, P. A. Jeggo, Factors determining DNA double-strand break repair pathway choice in G2 phase. *The EMBO journal* **30**, 1079-1092 (2011); published online EpubMar 16 (10.1038/emboj.2011.27
emboj201127 [pii]).
185. L. S. Teng, Y. Zheng, H. H. Wang, BRCA1/2 associated hereditary breast cancer. *J Zhejiang Univ Sci B* **9**, 85-89 (2008); published online EpubFeb (10.1631/jzus.B0710617).
186. C. X. Deng, S. G. Brodie, Roles of BRCA1 and its interacting proteins. *Bioessays* **22**, 728-737 (2000); published online EpubAug (10.1002/1521-1878(200008)22:8<728::AID-BIES6>3.0.CO;2-B [pii]
10.1002/1521-1878(200008)22:8<728::AID-BIES6>3.0.CO;2-B).
187. A. R. Venkitaraman, Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* **108**, 171-182 (2002); published online EpubJan 25 (S0092867402006153 [pii]).
188. J. H. Lee, A. A. Goodarzi, P. A. Jeggo, T. T. Paull, 53BP1 promotes ATM activity through direct interactions with the MRN complex. *The EMBO journal* **29**, 574-585 (2010); published online EpubFeb 3 (10.1038/emboj.2009.372
emboj2009372 [pii]).
189. S. Panier, S. J. Boulton, Double-strand break repair: 53BP1 comes into focus. *Nature reviews. Molecular cell biology* **15**, 7-18 (2014); published online EpubJan (10.1038/nrm3719
nrm3719 [pii]).
190. D. C. van Gent, M. van der Burg, Non-homologous end-joining, a sticky affair. *Oncogene* **26**, 7731-7740 (2007); published online EpubDec 10 (10.1038/sj.onc.1210871).

191. L. Krejci, V. Altmannova, M. Spirek, X. Zhao, Homologous recombination and its regulation. *Nucleic acids research* **40**, 5795-5818 (2012); published online EpubJul (10.1093/nar/gks270
gks270 [pii]).
192. M. van den Bosch, R. T. Bree, N. F. Lowndes, The MRN complex: coordinating and mediating the response to broken chromosomes. *EMBO Rep* **4**, 844-849 (2003); published online EpubSep (10.1038/sj.embor.embor925
embor925 [pii]).
193. N. Suwaki, K. Klare, M. Tarsounas, RAD51 paralogs: roles in DNA damage signalling, recombinational repair and tumorigenesis. *Semin Cell Dev Biol* **22**, 898-905 (2011); published online EpubOct (10.1016/j.semcdb.2011.07.019
S1084-9521(11)00107-8 [pii]).
194. A. Shinohara, M. Shinohara, Roles of RecA homologues Rad51 and Dmc1 during meiotic recombination. *Cytogenet Genome Res* **107**, 201-207 (2004)80598 [pii]
10.1159/000080598).
195. P. Sung, L. Krejci, S. Van Komen, M. G. Sehorn, Rad51 recombinase and recombination mediators. *The Journal of biological chemistry* **278**, 42729-42732 (2003); published online EpubOct 31 (10.1074/jbc.R300027200
R300027200 [pii]).
196. M. Jasin, R. Rothstein, Repair of strand breaks by homologous recombination. *Cold Spring Harb Perspect Biol* **5**, a012740 (2013); published online EpubNov (10.1101/cshperspect.a012740
a012740 [pii]
cshperspect.a012740 [pii]).
197. P. Swuec, A. Costa, Molecular mechanism of double Holliday junction dissolution. *Cell Biosci* **4**, 36 (2014)10.1186/2045-3701-4-36
2045-3701-4-36 [pii]).
198. A. Malkova, G. Ira, Break-induced replication: functions and molecular mechanism. *Curr Opin Genet Dev* **23**, 271-279 (2013); published online EpubJun (10.1016/j.gde.2013.05.007
S0959-437X(13)00084-1 [pii]).
199. S. H. Yang, A. Galanis, J. Witty, A. D. Sharrocks, An extended consensus motif enhances the specificity of substrate modification by SUMO. *The EMBO journal* **25**, 5083-5093 (2006); published online EpubNov 1 (7601383 [pii]
10.1038/sj.emboj.7601383).
200. H. Saitoh, J. Hinchey, Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. *The Journal of biological chemistry* **275**, 6252-6258 (2000); published online EpubMar 3 (
201. K. I. Kim, S. H. Baek, C. H. Chung, Versatile protein tag, SUMO: its enzymology and biological function. *J Cell Physiol* **191**, 257-268 (2002); published online EpubJun (10.1002/jcp.10100).

202. Y. Wang, M. Dasso, SUMOylation and deSUMOylation at a glance. *Journal of cell science* **122**, 4249-4252 (2009); published online EpubDec 1 (10.1242/jcs.050542
122/23/4249 [pii]).
203. T. Hayashi, M. Seki, D. Maeda, W. Wang, Y. Kawabe, T. Seki, H. Saitoh, T. Fukagawa, H. Yagi, T. Enomoto, Ubc9 is essential for viability of higher eukaryotic cells. *Exp Cell Res* **280**, 212-221 (2002); published online EpubNov 1 (S001448270295634X [pii]).
204. M. Hochstrasser, SP-RING for SUMO: new functions bloom for a ubiquitin-like protein. *Cell* **107**, 5-8 (2001); published online EpubOct 5 (S0092-8674(01)00519-0 [pii]).
205. A. Pichler, A. Gast, J. S. Seeler, A. Dejean, F. Melchior, The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell* **108**, 109-120 (2002); published online EpubJan 11 (S009286740100633X [pii]).
206. M. H. Kagey, T. A. Melhuish, D. Wotton, The polycomb protein Pc2 is a SUMO E3. *Cell* **113**, 127-137 (2003); published online EpubApr 4 (S0092867403001594 [pii]).
207. M. H. Tatham, E. Jaffray, O. A. Vaughan, J. M. Desterro, C. H. Botting, J. H. Naismith, R. T. Hay, Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *The Journal of biological chemistry* **276**, 35368-35374 (2001); published online EpubSep 21 (10.1074/jbc.M104214200).
208. S. J. Li, M. Hochstrasser, A new protease required for cell-cycle progression in yeast. *Nature* **398**, 246-251 (1999); published online EpubMar 18 (10.1038/18457).
209. E. S. Johnson, I. Schwienhorst, R. J. Dohmen, G. Blobel, The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. *The EMBO journal* **16**, 5509-5519 (1997); published online EpubSep 15 (10.1093/emboj/16.18.5509).
210. F. Golebiowski, M. H. Tatham, A. Nakamura, R. T. Hay, High-stringency tandem affinity purification of proteins conjugated to ubiquitin-like moieties. *Nat Protoc* **5**, 873-882 (2010); published online EpubMay (10.1038/nprot.2010.40
nprot.2010.40 [pii]).
211. J. S. Seeler, A. Dejean, Nuclear and unclear functions of SUMO. *Nature reviews. Molecular cell biology* **4**, 690-699 (2003); published online EpubSep (10.1038/nrm1200
nrm1200 [pii]).
212. R. S. Hilgarth, L. A. Murphy, H. S. Skaggs, D. C. Wilkerson, H. Xing, K. D. Sarge, Regulation and function of SUMO modification. *The Journal of biological chemistry* **279**, 53899-53902 (2004); published online EpubDec 24 (10.1074/jbc.R400021200
R400021200 [pii]).
213. E. Duprez, A. J. Saurin, J. M. Desterro, V. Lallemand-Breitenbach, K. Howe, M. N. Boddy, E. Solomon, H. de The, R. T. Hay, P. S. Freemont, SUMO-1 modification of the acute promyelocytic leukaemia protein PML: implications

for nuclear localisation. *Journal of cell science* **112 (Pt 3)**, 381-393 (1999); published online EpubFeb (
214. T. Kamitani, K. Kito, H. P. Nguyen, H. Wada, T. Fukuda-Kamitani, E. T. Yeh, Identification of three major sentrinization sites in PML. *The Journal of biological chemistry* **273**, 26675-26682 (1998); published online EpubOct 9 (
215. D. Ungureanu, S. Vanhatupa, N. Kotaja, J. Yang, S. Aittomaki, O. A. Janne, J. J. Palvimo, O. Silvennoinen, PIAS proteins promote SUMO-1 conjugation to STAT1. *Blood* **102**, 3311-3313 (2003); published online EpubNov 1 (10.1182/blood-2002-12-3816
2002-12-3816 [pii]).
216. S. H. Yang, E. Jaffray, R. T. Hay, A. D. Sharrocks, Dynamic interplay of the SUMO and ERK pathways in regulating Elk-1 transcriptional activity. *Mol Cell* **12**, 63-74 (2003); published online EpubJul (S109727650300265X [pii]).
217. S. Ross, J. L. Best, L. I. Zon, G. Gill, SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol Cell* **10**, 831-842 (2002); published online EpubOct (S1097276502006822 [pii]).
218. J. Bies, J. Markus, L. Wolff, Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. *The Journal of biological chemistry* **277**, 8999-9009 (2002); published online EpubMar 15 (10.1074/jbc.M110453200
M110453200 [pii]).
219. K. L. Hari, K. R. Cook, G. H. Karpen, The Drosophila Su(var)2-10 locus regulates chromosome structure and function and encodes a member of the PIAS protein family. *Genes Dev* **15**, 1334-1348 (2001); published online EpubJun 1 (10.1101/gad.877901).
220. A. V. Strunnikov, L. Aravind, E. V. Koonin, Saccharomyces cerevisiae SMT4 encodes an evolutionarily conserved protease with a role in chromosome condensation regulation. *Genetics* **158**, 95-107 (2001); published online EpubMay (
221. A. F. Pluta, W. C. Earnshaw, I. G. Goldberg, Interphase-specific association of intrinsic centromere protein CENP-C with HDaxx, a death domain-binding protein implicated in Fas-mediated cell death. *Journal of cell science* **111 (Pt 14)**, 2029-2041 (1998); published online EpubJul 30 (
222. J. Joseph, S. H. Tan, T. S. Karpova, J. G. McNally, M. Dasso, SUMO-1 targets RanGAP1 to kinetochores and mitotic spindles. *The Journal of cell biology* **156**, 595-602 (2002); published online EpubFeb 18 (10.1083/jcb.200110109
jcb.200110109 [pii]).
223. R. D. Everett, W. C. Earnshaw, J. Findlay, P. Lomonte, Specific destruction of kinetochore protein CENP-C and disruption of cell division by herpes simplex virus immediate-early protein Vmw110. *The EMBO journal* **18**, 1526-1538 (1999); published online EpubMar 15 (10.1093/emboj/18.6.1526).
224. R. Mahajan, C. Delphin, T. Guan, L. Gerace, F. Melchior, A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* **88**, 97-107 (1997); published online EpubJan 10 (S0092-8674(00)81862-0 [pii]).

225. K. Weis, Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. *Cell* **112**, 441-451 (2003); published online EpubFeb 21 (S0092867403000825 [pii]).
226. D. Baba, N. Maita, J. G. Jee, Y. Uchimura, H. Saitoh, K. Sugasawa, F. Hanaoka, H. Tochio, H. Hiroaki, M. Shirakawa, Crystal structure of thymine DNA glycosylase conjugated to SUMO-1. *Nature* **435**, 979-982 (2005); published online EpubJun 16 (nature03634 [pii]
10.1038/nature03634).
227. R. Steinacher, P. Schar, Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. *Curr Biol* **15**, 616-623 (2005); published online EpubApr 12 (S0960-9822(05)00227-7 [pii]
10.1016/j.cub.2005.02.054).
228. C. Smet-Nocca, J. M. Wieruszeski, H. Leger, S. Eilebrecht, A. Benecke, SUMO-1 regulates the conformational dynamics of thymine-DNA Glycosylase regulatory domain and competes with its DNA binding activity. *BMC Biochem* **12**, 4 (2011)10.1186/1471-2091-12-4
1471-2091-12-4 [pii]).
229. Y. Mao, S. D. Desai, L. F. Liu, SUMO-1 conjugation to human DNA topoisomerase II isozymes. *The Journal of biological chemistry* **275**, 26066-26073 (2000); published online EpubAug 25 (10.1074/jbc.M001831200
M001831200 [pii]).
230. Y. Mao, M. Sun, S. D. Desai, L. F. Liu, SUMO-1 conjugation to topoisomerase I: A possible repair response to topoisomerase-mediated DNA damage. *Proc Natl Acad Sci U S A* **97**, 4046-4051 (2000); published online EpubApr 11 (10.1073/pnas.080536597
080536597 [pii]).
231. Y. Galanty, R. Belotserkovskaya, J. Coates, S. Polo, K. M. Miller, S. P. Jackson, Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. *Nature* **462**, 935-939 (2009); published online EpubDec 17 (10.1038/nature08657
nature08657 [pii]).
232. J. R. Morris, C. Boutell, M. Keppler, R. Densham, D. Weekes, A. Alamshah, L. Butler, Y. Galanty, L. Pangon, T. Kiuchi, T. Ng, E. Solomon, The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. *Nature* **462**, 886-890 (2009); published online EpubDec 17 (10.1038/nature08593
nature08593 [pii]).
233. C. A. Cremona, P. Sarangi, Y. Yang, L. E. Hang, S. Rahman, X. Zhao, Extensive DNA damage-induced sumoylation contributes to replication and repair and acts in addition to the mec1 checkpoint. *Mol Cell* **45**, 422-432 (2012); published online EpubFeb 10 (10.1016/j.molcel.2011.11.028
S1097-2765(11)00995-6 [pii]).
234. J. Torres-Rosell, I. Sunjevaric, G. De Piccoli, M. Sacher, N. Eckert-Boulet, R. Reid, S. Jentsch, R. Rothstein, L. Aragon, M. Lisby, The Smc5-Smc6 complex and SUMO modification of Rad52 regulates recombinational repair

at the ribosomal gene locus. *Nat Cell Biol* **9**, 923-931 (2007); published online EpubAug (ncb1619 [pii]
10.1038/ncb1619).
235. I. Onn, J. M. Heidinger-Pauli, V. Guacci, E. Unal, D. E. Koshland, Sister chromatid cohesion: a simple concept with a complex reality. *Annu Rev Cell Dev Biol* **24**, 105-129 (2008)10.1146/annurev.cellbio.24.110707.175350).
236. A. McAleenan, V. Cordon-Preciado, A. Clemente-Blanco, I. C. Liu, N. Sen, J. Leonard, A. Jarmuz, L. Aragon, SUMOylation of the alpha-kleisin subunit of cohesin is required for DNA damage-induced cohesion. *Curr Biol* **22**, 1564-1575 (2012); published online EpubSep 11 (10.1016/j.cub.2012.06.045
S0960-9822(12)00721-X [pii]).
237. C. Guo, J. M. Henley, Wrestling with stress: roles of protein SUMOylation and deSUMOylation in cell stress response. *IUBMB Life* **66**, 71-77 (2014); published online EpubFeb (10.1002/iub.1244).
238. J. R. Danielsen, L. K. Povlsen, B. H. Villumsen, W. Streicher, J. Nilsson, M. Wikstrom, S. Bekker-Jensen, N. Mailand, DNA damage-inducible SUMOylation of HERC2 promotes RNF8 binding via a novel SUMO-binding Zinc finger. *The Journal of cell biology* **197**, 179-187 (2012); published online EpubApr 16 (10.1083/jcb.201106152
jcb.201106152 [pii]).
239. J. J. Hudson, S. C. Chiang, O. S. Wells, C. Rookyard, S. F. El-Khamisy, SUMO modification of the neuroprotective protein TDP1 facilitates chromosomal single-strand break repair. *Nat Commun* **3**, 733 (2012)10.1038/ncomms1739
ncomms1739 [pii]).
240. H. Dou, C. Huang, M. Singh, P. B. Carpenter, E. T. Yeh, Regulation of DNA repair through deSUMOylation and SUMOylation of replication protein A complex. *Mol Cell* **39**, 333-345 (2010); published online EpubAug 13 (10.1016/j.molcel.2010.07.021
S1097-2765(10)00567-8 [pii]).
241. K. J. Ouyang, L. L. Woo, J. Zhu, D. Huo, M. J. Matunis, N. A. Ellis, SUMO modification regulates BLM and RAD51 interaction at damaged replication forks. *PLoS Biol* **7**, e1000252 (2009); published online EpubDec (10.1371/journal.pbio.1000252).
242. J. J. Perry, J. A. Tainer, M. N. Boddy, A SIM-ultaneous role for SUMO and ubiquitin. *Trends in biochemical sciences* **33**, 201-208 (2008); published online EpubMay (10.1016/j.tibs.2008.02.001).
243. C. J. Chang, D. J. Mulholland, B. Valamehr, S. Mosessian, W. R. Sellers, H. Wu, PTEN nuclear localization is regulated by oxidative stress and mediates p53-dependent tumor suppression. *Molecular and cellular biology* **28**, 3281-3289 (2008); published online EpubMay (10.1128/MCB.00310-08
MCB.00310-08 [pii]).
244. J. Anckar, L. Sistonen, SUMO: getting it on. *Biochem Soc Trans* **35**, 1409-1413 (2007); published online EpubDec (BST0351409 [pii]
10.1042/BST0351409).

245. S. M. Jeram, T. Srikumar, X. D. Zhang, H. Anne Eisenhauer, R. Rogers, P. G. Pedrioli, M. Matunis, B. Raught, An improved SUMmOn-based methodology for the identification of ubiquitin and ubiquitin-like protein conjugation sites identifies novel ubiquitin-like protein chain linkages. *Proteomics* **10**, 254-265 (2010); published online EpubJan (10.1002/pmic.200900648).
246. P. G. Pedrioli, B. Raught, X. D. Zhang, R. Rogers, J. Aitchison, M. Matunis, R. Aebersold, Automated identification of SUMOylation sites using mass spectrometry and SUMmOn pattern recognition software. *Nat Methods* **3**, 533-539 (2006); published online EpubJul (nmeth891 [pii]
10.1038/nmeth891).
247. Y. Samuels, L. A. Diaz, Jr., O. Schmidt-Kittler, J. M. Cummins, L. Delong, I. Cheong, C. Rago, D. L. Huso, C. Lengauer, K. W. Kinzler, B. Vogelstein, V. E. Velculescu, Mutant PIK3CA promotes cell growth and invasion of human cancer cells. *Cancer cell* **7**, 561-573 (2005); published online EpubJun (S1535-6108(05)00160-1 [pii]
10.1016/j.ccr.2005.05.014).
248. B. Wang, S. Matsuoka, B. A. Ballif, D. Zhang, A. Smogorzewska, S. P. Gygi, S. J. Elledge, Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. *Science* **316**, 1194-1198 (2007); published online EpubMay 25 (316/5828/1194 [pii]
10.1126/science.1139476).
249. M. V. Botuyan, J. Lee, I. M. Ward, J. E. Kim, J. R. Thompson, J. Chen, G. Mer, Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. *Cell* **127**, 1361-1373 (2006); published online EpubDec 29 (S0092-8674(06)01525-X [pii]
10.1016/j.cell.2006.10.043).
250. L. H. Thompson, Recognition, signaling, and repair of DNA double-strand breaks produced by ionizing radiation in mammalian cells: The molecular choreography. *Mutat Res*, (2012); published online EpubJun 26 (S1383-5742(12)00045-2 [pii]
10.1016/j.mrrev.2012.06.002).
251. V. Costanzo, Brca2, Rad51 and Mre11: performing balancing acts on replication forks. *DNA repair* **10**, 1060-1065 (2011); published online EpubOct 10 (S1568-7864(11)00200-X [pii]
10.1016/j.dnarep.2011.07.009).
252. M. Fraser, H. Zhao, K. R. Luoto, C. Lundin, C. Coackley, N. Chan, A. M. Joshua, T. A. Bismar, A. Evans, T. Helleday, R. G. Bristow, PTEN deletion in prostate cancer cells does not associate with loss of RAD51 function: implications for radiotherapy and chemotherapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* **18**, 1015-1027 (2012); published online EpubFeb 15 (1078-0432.CCR-11-2189 [pii]
10.1158/1078-0432.CCR-11-2189).
253. D. Kalderon, W. D. Richardson, A. F. Markham, A. E. Smith, Sequence requirements for nuclear location of simian virus 40 large-T antigen. *Nature* **311**, 33-38 (1984); published online EpubSep 6-11 (

254. B. Elliott, M. Jasin, Repair of double-strand breaks by homologous recombination in mismatch repair-defective mammalian cells. *Molecular and cellular biology* **21**, 2671-2682 (2001); published online EpubApr (10.1128/MCB.21.8.2671-2682.2001).
255. P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, M. R. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **82**, 1107-1112 (1990); published online EpubJul 4 (
256. J. Bartkova, Z. Horejsi, K. Koed, A. Kramer, F. Tort, K. Zieger, P. Guldberg, M. Sehested, J. M. Nesland, C. Lukas, T. Orntoft, J. Lukas, J. Bartek, DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* **434**, 864-870 (2005); published online EpubApr 14 (nature03482 [pii]
10.1038/nature03482).
257. S. Matsuoka, B. A. Ballif, A. Smogorzewska, E. R. McDonald, 3rd, K. E. Hurov, J. Luo, C. E. Bakalarski, Z. Zhao, N. Solimini, Y. Lerenthal, Y. Shiloh, S. P. Gygi, S. J. Elledge, ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* **316**, 1160-1166 (2007); published online EpubMay 25 (316/5828/1160 [pii]
10.1126/science.1140321).
258. S. Bergink, S. Jentsch, Principles of ubiquitin and SUMO modifications in DNA repair. *Nature* **458**, 461-467 (2009); published online EpubMar 26 (nature07963 [pii]
10.1038/nature07963).
259. M. A. Park, Y. J. Seok, G. Jeong, J. S. Lee, SUMO1 negatively regulates BRCA1-mediated transcription, via modulation of promoter occupancy. *Nucleic acids research* **36**, 263-283 (2008); published online EpubJan (gkm969 [pii]
10.1093/nar/gkm969).
260. W. Li, B. Hesabi, A. Babbo, C. Pacione, J. Liu, D. J. Chen, J. A. Nickoloff, Z. Shen, Regulation of double-strand break-induced mammalian homologous recombination by UBL1, a RAD51-interacting protein. *Nucleic acids research* **28**, 1145-1153 (2000); published online EpubMar 1 (gkd225 [pii]).
261. Z. Shen, P. E. Pardington-Purtymun, J. C. Comeaux, R. K. Moyzis, D. J. Chen, UBL1, a human ubiquitin-like protein associating with human RAD51/RAD52 proteins. *Genomics* **36**, 271-279 (1996); published online EpubSep 1 (S0888-7543(96)90462-0 [pii]
10.1006/geno.1996.0462).
262. K. K. Wong, J. A. Engelman, L. C. Cantley, Targeting the PI3K signaling pathway in cancer. *Curr Opin Genet Dev* **20**, 87-90 (2010); published online EpubFeb (S0959-437X(09)00178-6 [pii]
10.1016/j.gde.2009.11.002).
263. P. Liu, H. Cheng, T. M. Roberts, J. J. Zhao, Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat Rev Drug Discov* **8**, 627-644 (2009); published online EpubAug (nrd2926 [pii]
10.1038/nrd2926).

264. C. J. Lord, A. Ashworth, The DNA damage response and cancer therapy. *Nature* **481**, 287-294 (2012); published online EpubJan 19 (10.1038/nature10760
nature10760 [pii]).
265. J. P. Morgenstern, H. Land, Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res* **18**, 3587-3596 (1990); published online EpubJun 25 (
266. V. Vichai, K. Kirtikara, Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc* **1**, 1112-1116 (2006)nprot.2006.179 [pii]
10.1038/nprot.2006.179).
267. Y. Sun, X. Jiang, S. Chen, N. Fernandes, B. D. Price, A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. *Proc Natl Acad Sci U S A* **102**, 13182-13187 (2005); published online EpubSep 13 (0504211102 [pii]
10.1073/pnas.0504211102).
268. M. R. Stratton, P. J. Campbell, P. A. Futreal, The cancer genome. *Nature* **458**, 719-724 (2009); published online EpubApr 9 (10.1038/nature07943
nature07943 [pii]).
269. H. Varmus, The new era in cancer research. *Science* **312**, 1162-1165 (2006); published online EpubMay 26 (10.1126/science.1126758).
270. P. Workman, Drugging the cancer kinome: progress and challenges in developing personalized molecular cancer therapeutics. *Cold Spring Harbor symposia on quantitative biology* **70**, 499-515 (2005)10.1101/sqb.2005.70.020).
271. S. M. Maira, S. Pecchi, A. Huang, M. Burger, M. Knapp, D. Sterker, C. Schnell, D. Guthy, T. Nagel, M. Wiesmann, S. Brachmann, C. Fritsch, M. Dorsch, P. Chene, K. Shoemaker, A. De Pover, D. Menezes, G. Martiny-Baron, D. Fabbro, C. J. Wilson, R. Schlegel, F. Hofmann, C. Garcia-Echeverria, W. R. Sellers, C. F. Voliva, Identification and characterization of NVP-BKM120, an orally available pan-class I PI3-kinase inhibitor. *Mol Cancer Ther* **11**, 317-328 (2012); published online EpubFeb (10.1158/1535-7163.MCT-11-0474
1535-7163.MCT-11-0474 [pii]).
272. C. O'Brien, J. J. Wallin, D. Sampath, D. GuhaThakurta, H. Savage, E. A. Punnoose, J. Guan, L. Berry, W. W. Prior, L. C. Amler, M. Belvin, L. S. Friedman, M. R. Lackner, Predictive biomarkers of sensitivity to the phosphatidylinositol 3' kinase inhibitor GDC-0941 in breast cancer preclinical models. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**, 3670-3683 (2010); published online EpubJul 15 (10.1158/1078-0432.CCR-09-2828
1078-0432.CCR-09-2828 [pii]).
273. L. W. Cheung, B. T. Hennessy, J. Li, S. Yu, A. P. Myers, B. Djordjevic, Y. Lu, K. Stemke-Hale, M. D. Dyer, F. Zhang, Z. Ju, L. C. Cantley, S. E. Scherer, H. Liang, K. H. Lu, R. R. Broaddus, G. B. Mills, High frequency of PIK3R1 and PIK3R2 mutations in endometrial cancer elucidates a novel mechanism for

regulation of PTEN protein stability. *Cancer Discov* **1**, 170-185 (2011); published online EpubJul (10.1158/2159-8290.CD-11-0039
2159-8290.CD-11-0039 [pii]).
274. N. T. Ihle, G. Paine-Murrieta, M. I. Berggren, A. Baker, W. R. Tate, P. Wipf, R. T. Abraham, D. L. Kirkpatrick, G. Powis, The phosphatidylinositol-3-kinase inhibitor PX-866 overcomes resistance to the epidermal growth factor receptor inhibitor gefitinib in A-549 human non-small cell lung cancer xenografts. *Mol Cancer Ther* **4**, 1349-1357 (2005); published online EpubSep (4/9/1349 [pii]
10.1158/1535-7163.MCT-05-0149).
275. A. J. Folkes, K. Ahmadi, W. K. Alderton, S. Alix, S. J. Baker, G. Box, I. S. Chuckowree, P. A. Clarke, P. Depledge, S. A. Eccles, L. S. Friedman, A. Hayes, T. C. Hancox, A. Kugendradas, L. Lensun, P. Moore, A. G. Olivero, J. Pang, S. Patel, G. H. Pergl-Wilson, F. I. Raynaud, A. Robson, N. Saghir, L. Salphati, S. Sohal, M. H. Ultsch, M. Valenti, H. J. Wallweber, N. C. Wan, C. Wiesmann, P. Workman, A. Zhyvoloup, M. J. Zvelebil, S. J. Shuttleworth, The identification of 2-(1H-indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-t hieno[3,2-d]pyrimidine (GDC-0941) as a potent, selective, orally bioavailable inhibitor of class I PI3 kinase for the treatment of cancer. *J Med Chem* **51**, 5522-5532 (2008); published online EpubSep 25 (10.1021/jm800295d).
276. S. M. Maira, C. Voliva, C. Garcia-Echeverria, Class IA phosphatidylinositol 3-kinase: from their biologic implication in human cancers to drug discovery. *Expert Opin Ther Targets* **12**, 223-238 (2008); published online EpubFeb (10.1517/14728222.12.2.223).
277. F. Janku, D. S. Hong, S. Fu, S. A. Piha-Paul, A. Naing, G. S. Falchook, A. M. Tsimberidou, V. M. Stepanek, S. L. Moulder, J. J. Lee, R. Luthra, R. G. Zinner, R. R. Broaddus, J. J. Wheler, R. Kurzrock, Assessing PIK3CA and PTEN in early-phase trials with PI3K/AKT/mTOR inhibitors. *Cell Rep* **6**, 377-387 (2014); published online EpubJan 30 (10.1016/j.celrep.2013.12.035
S2211-1247(13)00793-6 [pii]).
278. A. Carracedo, L. Ma, J. Teruya-Feldstein, F. Rojo, L. Salmena, A. Alimonti, A. Egia, A. T. Sasaki, G. Thomas, S. C. Kozma, A. Papa, C. Nardella, L. C. Cantley, J. Baselga, P. P. Pandolfi, Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *J Clin Invest* **118**, 3065-3074 (2008); published online EpubSep (10.1172/JCI34739).
279. D. Watters, Molecular mechanisms of ionizing radiation-induced apoptosis. *Immunol Cell Biol* **77**, 263-271 (1999); published online EpubJun (10.1046/j.1440-1711.1999.00824.x).
280. R. L. Fisher, J. T. Sanuik, A. J. Gandolfi, K. Brendel, Toxicity of cisplatin and mercuric chloride in human kidney cortical slices. *Hum Exp Toxicol* **13**, 517-523 (1994); published online EpubAug (
281. C. Deans, S. J. Wigmore, Systemic inflammation, cachexia and prognosis in patients with cancer. *Curr Opin Clin Nutr Metab Care* **8**, 265-269 (2005); published online EpubMay (00075197-200505000-00005 [pii]).

282. M. Maltoni, O. Nanni, M. Pirovano, E. Scarpi, M. Indelli, C. Martini, M. Monti, E. Arnoldi, L. Piva, A. Ravaioli, G. Cruciani, R. Labianca, D. Amadori, Successful validation of the palliative prognostic score in terminally ill cancer patients. Italian Multicenter Study Group on Palliative Care. *J Pain Symptom Manage* **17**, 240-247 (1999); published online EpubApr (S0885392498001468 [pii]).
283. R. A. Adams, A. M. Meade, M. T. Seymour, R. H. Wilson, A. Madi, D. Fisher, S. L. Kenny, E. Kay, E. Hodgkinson, M. Pope, P. Rogers, H. Wasan, S. Falk, S. Gollins, T. Hickish, E. M. Bessell, D. Propper, M. J. Kennedy, R. Kaplan, T. S. Maughan, Intermittent versus continuous oxaliplatin and fluoropyrimidine combination chemotherapy for first-line treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. *Lancet Oncol* **12**, 642-653 (2011); published online EpubJul (10.1016/S1470-2045(11)70102-4
S1470-2045(11)70102-4 [pii]).
284. A. R. Gottschalk, A. Doan, J. L. Nakamura, D. Stokoe, D. A. Haas-Kogan, Inhibition of phosphatidylinositol-3-kinase causes increased sensitivity to radiation through a PKB-dependent mechanism. *Int J Radiat Oncol Biol Phys* **63**, 1221-1227 (2005); published online EpubNov 15 (S0360-3016(05)02381-3 [pii]
10.1016/j.ijrobp.2005.08.014).
285. A. K. Gupta, G. J. Cerniglia, R. Mick, M. S. Ahmed, V. J. Bakanauskas, R. J. Muschel, W. G. McKenna, Radiation sensitization of human cancer cells in vivo by inhibiting the activity of PI3K using LY294002. *Int J Radiat Oncol Biol Phys* **56**, 846-853 (2003); published online EpubJul 1 (S0360301603002141 [pii]).
286. I. A. Kim, S. S. Bae, A. Fernandes, J. Wu, R. J. Muschel, W. G. McKenna, M. J. Birnbaum, E. J. Bernhard, Selective inhibition of Ras, phosphoinositide 3 kinase, and Akt isoforms increases the radiosensitivity of human carcinoma cell lines. *Cancer Res* **65**, 7902-7910 (2005); published online EpubSep 1 (65/17/7902 [pii]
10.1158/0008-5472.CAN-05-0513).
287. C. M. Lee, C. B. Fuhrman, V. Planelles, M. R. Peltier, D. K. Gaffney, A. P. Soisson, M. K. Dodson, H. D. Tolley, C. L. Green, K. A. Zempolich, Phosphatidylinositol 3-kinase inhibition by LY294002 radiosensitizes human cervical cancer cell lines. *Clinical cancer research : an official journal of the American Association for Cancer Research* **12**, 250-256 (2006); published online EpubJan 1 (12/1/250 [pii]
10.1158/1078-0432.CCR-05-1084).
288. B. Li, M. Yuan, I. A. Kim, C. M. Chang, E. J. Bernhard, H. K. Shu, Mutant epidermal growth factor receptor displays increased signaling through the phosphatidylinositol-3 kinase/AKT pathway and promotes radioresistance in cells of astrocytic origin. *Oncogene* **23**, 4594-4602 (2004); published online EpubJun 3 (10.1038/sj.onc.1207602
1207602 [pii]).

289. K. Okkenhaug, B. Vanhaesebroeck, PI3K-signalling in B- and T-cells: insights from gene-targeted mice. *Biochem Soc Trans* **31**, 270-274 (2003); published online EpubFeb (10.1042/).
290. S. Gupta, A. R. Ramjaun, P. Haiko, Y. Wang, P. H. Warne, B. Nicke, E. Nye, G. Stamp, K. Alitalo, J. Downward, Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. *Cell* **129**, 957-968 (2007); published online EpubJun 1 (S0092-8674(07)00520-X [pii]
10.1016/j.cell.2007.03.051).
291. C. Fritsch, A. Huang, C. Chatenay-Rivauday, C. Schnell, A. Reddy, M. Liu, A. Kauffmann, D. Guthy, D. Erdmann, A. De Pover, P. Furet, H. Gao, S. Ferretti, Y. Wang, J. Trappe, S. M. Brachmann, S. M. Maira, C. Wilson, M. Boehm, C. Garcia-Echeverria, P. Chene, M. Wiesmann, R. Cozens, J. Lehar, R. Schlegel, G. Caravatti, F. Hofmann, W. R. Sellers, Characterization of the novel and specific PI3Kalpha inhibitor NVP-BYL719 and development of the patient stratification strategy for clinical trials. *Mol Cancer Ther* **13**, 1117-1129 (2014); published online EpubMay (10.1158/1535-7163.MCT-13-0865
1535-7163.MCT-13-0865 [pii]).
292. J. Ni, Q. Liu, S. Xie, C. Carlson, T. Von, K. Vogel, S. Riddle, C. Benes, M. Eck, T. Roberts, N. Gray, J. Zhao, Functional characterization of an isoform-selective inhibitor of PI3K-p110beta as a potential anticancer agent. *Cancer Discov* **2**, 425-433 (2012); published online EpubMay (10.1158/2159-8290.CD-12-0003
2159-8290.CD-12-0003 [pii]).
293. K. A. Edgar, J. J. Wallin, M. Berry, L. B. Lee, W. W. Prior, D. Sampath, L. S. Friedman, M. Belvin, Isoform-specific phosphoinositide 3-kinase inhibitors exert distinct effects in solid tumors. *Cancer Res* **70**, 1164-1172 (2010); published online EpubFeb 1 (10.1158/0008-5472.CAN-09-2525
0008-5472.CAN-09-2525 [pii]).
294. I. M. Berenjeno, J. Guillermet-Guibert, W. Pearce, A. Gray, S. Fleming, B. Vanhaesebroeck, Both p110alpha and p110beta isoforms of PI3K can modulate the impact of loss-of-function of the PTEN tumour suppressor. *Biochem J* **442**, 151-159 (2012); published online EpubFeb 15 (10.1042/BJ20111741
BJ20111741 [pii]).
295. E. Razis, E. Briasoulis, E. Vrettou, D. V. Skarlos, D. Papamichael, I. Kostopoulos, E. Samantas, I. Xanthakis, M. Bobos, E. Galanidi, M. Bai, I. Gikonti, A. Koukouma, G. Kafiri, P. Papakostas, K. T. Kalogeras, P. Kosmidis, G. Fountzilas, Potential value of PTEN in predicting cetuximab response in colorectal cancer: an exploratory study. *BMC Cancer* **8**, 234 (2008)10.1186/1471-2407-8-234
1471-2407-8-234 [pii]).
296. S. Gori, A. Sidoni, M. Colozza, I. Ferri, M. G. Mameli, D. Fenocchio, L. Stocchi, J. Foglietta, V. Ludovini, E. Minenza, V. De Angelis, L. Crino, EGFR, pMAPK, pAkt and PTEN status by immunohistochemistry: correlation with clinical outcome in HER2-positive metastatic breast cancer patients treated

with trastuzumab. *Ann Oncol* **20**, 648-654 (2009); published online EpubApr (10.1093/annonc/mdn681
mdn681 [pii]).
297. Y. S. DeRose, G. Wang, Y. C. Lin, P. S. Bernard, S. S. Buys, M. T. Ebbert, R. Factor, C. Matsen, B. A. Milash, E. Nelson, L. Neumayer, R. L. Randall, I. J. Stijleman, B. E. Welm, A. L. Welm, Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes. *Nature medicine* **17**, 1514-1520 (2011)10.1038/nm.2454).
298. P. Loukopoulos, K. Kanetaka, M. Takamura, T. Shibata, M. Sakamoto, S. Hirohashi, Orthotopic transplantation models of pancreatic adenocarcinoma derived from cell lines and primary tumors and displaying varying metastatic activity. *Pancreas* **29**, 193-203 (2004); published online EpubOct (
299. X. Zhao, Z. Liu, L. Yu, Y. Zhang, P. Baxter, H. Voicu, S. Gurusiddappa, J. Luan, J. M. Su, H. C. Leung, X. N. Li, Global gene expression profiling confirms the molecular fidelity of primary tumor-based orthotopic xenograft mouse models of medulloblastoma. *Neuro-oncology* **14**, 574-583 (2012); published online EpubMay (10.1093/neuonc/nos061).
300. J. J. Tentler, A. C. Tan, C. D. Weekes, A. Jimeno, S. Leong, T. M. Pitts, J. J. Arcaroli, W. A. Messersmith, S. G. Eckhardt, Patient-derived tumour xenografts as models for oncology drug development. *Nature reviews. Clinical oncology* **9**, 338-350 (2012); published online EpubJun (10.1038/nrclinonc.2012.61
nrclinonc.2012.61 [pii]).
301. M. Utsuyama, K. Hirokawa, Radiation-induced-thymic lymphoma occurs in young, but not in old mice. *Exp Mol Pathol* **74**, 319-325 (2003); published online EpubJun (S0014480003000261 [pii]).
302. C. T. Guy, M. A. Webster, M. Schaller, T. J. Parsons, R. D. Cardiff, W. J. Muller, Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A* **89**, 10578-10582 (1992); published online EpubNov 15 (
303. J. I. Herschkowitz, K. Simin, V. J. Weigman, I. Mikaelian, J. Usary, Z. Hu, K. E. Rasmussen, L. P. Jones, S. Assefnia, S. Chandrasekharan, M. G. Backlund, Y. Yin, A. I. Khramtsov, R. Bastein, J. Quackenbush, R. I. Glazer, P. H. Brown, J. E. Green, L. Kopelovich, P. A. Furth, J. P. Palazzo, O. I. Olopade, P. S. Bernard, G. A. Churchill, T. Van Dyke, C. M. Perou, Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome biology* **8**, R76 (2007)10.1186/gb-2007-8-5-r76).
304. J. Usary, W. Zhao, D. Darr, P. J. Roberts, M. Liu, L. Balletta, O. Karginova, J. Jordan, A. Combest, A. Bridges, A. Prat, M. C. Cheang, J. I. Herschkowitz, J. M. Rosen, W. Zamboni, N. E. Sharpless, C. M. Perou, Predicting drug responsiveness in human cancers using genetically engineered mice. *Clin Cancer Res* **19**, 4889-4899 (2013); published online EpubSep 1 (10.1158/1078-0432.CCR-13-0522).
305. B. Li, J. M. Rosen, J. McMenamin-Balano, W. J. Muller, A. S. Perkins, neu/ERBB2 cooperates with p53-172H during mammary tumorigenesis in

transgenic mice. *Molecular and cellular biology* **17**, 3155-3163 (1997); published online EpubJun (
306. D. Figeys, L. D. McBroom, M. F. Moran, Mass spectrometry for the study of protein-protein interactions. *Methods* **24**, 230-239 (2001); published online EpubJul (10.1006/meth.2001.1184
S1046-2023(01)91184-3 [pii]).
307. W. D. Heyer, K. T. Ehmsen, J. Liu, Regulation of homologous recombination in eukaryotes. *Annu Rev Genet* **44**, 113-139 (2010)10.1146/annurev-genet-051710-150955).
308. I. Weibrecht, K. J. Leuchowius, C. M. Clausson, T. Conze, M. Jarvius, W. M. Howell, M. Kamali-Moghaddam, O. Soderberg, Proximity ligation assays: a recent addition to the proteomics toolbox. *Expert Rev Proteomics* **7**, 401-409 (2010); published online EpubJun (10.1586/epr.10.10).
309. M. C. Hollander, G. M. Blumenthal, P. A. Dennis, PTEN loss in the continuum of common cancers, rare syndromes and mouse models. *Nature reviews* **11**, 289-301 (2011); published online EpubApr (10.1038/nrc3037
nrc3037 [pii]).
310. R. Endersby, S. J. Baker, PTEN signaling in brain: neuropathology and tumorigenesis. *Oncogene* **27**, 5416-5430 (2008); published online EpubSep 18 (10.1038/onc.2008.239
onc2008239 [pii]).
311. P. L. Depowski, S. I. Rosenthal, J. S. Ross, Loss of expression of the PTEN gene protein product is associated with poor outcome in breast cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **14**, 672-676 (2001); published online EpubJul (10.1038/modpathol.3880371).
312. M. H. Roh, Y. Yassin, A. Miron, K. K. Mehra, M. Mehrad, N. M. Monte, G. L. Mutter, M. R. Nucci, G. Ning, F. D. McKeon, M. S. Hirsch, X. Wa, C. P. Crum, High-grade fimbrial-ovarian carcinomas are unified by altered p53, PTEN and PAX2 expression. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **23**, 1316-1324 (2010); published online EpubOct (10.1038/modpathol.2010.119
modpathol2010119 [pii]).
313. J. C. Bendell, J. Rodon, H. A. Burris, M. de Jonge, J. Verweij, D. Birle, D. Demanse, S. S. De Buck, Q. C. Ru, M. Peters, M. Goldbrunner, J. Baselga, Phase I, dose-escalation study of BKM120, an oral pan-Class I PI3K inhibitor, in patients with advanced solid tumors. *J Clin Oncol* **30**, 282-290 (2012); published online EpubJan 20 (10.1200/JCO.2011.36.1360
JCO.2011.36.1360 [pii]).
314. B. McEllin, C. V. Camacho, B. Mukherjee, B. Hahm, N. Tomimatsu, R. M. Bachoo, S. Burma, PTEN loss compromises homologous recombination repair in astrocytes: implications for glioblastoma therapy with temozolomide or poly(ADP-ribose) polymerase inhibitors. *Cancer Res* **70**, 5457-5464 (2010); published online EpubJul 1 (10.1158/0008-5472.CAN-09-4295
0008-5472.CAN-09-4295 [pii]).

315. J. Puc, M. Keniry, H. S. Li, T. K. Pandita, A. D. Choudhury, L. Memeo, M. Mansukhani, V. V. Murty, Z. Gaciong, S. E. Meek, H. Piwnica-Worms, H. Hibshoosh, R. Parsons, Lack of PTEN sequesters CHK1 and initiates genetic instability. *Cancer cell* **7**, 193-204 (2005); published online EpubFeb (S1535-6108(05)00030-9 [pii]
10.1016/j.ccr.2005.01.009).
316. C. Bassi, J. Ho, T. Srikumar, R. J. Dowling, C. Gorrini, S. J. Miller, T. W. Mak, B. G. Neel, B. Raught, V. Stambolic, Nuclear PTEN controls DNA repair and sensitivity to genotoxic stress. *Science* **341**, 395-399 (2013); published online EpubJul 26 (10.1126/science.1236188
341/6144/395 [pii]).
317. G. Perez-Tenorio, L. Alkhori, B. Olsson, M. A. Waltersson, B. Nordenskjold, L. E. Rutqvist, L. Skoog, O. Stal, PIK3CA mutations and PTEN loss correlate with similar prognostic factors and are not mutually exclusive in breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 3577-3584 (2007); published online EpubJun 15 (13/12/3577 [pii]
10.1158/1078-0432.CCR-06-1609).
318. Z. H. Siddik, Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22**, 7265-7279 (2003); published online EpubOct 20 (10.1038/sj.onc.1206933
1206933 [pii]).