

**Regulation of Protein kinase B (PKB/Akt) by
DNA-dependent protein kinase (DNA-PK)
under physiological conditions**

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Banu Sürücü
aus Ankara, Türkei

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Antrag von Dr. Brian Hemmings, Prof. Dr. Markus Affolter, Prof. Dr.
Patrick Matthias.

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Prof. Dr. Hans-Peter Hauri
(Dekan)

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i. SUMMARY

The serine/threonine protein kinase B (PKB/Akt) is a downstream effector of phosphatidylinositol 3-kinase (PI3K) and a major regulator of a variety of cellular processes, including metabolism, transcription, survival, proliferation, and growth. PKB is activated by several stimuli, including hormones, growth factors, cytokines and, as recently reported, also by DNA damage. Activation of PKB requires phosphorylation at two key regulatory sites: Thr308 and Ser473 (of PKB α). Phosphorylation by 3-phosphoinositide-dependent kinase-1 (PDK1) occurs on Thr308 in the activation loop of PKB. The phosphorylation on Ser473 within a C-terminal hydrophobic motif leads to full activation of PKB and mediated by two members of the PI3K-related kinase (PIKK) family, mTOR/riCTOR complex (mTORC2) or DNA-dependent protein kinase (DNA-PK) in a stimulus specific manner. Insulin or growth factor induced PKB Ser473 phosphorylation is regulated by mTORC2. In contrast, DNA damage-induced phosphorylation of PKB Ser473 is mediated by DNA-PK.

The present study made use of genetically modified mouse models to investigate PKB regulation by DNA-PK, as phosphorylation of Ser473 may be stimulus-, signalling pathway- and/or cell type-specific. In this study, we investigated the role of DNA-PK in basal, insulin-induced, and DNA damage-induced phosphorylation of PKB Ser473 under physiological conditions. We report that DNA-PK phosphorylated PKB on Ser473 upon DNA damage induced by γ -irradiation *in vivo*. In contrast, DNA-PK was dispensable for

insulin and growth factor-induced PKB activation. Interestingly, analysis of basal PKB Ser473 phosphorylation in DNA-PKcs^{-/-} mice showed tissue-specific deregulation of the PKB/FoxO pathway. In particular, we provide evidence that persistent PKB hyperactivity in the thymus apparently contributes to spontaneous tumourigenesis in DNA-PKcs^{-/-} mice. Lymphomagenesis could be prevented by the deletion of PKB α and implies deregulation of PKB in DNA-PKcs^{-/-} thymi.

Deregulation of PKB is implicated in various types of cancer and PI3K/PKB pathway is one of the most deregulated pathways in human malignancies. Therefore PI3K/PKB pathway is a major focus of current efforts for the treatment of cancer. In the second part of the study we made use of differential activation of PKB by upstream kinases in response to specific stimuli as a tool to dissect the mode of action of a small molecule inhibitor BBD130.

ii. ABBREVIATIONS

DDR	DNA damage response
DNA DSBs	DNA double strand breaks
DNA-PKcs	DNA dependent protein kinase catalytical subunit
DN	double negative
DP	double positive
FoxO	Forkhead box, class O
GSK3	glycogen synthase kinase
HM	hydrophobic motif
IGF1	insulin-like growth factor
mTOR	mammalian target of rapamycin
mTORC2	mTOR/riCTOR complex
PDK1	3-phosphoinositide-dependent kinase-1
PI3K	phosphatidylinositol-3-kinase
PI(3,4,5)P3	phosphatidylinositol 3,4,5-triphosphate
PIKK	PI3K-related kinase
PH	pleckstrin homology
pre-TCR	pre-T cell receptor
riCTOR	rapamycin insensitive companion of mTOR
RTK	receptor tyrosine kinase
S6K1	p70 ribosomal protein S6 kinase 1

I. INTRODUCTION

I. INTRODUCTION

1. Protein kinase B (PKB/Akt)

1.1 PKB structure

PKB/Akt belongs to class of AGC kinases (related to AMP/GMP kinase and protein kinase C). PKB was identified by homology cloning as a serine/threonine protein kinase of the second-messenger subfamily (1,2) and concurrently as a cellular homolog of the v-AKT oncogene within the mouse leukaemia virus AKT8 (3,4). There are three isoforms of PKB in mammals, termed PKB α /Akt1, PKB β /Akt2 and PKB γ /Akt3, which are products of distinct genes yet comprise more than 80% sequence identity and share a conserved structural organization that includes three functional domains.

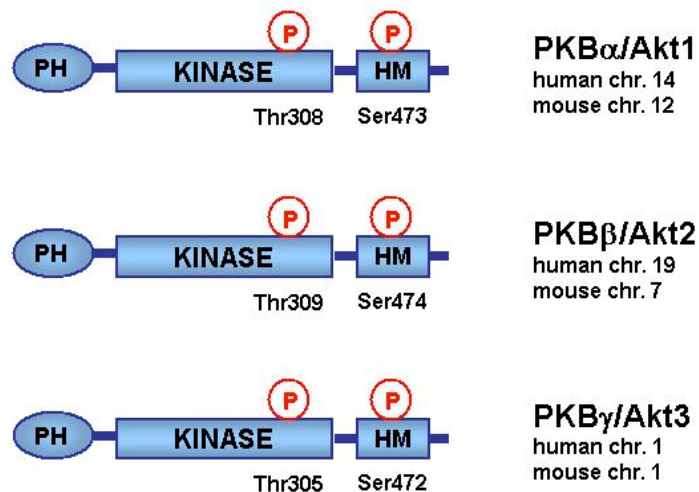


Figure 1. Domain structure and phosphorylation sites of PKB isoforms. PH: pleckstrin homology; HM: hydrophobic motif. Adapted from (5).

All PKB isoforms consist of an N-terminal pleckstrin homology (PH) domain, followed by a short α -helical linker and a catalytic (kinase) domain (Figure 1). The conserved threonine residue (Thr308 of PKB α /Akt1), one of the two crucial phosphorylation sites for activation of PKB, is located in the activation loop of the kinase domain. Like in other AGC kinases, a C-terminal tail follows the kinase domain and this regulatory domain contains hydrophobic motif (HM) that is a characteristic of AGC kinase family (5). In mammalian PKB isoforms this motif is identical [FPQFSY] and comprise the second conserved phosphorylation site, Ser473 (of PKB α /Akt1).

Phosphorylation of both Thr308 in the activation loop and Ser473 in the hydrophobic motif is required for full activation of PKB. The crystal structures of PKB kinase domain in inactive and active states were solved (6), and provided an explanation of how these two phosphorylation sites contributes to enzymatic activation of the kinase. The phosphorylation of Thr308 induces a catalytically active conformation of PKB and full activation is achieved by phosphorylation of Ser473, which leads to stabilization of the active conformation. This stabilization is due to the intramolecular interaction between the hydrophobic motif and its acceptor structure within the kinase domain, named the hydrophobic groove and phosphorylation of the hydrophobic motif residue leads to a disorder to order transition of the kinase domain (6).

1.2. Activation of PKB

The key role of PKB in signalling became obvious when it was shown to be a downstream effector of phosphatidylinositol 3-kinase (PI3K) pathway that is activated upon autophosphorylation of receptor tyrosine kinases induced by insulin or other growth factors; stimulation of G-protein coupled receptors or activation of integrin signalling (7-9). PKB is activated by several stimuli, including hormones, growth factors, cytokines and, as recently reported, also by DNA damage (8,10-12).

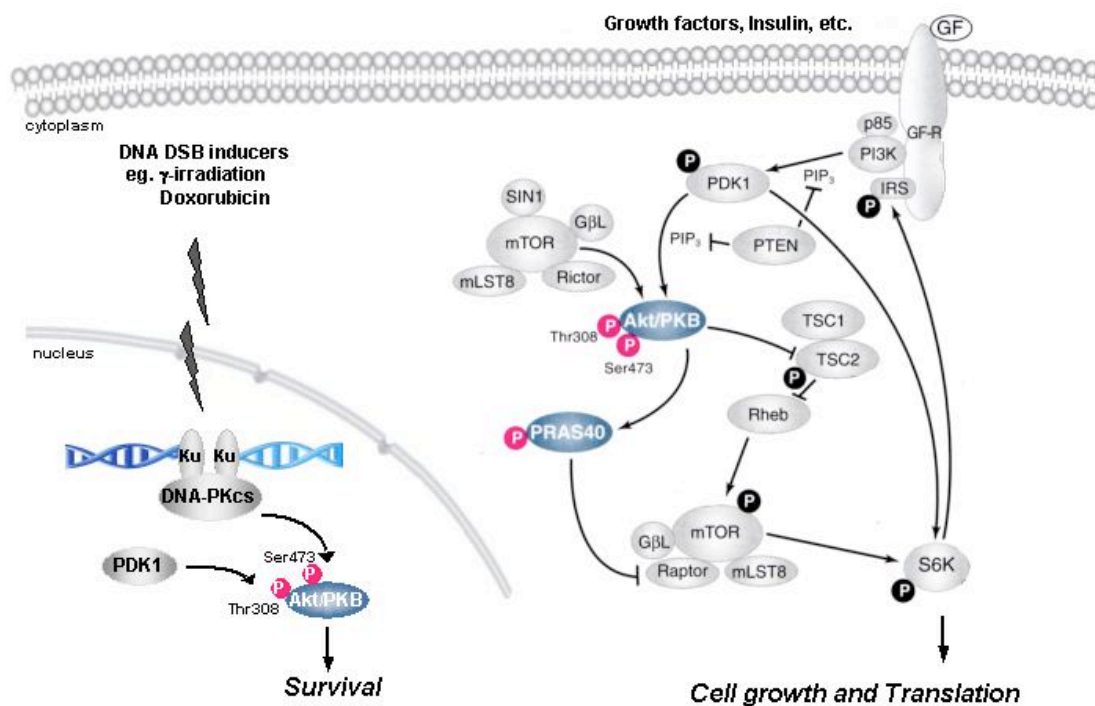


Figure 3. Activation of PI3K-PKB pathway in response to several stimuli. Adapted from Cell Signaling.

The typical route of PKB activation is via receptor tyrosine kinases. Upon ligand binding and PI3K activation, phosphatidylinositol 3,4,5-triphosphate (PIP3) is produced. Inactive PKB is recruited from cytosol to the membrane via binding of its PH domain to PIP3. Membrane recruitment brings PKB to close proximity to 3-phosphoinositide-dependent kinase-1 (PDK1), and co-localization of the proteins and conformational change in PKB upon PIP3 binding lead to phosphorylation of Thr308 residue of PKB by PDK1 (Figure 3). Recently, PKB and PDK1 were found as a preactivation complex, which is maintained in an inactive state through a PKB intramolecular interaction (13). Full activation of PKB is achieved by phosphorylation of Ser473 residue within a C-terminal hydrophobic motif (Figure 3). Several candidates were proposed to be the kinase responsible for phosphorylation of PKB Ser473 residue including PKB itself (14), PDK1 (15), integrin-linked kinase-1 (ILK1) (16), mitogen activated protein kinase activated protein kinase 2 (MAPKAP-K2) (17), protein kinase C β II (PKC β II) (18), and the members of the PI3K-related protein kinase family (also referred to as class IV PI3Ks) including DNA dependent protein kinase (DNA-PK) (19), Ataxia telangiectasia mutated (ATM) (20), and mTOR/ricor complex (mTORC2) (21). However, recent studies indicated that DNA-dependent protein kinase (DNA-PK) (11,19,22) and mTOR/ricor complex (mTORC2) (21), both of which are members of the PI3K-related protein kinase family (also referred to as class IV PI3Ks) are the most relevant ones at present and regulate PKB Ser473 phosphorylation in a stimulus specific manner. Insulin or growth factor induced PKB Ser473 phosphorylation is regulated by mTORC2 (21). In contrast, DNA damage-induced phosphorylation of PKB Ser473 is mediated by DNA-PK (11) (Figure 3).

Once activated, PKB phosphorylates a plethora of substrates and PKB-mediated phosphorylation of these proteins lead to their activation or inhibition (Figure 4). Regulation of these substrates by PKB results in multiple cellular outcomes affecting survival, growth, proliferation, and metabolism (8).

1.3. The role of PKB in cell survival and proliferation

Several lines of evidence demonstrated the crucial role of PKB in promoting cell survival and induce proliferation downstream of growth factors and cell stress (8,23).

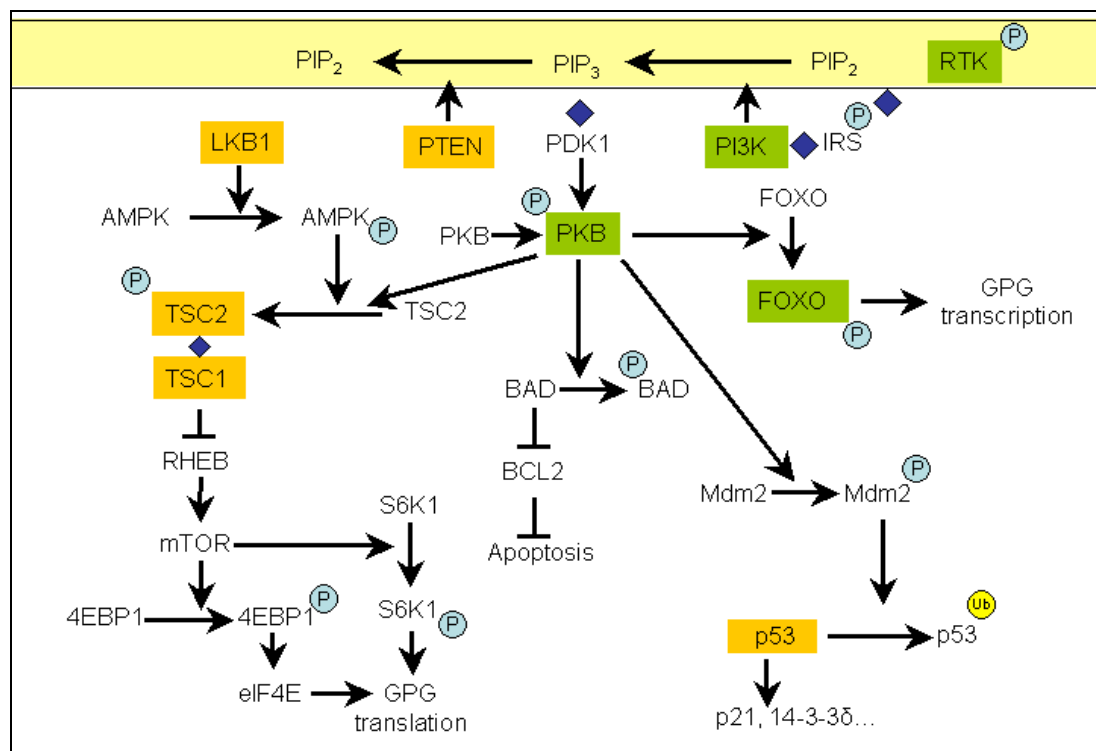


Figure 4. PI3-kinase/PKB pathway. Adapted from (24). Yellow boxes indicate tumor suppressor genes and green boxes indicate oncogenes). 'GPG' denotes growth-promoting genes—that is, genes that stimulate cell proliferation or inhibit the rate of cell death or arrest. Diamonds (♦) indicate protein-protein interactions. Arrows and T-bars indicate transcriptional induction and repression, respectively. Small-circled 'P' and 'Ub' represent covalently attached phosphate and ubiquitin, respectively.

PKB enhances the survival of cells by blocking the function of proapoptotic proteins and processes. PKB negatively regulates the function of proapoptotic protein BAD by phosphorylation on Ser136, which creates a binding site for 14-3-3 proteins and this, leads to release of BAD from its target proteins (25). PKB also inhibits the expression of proapoptotic proteins through nuclear exclusion of Forkhead (FoxO) transcription factors (26). Phosphorylation of FoxOs (FoxO1, FoxO3a and FoxO4) by PKB which as well lead to 14-3-3 binding and release from their targets and trigger their export from nucleus. Through this mechanism PKB blocks FoxO-mediated transcription of target genes that promote apoptosis and cell-cycle arrest including pro-apoptotic BIM (27,28) and proapoptotic cytokine Fas ligand (Fas-L) (29), and cyclin-dependent kinase inhibitor p27^{Kip1} (30). PKB also phosphorylates p27 (31-33) and this leads to 14-3-3 binding and cytosolic sequestration (34). Another mechanism that PKB promotes survival is through phosphorylation of MDM2, an E3 ubiquitin ligase that triggers p53 degradation. MDM2 phosphorylation by PKB induces translocation of MDM2 to nucleus where it negatively regulates p53 function (35,36). Phosphorylation by PKB also results in stabilization of MDM2 via decreased ubiquitination (37). Another direct target of PKB, Glycogen synthase kinase (GSK3) is inactivated upon phosphorylation (38). A pro-survival protein MCL-1 is a direct target inhibited by GSK3 (39). Further, GSK3 is likely to drive cell proliferation through regulating the stability of proteins involved in cell-cycle entry. GSK3 mediated phosphorylation of G1 cyclins, cyclin D and cyclin E and transcription factors,

c-myc and c-jun targets these proteins for proteosomal degradation (40-43). Therefore PKB mediated phosphorylation and inhibition of GSK3 could result in cell cycle progression by stabilization of these proteins.

1.4. Role of PKB in cell growth

A crucial function of PKB in cell is induction of growth. Predominant mechanism appears to be via activation of mTOR/Raptor (mTORC1) signaling, which is regulated by growth factors and nutrients, and mediate translation initiation and ribosome biogenesis through S6K and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (44) (Figure 3). Tuberous sclerosis complex 2 (TSC2) is a negative regulator of mTORC1 signalling (45,46). PKB phosphorylates and inhibits TSC2 thereby activates mTORC1 signalling (47-49). Recently, a further substrate of PKB, the proline-rich Akt substrate of 40 kDa (PRAS40) was found to negatively regulate mTORC1 signalling (50,51).

1.5. Deregulation of PKB in cancer

The PI3K/PKB signalling pathway is crucial to many aspects of cell growth and survival. As PKB regulated responses could favor tumor initiation and/or progression it is not surprising that PI3K/PKB pathway is one of the most deregulated pathways in human cancers. Several cancer types showed deregulation of this pathway due mutations, amplifications or overexpression of PKB or PI3K isoforms (Table 1) (52-55). PKB Ser473 phosphorylation is used as a biomarker and strongly correlated with poor prognosis of several

cancers (56-58) rendering inhibition of PKB by targeting the components of PI3K/PKB pathway as an effective strategy for cancer treatment (59-62).

Table 1. PI3K/PTEN/PKB signaling deregulation in human malignancies

Cancer Type	Type of alteration
Brain	<i>PTEN</i> mutation (glioblastoma) PI3K p110 α mutation PI3K p85 α mutation
Ovarian	Allelic imbalance and mutations of <i>PTEN</i> gene PI3K p110 α amplification and overexpression PI3K p85 α mutation PKB α mutation Elevated PKB α kinase activity and amplification <i>PKBβ</i> amplification and overexpression
Breast	Loss of heterozygosity at <i>PTEN</i> locus PI3K p110 α mutation PI3K p110 β amplification PI3K and <i>PKBβ</i> overactivation Elevated PKB α kinase activity <i>PKBβ</i> amplification and overexpression PKB α mutation
Endometrial	<i>PTEN</i> mutations and deletions <i>PTEN</i> silencing PI3K p110 α mutation
Hepatocellular carcinoma	<i>PTEN</i> mutation Aberrant <i>PTEN</i> promotor methylation PKB β overexpression
Melanoma	<i>PTEN</i> mutation and deletion

	<i>PTEN</i> silencing
Digestive tract	Aberrant <i>PTEN</i> transcripts Loss of <i>PTEN</i> expression <i>PTEN</i> mutation and deletions PI3K <i>p85α</i> mutation PI3K <i>p110α</i> mutation PKB α mutation PKB β overexpression and amplification
Lung	<i>PTEN</i> inactivation, deletion and mutation PI3K <i>p110α</i> mutation PKB α mutation
Renal-cell carcinoma	<i>PTEN</i> mutations
Thyroid	<i>PTEN</i> mutations and deletions PKB overexpression and activation
Lymphoid	<i>PTEN</i> mutation
Prostate	<i>PTEN</i> mutations and deletions PKB γ overexpression Elevated PKB α activity
Head&Neck cancers	<i>PTEN</i> deletion PI3K <i>p110α</i> mutation
Pancreas cancer	PKB α amplification PKB β amplification

Adapted from (24,54,55,63-65)

2. DNA dependent protein kinase

2.1. DNA-PKcs structure and DNA binding

DNA dependent protein kinase catalytical subunit (DNA-PKcs) is a serine/threonine kinase that belongs to the phosphatidylinositol-3-kinase (PI3-K)-related kinase family (PIKKs) (also referred as Class IV PI3Ks), which includes other DNA damage-sensor enzymes Ataxia-telangiectasia mutated (ATM), ATM-Rad3-related (ATR) as well as nutrient-sensor kinase, mammalian target of rapamycin (mTOR). A common feature of this family is their large size. DNA-PKcs gene spans about 250 kb of the murine genome, encoded by a 14 kb cDNA composed of 86 exons and maps to mouse chromosome 16 and human chromosome 8. DNA-PKcs protein is composed of 4128 aminoacids and it is highly conserved between murine and human with 78.9% homology. The 467-kDa catalytical subunit DNA-PKcs and the Ku antigen complex, Ku70/K080 form the holoenzyme, DNA-PK.

The cryo-EM structure of DNA-PKcs shows the organization of the protein into a bulky and largely globular “head” structure connected to a flat tubular “arm” segment terminating in two projecting “claws” (66) (Figure 3). The long and distinctive N-terminal region of DNA-PKcs, probably formed by an extended series of HEAT¹ and related helical repeats (68), maps into the long curved

¹ Huntington elongation A subunit TOR (HEAT) repeats, originally identified in PR65a subunit of PP2A (45) are ~40 residue long and are arranged as a pair of antiparallel α -helices separated by a tight turn. Multiple copies of these helix pairs stack in parallel to create flattened tubular structures composed of double layers of α helices that typically curve to form a convex and a concave face (67). Groves, M. R., and Barford, D. (1999) *Curr Opin Struct Biol* **9**, 383-389

tubular- shaped domains within the arm region (Figure 3,orange). The C terminus containing the conserved PI3K-related catalytic domain (residues 3649–4011 in DNA-PKcs), a weakly conserved ~500 residue helical repeat region immediately N-terminal of this (the FAT domain), and a narrow ~100 residue C-terminal extension at the end of the catalytic domain (FATC domain) (69) all locate in the head region (Figure 3, pink, green, and blue).

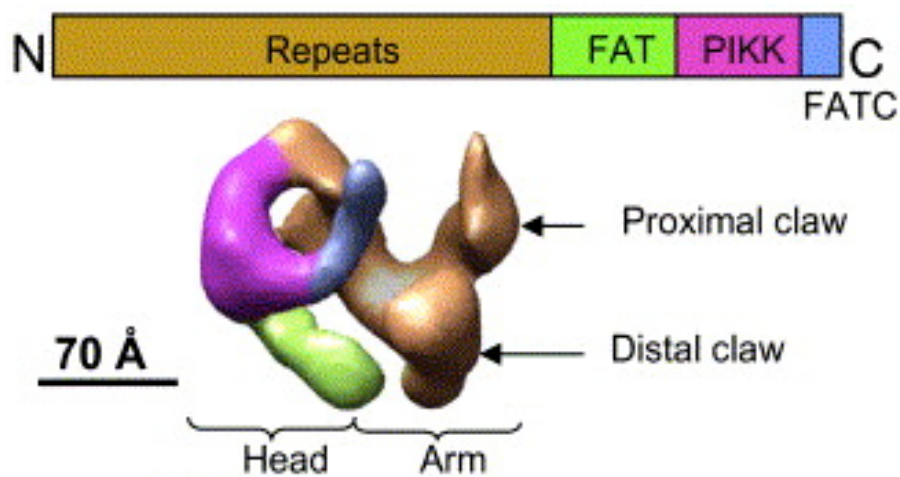


Figure 3. 3D structure of DNA-PKcs taken from Rivera-Calzada et al. (66). Coloring depicts the assignment of domains in the sequence of DNA-PKcs (top row) into the 3D structure. The scale bar represents 70 Å.

Ser3205 autophosphorylation site lies in the head region at the central part of the FAT domain. The rest of the autophosphorylation sites namely, Thr2609, Ser2613, Thr2620, Ser2624, Thr2638 and Thr2647, is located as a cluster in the shoulder region. Based on docking of a PI3K γ -based modeling, ATP binding site lies on the outer surface of head region of DNA-PKcs and would be accessible in the presence of bound DNA. The main autophosphorylation

sites are predicted to lie within the shoulder region and would be inaccessible to ATP binding site in the same molecule and would require phosphorylation by a second DNA-PKcs molecule in trans (66,70).

2.2. The role of DNA-PK in DNA damage response

Maintenance of genomic integrity is one of the essential aspects of life. The genome surveillance machinery encounters diverse genotoxic insults including ultraviolet or ionizing radiation and various chemicals all of which share the property of causing mutagenic damage to DNA (71). DNA double strand breaks are the major form of DNA damage induced by ionizing radiation and radiomimetic drugs and represent the most lethal form of DNA damage. A complex signaling network named DNA damage response (DDR) guards the genomic integrity through integration of several processes initiated by sensing the damage by DNA damage sensor proteins (Figure 4). DDR orchestrates this network through the action of sensors, transducers and effectors and coordination of these processes with ongoing cellular physiology (72).

Pathways that are elicited by DDR to cope with the harmful damage effects include DNA repair, transcriptional response, DNA damage checkpoints and apoptosis. These responses may function independently however frequently a protein primarily involved in one response may participate in others (73). The effectiveness relies on proper spatiotemporal dynamics of the components of this signaling network and these dynamics is influenced by modifications of

the proteins such as phosphorylations and subsequent recognition by the components of the network itself (74).

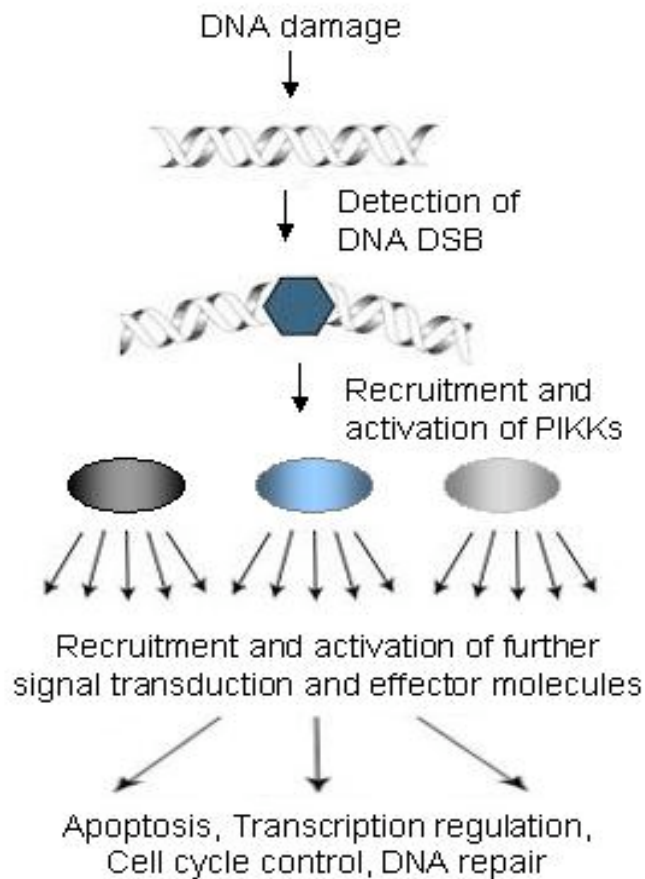


Figure 4. Adapted from (75). Sensing and responding to DNA double strand breaks (DSBs).

The kinase activity of DNA-PKcs is strongly stimulated by DNA binding. Double-stranded DNA ends produced by ionizing radiation or radiomimetic drugs activate DNA-PK. Double stranded DNA bind to the open cavity between head and the palm and DNA binding elicits a substantial change in the overall conformation so that the palm and the head are brought in intimate contact (76). The FAT and the FATC domains, directly attached on the

opposite ends of the kinase core, may together act as a sensor that couples conformational changes upon DNA binding to directly activate the catalytic center. In line with this prediction, a widely used mouse model with severe combined immunodeficiency (SCID) bears a nonsense mutation on Tyr4046 of the DNA-PKcs protein, which lead to loss of last 70 aminoacids of the FATC domain and a drastic reduction in its kinase activity (77).

One distinctive feature of the large PIKK proteins in addition to their kinase activity is their role as scaffolds recruiting other proteins involved in downstream signaling. N-terminal repeats present in the PIKKs including DNA-PKcs suggest that they may play a role in assembling and regulating multiprotein complexes.

Primary recognition of free DNA ends is mediated by Ku proteins (Figure 6), which is in a preformed ring structure that can sterically encircle the DNA without establishing sequence specific contacts (70). DNA bound Ku direct the recruitment of DNA-PKcs via the C terminus of Ku80. Ku interacts with DNA-PKcs with contact points that expand from back of the head region to the tubular N terminal arm, making extensive interactions with several distinct regions of DNA-PKcs including HEAT repeats and their projecting claws and contacts the head close to the expected position of the kinase domain. On the other hand DNA-PKcs can also bind to DNA ends independently of Ku with concomitant stimulation of its kinase activity (78). Upon DNA binding, dimeric DNA-PKcs/Ku70/Ku80 holoenzymes interact through the N-terminal HEAT repeats and maintain the broken DNA ends in proximity while providing a

platform for access of the various enzymes required for end processing and ligation.

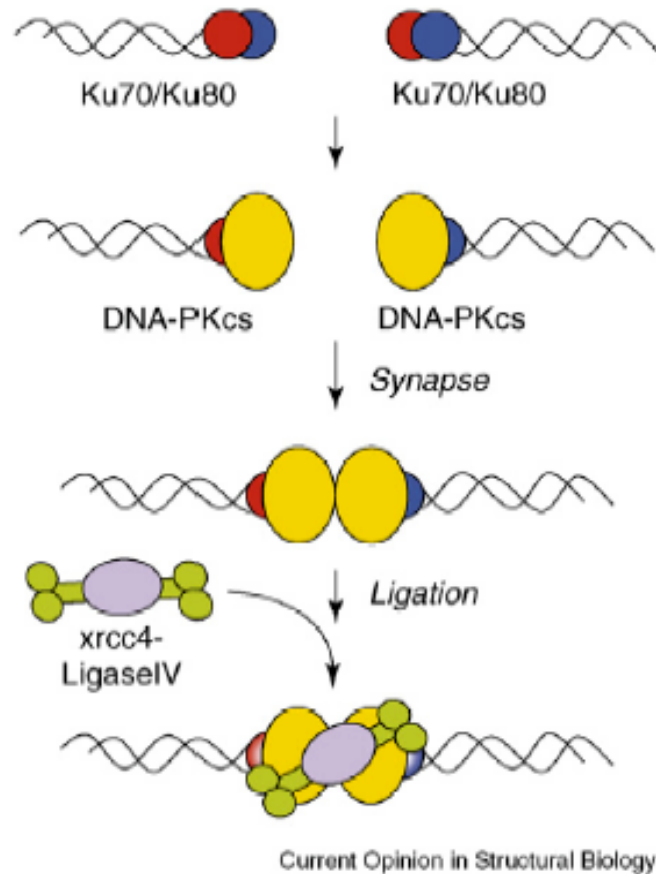


Figure 5. Ku70/Ku80 recognizes the DNA break and recruits DNA-PKcs. DNA-PKcs forms a synaptic complex between the two broken ends of DNA and helps to assemble the Xrcc4-ligaseIV complex, which performs the final step of DNA ligation. Phosphorylation of Ku70/Ku80 and Xrcc4/ligaseIV by DNA-PKcs, and also DNA-PKcs auto-phosphorylation, regulates the progress through the NHEJ reaction Adapted from (79).

Activated DNA-PKcs phosphorylates itself and a variety of other proteins including other NHEJ components. Autophosphorylation negatively regulates DNA binding of DNA-PKcs leading to dissociation from DNA (80,81). DNA-

PKcs phosphorylation/ autophosphorylation facilitates NHEJ by destabilizing the DNA–DNA-PKcs complex, which, in turn, enables efficient ligation.

3. Roles of PKB and DNA-PK in thymus

Mammalian T cells with various functions share a complex developmental history. T cells develop from bone marrow stem cells but their progenitors migrate to the thymus where they mature. Thymus is a lobular organ and consists of two regions: outer cortical region - thymic cortex-, and inner medulla. Most T cell development takes place in the cortex; thymic medulla contains mainly mature T cells. Rather few T cell progenitors migrate into thymus per day but during their differentiation with an elaborate transcriptional programme, they undergo extensive proliferation.

In the thymus, the immature T cells, or thymocytes, proliferate and differentiate, passing through a series of discrete phenotypic changes that can be identified by distinctive patterns of expression of various cell-surface proteins. T cell development requires an integration of extracellular signals to enforce lineage commitment at multiple defined checkpoints in a stage-specific manner. Thymocyte progenitors enter the thymus from the venules and migrate to the subcapsular region (Figure 6). At this stage, they express neither the antigen receptor nor the two co-receptors CD4 and CD8, and hence called double-negative (DN) thymocytes. These cells proliferate in the sub-capsular region of the thymus and begin the process of gene arrangement.

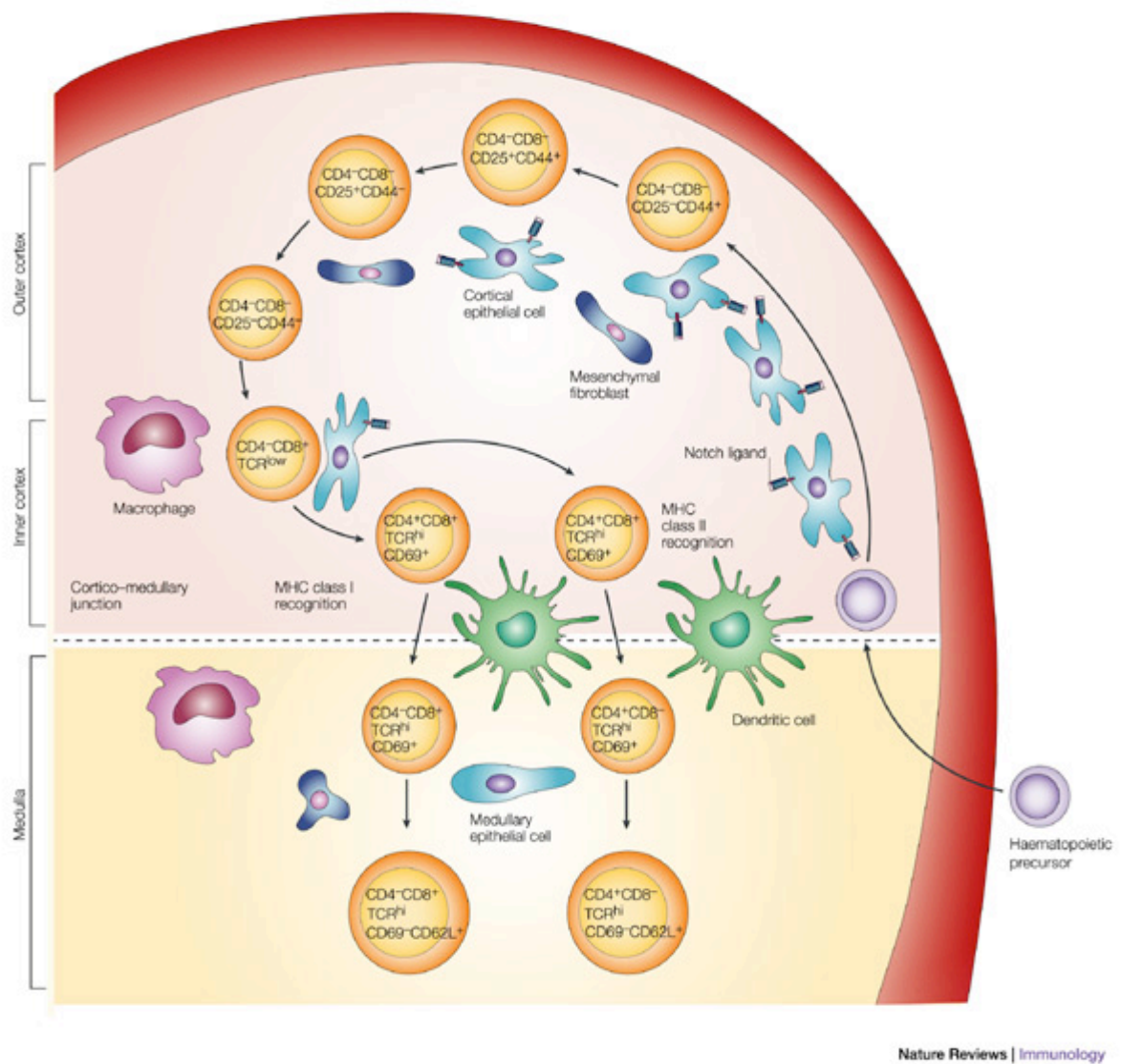


Figure 6. T cell development. Adapted from (82).

This programmed series of arrangements, namely V(D)J recombination² produces a large number of immature T cells each expressing a receptor of a different antigen. DNA-PK deficiency lead to defective V(D)J recombination and as a consequence which thymocytes lacking DNA-PK are blocked at DN stage. Functional gene rearrangement results in the expression of pre-T cell receptor (pre-TCR), which is composed of the newly rearranged TCR β chain paired with a non-rearranged pre-T α chain. The suitability of the TCR β chain is assessed at this stage namely, β -selection checkpoint, where the cells with a functionally rearranged TCR β chain survive while thymocytes that fail to express a correctly rearranged receptor undergo apoptosis. Survival through this checkpoint requires that cells generate a signal from the pre-TCR (83). Growing evidence suggest that PI3K signal transduction cascade is essential for thymocyte survival at this checkpoint. PKB has been shown to play an important role in DN- to DP-stage transition and to be essential for thymocyte survival and differentiation (84-86). Past this stage, the expression of the co-receptors CD4 and CD8 on the cell surface produces double positive (DP) cells.

² V(D)J recombination is the specialized DNA rearrangement used by cells of the immune system to assemble immunoglobulin and T-cell receptor genes from the preexisting gene segments. Because there is a large choice of segments to join, this process accounts for much of the diversity of the immune response. Recombination is initiated by the lymphoid-specific RAG1 and RAG2 proteins, which cooperate to make double-strand breaks at specific recognition sequences (recombination signal sequences, RSSs). The neighboring coding DNA is converted to a hairpin during breakage. Broken ends are then processed and joined with the help of several factors also involved in repair of radiation-damaged DNA, including the DNA-dependent protein kinase.

As the cells mature they move deeper into the thymus, then rearrange α -chain genes followed by positive³ and negative⁴ selection where thymocytes are subject to elimination by massive apoptosis. Finally mature single positive (SP) T cells exit to the peripheral circulation from the medulla.

Integration of multiple inputs controlling the T-cell development points to PI3K/PKB pathway as a central player. Critical signals from the pre-TCR, Notch, and the receptor for interleukin-7 (IL-7) dictate cellular differentiation from the CD4⁻CD8⁻ (double negative) stage to CD4⁺CD8⁺ (double positive) stage (83,87). The PI3K/PKB signaling pathway is required to translate these extracellular signaling events into multiple functional outcomes including cellular survival, proliferation, and differentiation.

4. Studies of knock-out mice for PKB isoforms and DNA-PK

DNA-PKcs^{-/-} mice display growth retardation, hypersensitivity to ionizing radiation, and severe immunodeficiency (88-90). Due to defects in V(D)J recombination, the development of T and B cells is blocked at an early progenitor stage in DNA-PKcs^{-/-} mice. Moreover, DNA-PKcs^{-/-} thymus has

³ Positive selection: The rescue of DP cells from apoptosis allows their maturation into CD4 or CD8 positive cells and is known as positive selection. By favoring the survival of thymocytes whose receptors can interact with self peptide:self MHC complexes, positive selection ensures that mature T cells can recognize and respond to peptides presented by self MHC molecules on antigen presenting cells.

⁴ Negative selection: DP cells also undergo negative selection where the cells that recognize self-peptide:self MHC complexes too well are induced to undergo apoptosis thereby eliminating the potentially self-reactive cells before they mature.

decreased cellularity and displays hypotrophy. Further, DNA-PKcs^{-/-} mice display an increased rate of T-cell lymphoma (91).

Studies of mutant mice carrying a null mutation for each of the three PKB isoforms, PKB α , PKB β , and PKB γ , revealed differing phenotypes, some of which are shared by DNA-PKcs^{-/-} mice (Table 2). Similar to DNA-PKcs^{-/-} mice, PKB α ^{-/-} mice display growth retardation and hypersensitivity to DNA damage (37,92-94). Furthermore, PKB α ^{-/-} mice show accumulation of early thymocyte subsets (84-86). PKB β ^{-/-} mice display glucose intolerance and insulin resistance, while PKB γ ^{-/-} mice have a reduced brain size (95-98). Deletion of PKB β and PKB γ isoforms revealed that a single functional allele of PKB α is sufficient for mouse development and survival (99).

Table 2. Phenotypes of mice with single and combined ablations of PKB isoforms. Adapted from (84-86,92-102).

PKB α ^{-/-}	Hypotrophy and decreased vascularization of placenta, intrauterine growth retardation, increased postnatal mortality, postnatal growth retardation, hypersensitivity to DNA damage, thymic hypocellularity in neonates and accumulation of early thymocyte subsets in adult mice
PKB β ^{-/-}	Diabetes mellitus type II phenotype (hyperglycemia, hyperinsulinemia; glucose intolerance, insulin resistance), pancreatic β cell failure, age dependent loss of adipose tissue, mild growth deficiency
PKB γ ^{-/-}	Reduction in brain size and weight with decrease in both cell size and number, hypomyelination of corpus callosum, decrease in expression of genes involved in synaptic transmission
PKB α ^{-/-} β ^{-/-}	Develop to term but die shortly after birth; severe growth deficiency, impaired skin and bone development, impeded adipogenesis, skeletal muscle atrophy, reduction in thymic cellularity and partial block in the early thymocytes
PKB α ^{-/-} γ ^{-/-}	Lethal at around embryonic day 11 (E11); multiple developmental defects, such as increased apoptosis in developing nervous system, abnormalities in the cardiovascular system, decreased vasculature, impaired differentiation of early thymocytes
PKB β ^{-/-} γ ^{-/-}	Normal embryonic development and postnatal survival; growth deficiency, reduced brain and testis size, impaired glucose homeostasis
PKB α ^{+/-} β ^{-/-} γ ^{-/-}	Normal embryonic development and postnatal survival; severe growth deficiency

5. Aim of the thesis

The general aim of this thesis is to delineate the physiological role of DNA-PK in regulation of PKB. Given that PKB Ser473 phosphorylation is mediated by both DNA-PK and mTORC2 in a stimulus dependent manner, the emphasis is placed on investigation of the role of DNA-PK in basal, insulin- and DNA damage- induced PKB phosphorylation in a physiological context.

II. RESULTS

Part 1: *In vivo* analysis of PKB/Akt regulation in DNA-PKcs-null mice reveals a role for PKB/Akt in DNA damage response and tumorigenesis.

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Surucu B., L. Bozulic, D. Hynx, A. Parcellier, and B. A. Hemmings.

**IN VIVO ANALYSIS OF PKB/AKT REGULATION IN DNA-PKCS-NULL MICE
REVEALS A ROLE FOR PKB/AKT IN DNA DAMAGE RESPONSE AND
TUMORIGENESIS**

**Banu Surucu¹, Lana Bozulich¹, Debby Hynx¹, Arnaud Parcellier¹, and Brian A.
Hemmings^{1*}**

¹ Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, Basel,
CH 4058, Switzerland

* Corresponding author: Brian A. Hemmings

Tel: +41 61 697 4872

Fax: +41 61 697 3976

E-mail: brian.hemmings@fmi.ch

Running title: PKB regulation by upstream kinases

Summary

Full activation of protein kinase B (PKB/Akt) requires phosphorylation on Thr308 and Ser473. It is well established that Thr308 is phosphorylated by 3-phosphoinositide-dependent kinase-1 (PDK1). Ser473 phosphorylation is mediated by both mTOR/ricor complex (mTORC2) and DNA-dependent protein kinase (DNA-PK) depending on type of stimulus. However, the physiological role of DNA-PK in the regulation of PKB phosphorylation remains to be established. To address this, we analyzed basal, insulin-induced and DNA damage-induced PKB Ser473 phosphorylation in DNA-PK catalytic subunit-null DNA-PKcs^{-/-} mice. Our results revealed that DNA-PK is required for DNA damage-induced but dispensable for insulin- and growth factor-induced PKB Ser473 phosphorylation. Moreover, DNA-PKcs^{-/-} mice showed a tissue-specific increase in basal PKB phosphorylation. In particular, persistent PKB hyperactivity in the thymus apparently contributed to spontaneous lymphomagenesis in DNA-PKcs^{-/-} mice. Significantly, these tumors could be prevented by deletion of PKB α . These findings reveal stimulus-specific regulation of PKB activation by specific upstream kinases and provide genetic evidence of PKB deregulation in DNA-PKcs^{-/-} mice.

Introduction

The serine/threonine protein kinase B (PKB), also known as Akt, is a downstream effector of phosphatidylinositol 3-kinase (PI3K) and a major regulator of a variety of cellular processes, including metabolism, transcription, survival, proliferation, and growth (7,8,23,103). PKB acts on a plethora of substrates, including GSK3 β and

FoxO transcription factors (23,103-105). PKB is activated by several stimuli, including hormones, growth factors, cytokines and, as recently reported, also by DNA damage (10-12,19). Deregulation of PKB is implicated in carcinogenesis and diabetes (8,54,106).

Activation of PKB requires phosphorylation at two key regulatory sites: Thr308 and Ser473 (of PKB α). Phosphorylation by 3-phosphoinositide-dependent kinase-1 (PDK1) occurs on Thr308 in the activation loop of PKB. The phosphorylation on Ser473 within a C-terminal hydrophobic motif leads to full activation of PKB. The mTOR/riCTOR complex (mTORC2), a member of the PI3K-related kinase (PIKK) family, has been reported to regulate Ser473 phosphorylation (21,107-109). Significantly, it was shown that DNA-dependent protein kinase (DNA-PK), a further member of the PIKK family, also regulates PKB Ser473 phosphorylation (19). In addition, a role for DNA-PK in the activation of PKB by CpG-DNA has been established using bone marrow-derived macrophages (22). Moreover, Bozulic et al. (11) demonstrated recently that DNA-PK phosphorylates PKB Ser473 upon induction of DNA double-strand breaks. However, the regulation of PKB by DNA-PK under physiological conditions remained to be established. The present study made use of genetically modified mouse models to investigate PKB regulation by DNA-PK, as phosphorylation of Ser473 may be stimulus-, signalling pathway- and/or cell type-specific.

DNA-PK is composed of a 470-kDa catalytic subunit (DNA-PKcs) and the Ku antigen complex (Ku80/Ku70), and involved in V(D)J recombination, repair of DNA double-strand breaks by non-homologous end joining, apoptosis and transcription regulation (110). Double-stranded DNA ends produced by ionizing radiation or radiomimetic

drugs activate DNA-PK, which is a primary sensor of DNA damage (111). DNA-PKcs^{-/-} mice display growth retardation, hypersensitivity to ionizing radiation, and severe immunodeficiency (88-90). Due to defects in V(D)J recombination, the development of T and B cells is blocked at an early progenitor stage in DNA-PKcs^{-/-} mice. Moreover, DNA-PKcs^{-/-} thymus has decreased cellularity and displays hypotrophy. Further, DNA-PKcs^{-/-} mice display an increased rate of T-cell lymphoma (91).

Studies of mutant mice carrying a null mutation for each of the three PKB isoforms PKB α , PKB β , and PKB γ revealed differing phenotypes, some of which are shared by DNA-PKcs^{-/-} mice. Similar to DNA-PKcs^{-/-} mice, PKB α ^{-/-} mice display growth retardation and hypersensitivity to DNA damage (37,92-94). Furthermore, PKB α ^{-/-} mice show accumulation of early thymocyte subsets (84-86). PKB β ^{-/-} mice display glucose intolerance and insulin resistance, while PKB γ ^{-/-} mice have a reduced brain size (95-98). Deletion of PKB β and PKB γ isoforms revealed that a single functional allele of PKB α is sufficient for mouse development and survival (99).

In this study, we investigated the role of DNA-PK in basal, insulin-induced, and DNA damage-induced phosphorylation of PKB Ser473 under physiological conditions. We report that DNA-PK phosphorylated PKB on Ser473 upon DNA damage induced by γ -irradiation *in vivo*. In contrast, DNA-PK was dispensable for insulin and growth factor-induced PKB activation. Interestingly, analysis of basal PKB Ser473 phosphorylation in DNA-PKcs^{-/-} mice showed tissue-specific deregulation of the PKB/FoxO pathway. In particular, we provide evidence that persistent PKB hyperactivity in the thymus apparently contributes to spontaneous thymic

lymphomagenesis in DNA-PKcs^{-/-} mice. Lymphomagenesis could be prevented by the deletion of PKB α and implies deregulation of PKB in DNA-PKcs^{-/-} thymi.

Experimental procedures

Mice. DNA-PKcs^{-/-} mice (88) were kindly provided by Prof. Fredrick Alt (Howard Hughes Medical Institute, Harvard Medical School, USA). PKB α ^{-/-} mice were described previously (94). For the generation of DNA-PKcs^{-/-}PKB α ^{-/-} mice, DNA-PKcs^{-/-} mice were mated with PKB α ^{-/-} mice and the resulting DNA-PKcs^{+/-}PKB α ^{+/-} progeny intercrossed. All mice had a C57BL/6;129 mixed background. Mice were housed according to Swiss Animal Protection legislation under specific pathogen-free conditions. All procedures were conducted with the approval of the appropriate authorities.

Cell culture and treatments. Mouse embryonic fibroblasts (MEFs) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 μ g/ml streptomycin. For IGF-1 and serum stimulation, MEFs were starved overnight prior to treatment with IGF-1 (50ng/ml) or serum (10% FCS) for the indicated times.

Western blot analysis. Protein lysates were prepared by homogenization of various organs in lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 1% NP-40, 40 mM β -glycerophosphate, 10% glycerol, 0.05 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 50 mM NaF, 1 mM Na₃VO₄, 1 μ M Microcystin LR). Homogenates were

centrifuged twice (13,000 rpm for 15 min at 4°C) to remove cell debris. Protein concentrations were determined using the Bradford assay (BioRad). Proteins were separated by 6%, 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Antibodies against phospho-PKB (Ser473), phospho-PKB (Thr308), phospho-Foxo4 (Ser193), phospho-GSK3 β (Ser9), phospho-S6K (Thr389), phospho-PKC (Ser657) were purchased from Cell Signaling. PKC α antibody was from Santa Cruz Biotechnology. Phospho-FoxO3 (Thr32) and IRS-1 antibodies were from Upstate Biotechnology/Millipore. DNA-PKcs (Ab-4) and actin (Ab-5) antibodies were from NeoMarkers. PKB α isoform specific antibody and PKB antibody Ab10 were previously described (94,112). A rat monoclonal anti- α -tubulin (YL1/2)-producing hybridoma cell line was obtained from American Type Culture Collection. Quantification was performed using ImageQuant TL (Amersham Biosciences) software.

In vivo insulin stimulation. Insulin stimulation was performed on ~3-month-old DNA-PKcs^{-/-} mice and wild-type controls (n=8). Following an overnight fast, a bolus of insulin (1 U/kg body weight; human recombinant insulin; Sigma) or saline solution was injected via the inferior vena cava into terminally anesthetized mice. White adipose tissue, liver, skeletal muscle and heart were collected 20 min after stimulation and immediately snap frozen. Tissues were homogenized and lysed as described above.

Glucose and insulin tolerance tests. Three-month-old mice were fasted overnight (n=2 for wild-type; n=5 for DNA-PKcs^{-/-}). For glucose tolerance tests, glucose (2 g/kg of body weight; D-(+)-glucose anhydrous, Fluka) was given orally and, for insulin tolerance tests, insulin (1 U/kg; human recombinant insulin, Sigma) was administered by intraperitoneal injection as described previously (99). Blood samples were collected at the indicated times from tail veins and glucose levels determined using Glucometer Elite (Bayer).

Total body γ -irradiation of mice. Mice received a single exposure of 1 Gy total body irradiation with X-rays [Asteophysics Research Corp, TORREX 120D X-Ray system]. The tissues were collected after 30 min and immediately snap frozen. Tissues were homogenized and lysed as described above.

Flow cytometry. Thymocytes in suspension were stained at 4°C for 30 min in FACS buffer (2% FCS in PBS) with fluorescein isothiocyanate (FITC)-or phycoerythrin (PE)-conjugated antibodies to CD4 and CD8 cell surface markers. Antibodies were from Immunotools.

Histological Analysis. Organs were dissected and fixed in 4% paraformaldehyde-phosphate-buffered saline at 4°C. After dehydration in ethanol, samples were embedded in paraffin. Tissues were cut into 4- μ m-thick sections and stored for staining. For hematoxylin-eosin (Sigma) staining, sections were deparaffinized and stained.

Statistics. Data are provided as arithmetic means \pm standard errors of the means (SEM), and n represents the number of independent experiments. Data were tested for significance using one-way analysis of variance (ANOVA). The P values for the Kaplan-Meier survival curve were determined by LogRank tests with the Holm-Sidak multiple comparisons procedure using SigmaStat 3.11 (Systat Software, Inc.) statistics software. Results at $P < 0.05$ were considered significant.

Results

In vivo, DNA-PK is dispensable for PKB Ser473 phosphorylation upon insulin stimulation. To evaluate the involvement of DNA-PK in the regulation of PKB in response to insulin stimulation, we treated wild-type and DNA-PKcs^{-/-} mice with insulin. The mice were fasted overnight and then injected with a bolus of insulin (1 U/kg body weight) or a saline control. Twenty minutes after injection, liver, skeletal muscle, adipose and heart tissues were collected and analyzed by immunoblotting. PKB Ser473 was robustly (~ 20-fold for liver; ~25-fold for skeletal muscle and heart; ~10-fold for adipose tissue, data not shown) phosphorylated upon insulin stimulation in all four tissues from both wild-type and DNA-PKcs^{-/-} mice (Fig. 1A-D). Thus, we concluded that DNA-PK is dispensable for insulin-induced PKB Ser473 phosphorylation *in vivo*. Additionally, we treated wild-type and DNA-PKcs^{-/-} mouse embryonic fibroblasts (MEFs) with insulin-like growth factor (IGF-1) (Fig. 1E) or serum (Fig. 1F). Neither treatment resulted in impaired PKB Ser473 phosphorylation in DNA-PKcs^{-/-} MEFs, indicating that DNA-PK is not essential for PKB Ser473 phosphorylation upon IGF-1 or serum stimulation.

To investigate the role of DNA-PK in the maintenance of glucose metabolism, mice fasted overnight were subjected to an insulin or glucose tolerance test. To evaluate insulin sensitivity, insulin (1 U/kg) was injected intraperitoneally and blood glucose levels measured at the indicated time points. No significant differences in blood glucose levels were found between DNA-PKcs^{-/-} mice and wild-type controls (Fig. 2A). After oral application of glucose (2 g/kg), blood glucose levels were measured at the indicated times. Wild-type and DNA-PKcs^{-/-} mice displayed a similar response to the glucose treatment (Fig. 2B). Hence, DNA-PKcs^{-/-} mice display neither insulin resistance nor glucose intolerance. Taken together, the results show that DNA-PK is dispensable for PKB Ser473 phosphorylation in response to insulin and growth factor stimulation and is also not essential for the maintenance of glucose homeostasis.

DNA-PK is the physiological PKB Ser473 kinase upon γ -irradiation-induced DNA damage. DNA-PK is activated by DNA double-strand breaks induced by γ -irradiation and radiomimetic drugs (110,111). To investigate the role of DNA-PK in PKB Ser473 phosphorylation induced by γ -irradiation, we analyzed the PKB response to DNA damage in MEFs. Phosphorylation of PKB was promoted in wild-type MEFs in a dose-dependent manner (~4-fold for 5 Gy and 5-fold for 10 Gy), whereas this response was significantly impaired (p=0.0017 for 5 Gy and p<0.001 for 10 Gy) in DNA-PKcs^{-/-} MEFs (Fig. 3A). Moreover, the levels of phosphorylated FoxO4 Ser193 also increased in a dose-dependent manner, reaching a peak at 10 Gy (Fig. 3A). Further increase in DNA damage at 15 Gy irradiation led to compromised PKB and FoxO4 phosphorylation (Fig. 3A), which is reminiscent of the dependence of PKB activation on the extent of DNA damage. Further analysis showed that the

phosphorylation of PKB Ser473 and FoxO4 Ser193 remained impaired in DNA-PKcs^{-/-} MEFs compared to WT MEFs at additional time points analyzed (Supplementary Fig. 1). Subsequently, we investigated DNA-PK-dependent PKB activation induced by γ -radiation *in vivo*. Wild-type and DNA-PKcs^{-/-} mice were subjected to 1 Gy total body irradiation and tissues collected after 30 min. PKB was phosphorylated on both Thr308 and Ser473 (~3.5-fold) in the brains of wild-type animals upon γ -irradiation. Strikingly, this activation was compromised in the DNA-PKcs^{-/-} brain (Fig. 3B). Likewise, levels of phosphorylated FoxO4 increased in wild-type irradiated brain (~18-fold), whereas there was no induction of FoxO4 phosphorylation in DNA-PKcs^{-/-} irradiated brain (Fig. 3B). Immunoblot analysis with an antibody that preferentially recognizes the unphosphorylated form (due to the fact that the peptide used for production of the antibodies against phospho-protein and total protein were derived from the same sequence) showed a decrease in total FoxO4 protein upon irradiation. This indicates a robust phosphorylation of FoxO4 Ser193 upon DNA damage (Fig. 3B). In summary, *in vivo* and *ex vivo* results both indicate that PKB activation is promoted and that DNA-PK is required for phosphorylation of PKB Ser473 in response to DNA damage induced by γ -irradiation. Overall, this implies stimulus-specific regulation of PKB activation by DNA-PK.

PKB is hyperactivated in DNA-PKcs^{-/-} thymus. To investigate how loss of DNA-PK is reflected in basal PKB Ser473 phosphorylation, we analyzed a panel of tissues from wild-type and DNA-PKcs^{-/-} mice. No differences were observed in skeletal muscle, liver, spleen and brain (Supplementary Fig. 1A) and a mild increase was found in

adipose and brown fat (~2- and 2.5-fold, respectively) (Fig. 4A and B). However, a robust 8-fold increase in PKB Ser473 phosphorylation was found in DNA-PKcs^{-/-} thymus compared with wild-type (Fig. 4C). p70 ribosomal protein S6 kinase (S6K), acting downstream of PKB, has been shown to repress upstream signalling by induction of insulin receptor substrate-1 (IRS-1) degradation (113-115). In contrast, neither the phosphorylation of S6K1 on the hydrophobic motif residue Thr389 nor the IRS-1 protein levels were significantly different in DNA-PKcs^{-/-} and wild-type thymus and adipose (Fig. 4C and Supplementary Fig. 1B). Hence, we concluded that the increased PKB activity in the DNA-PKcs null background was not due to defective S6K/IRS-1-mediated feedback regulation in these tissues. Interestingly, in some cases we observed increase of both S6K Thr389 phosphorylation as well as IRS-1 protein levels in DNA-PKcs^{-/-} brown fat (Supplementary Fig. 1B). The marked increase in PKB phosphorylation upon loss of an upstream kinase prompted us to further investigate components of the PKB signalling pathway in DNA-PKcs^{-/-} thymus. Further analysis of PKB downstream targets revealed that, there was no significant difference in GSK3 β Ser9 phosphorylation, whereas the FoxO4 transcription factor was strongly phosphorylated in DNA-PKcs^{-/-} thymus compared with the wild-type. This suggests that the PKB/FoxO pathway is deregulated in DNA-PKcs^{-/-} thymus (Fig. 4C).

Constitutive PKB activity contributes to the development of spontaneous thymic lymphomas in DNA-PKcs^{-/-} mice and can be prevented by deletion of PKB α . PKB deregulation has been implicated in various types of cancer, including thymic lymphoma (101). Transgenic mice expressing a constitutively active PKB in T cells

develop T-cell lymphomas (116-118). Consistent with previous results (91), we observed an increased frequency of thymic lymphomas in DNA-PKcs^{-/-} mice (Fig. 5A). Thymic tumors already appeared in 3-month-old animals and were 30-fold larger than non-malignant DNA-PKcs^{-/-} thymi (0.010± 0.001 g. vs. 0.313± 0.106 g.; *P*=0.03) (Table 1). PKBα is highly expressed in thymocytes particularly at the early stages of development (84-86), where DNA-PKcs^{-/-} T cells are arrested (88-90). Reasoning that persistent PKB activity in DNA-PKcs^{-/-} thymus might contribute to thymic lymphoma formation, we investigated whether deletion of PKBα, prevented formation of thymic tumors in DNA-PKcs^{-/-} mice. About 27% (6/22) of DNA-PKcs^{-/-} mice aged 3-7 months displayed thymic tumors, as against zero in wild-type mice (0/14) (Fig. 5A). Significantly, none of the DNA-PKcs^{-/-}PKBα^{-/-} double knock-out (DKO) mice analyzed at 2-15 months (0/6) exhibited thymic tumors (Fig. 5A). The analysis of DNA-PKcs^{-/-}PKBα^{-/-} DKO mice over a wide age range ruled out delayed progression of thymic tumors. Further immunoblot analysis of DNA-PKcs^{-/-}PKBα^{-/-} DKO thymi revealed that, elevated PKB Ser473 and FoxO phosphorylation in DNA-PKcs^{-/-} thymi were restored to wild-type levels with deletion of PKBα (Fig. 5B). This suggests that increased PKB and FoxO phosphorylation in DNA-PKcs^{-/-} thymi is due to deregulation of PKBα. In line with previous reports (91), we observed reduced viability correlated with the occurrence of thymic tumors in DNA-PKcs^{-/-} mice (Fig. 6). Hence, we investigated whether deletion of PKBα gene improved survival of mice lacking DNA-PKcs. The survival of DNA-PKcs^{-/-}PKBα^{-/-} DKO mice significantly increased compared with DNA-PKcs^{-/-} mice (*P*<0.001). The longevity of mice increased from approximately 238 days for DNA-PKcs^{-/-} to 375 days for DNA-

PKcs^{-/-}PKB α ^{-/-} DKO supporting the involvement of PKB deregulation in the reduced life span of DNA-PK-deficient mice due to tumorigenesis (Fig. 6).

Histological analysis of thymi by hematoxylin-eosin and Ki67 staining showed tumors from DNA-PKcs^{-/-} mice to consist of atypical cells with a high proliferative index (Fig. 7A and data not shown). DNA-PKcs^{-/-}PKB α ^{-/-} DKO thymi showed disruption of the cortico-medullary boundary similar to DNA-PKcs^{-/-} thymi (see inset in Fig. 7A). To further investigate the role of PKB in DNA-PKcs^{-/-} tumors, thymocytes isolated from wild-type, DNA-PKcs^{-/-} and DNA-PKcs^{-/-}PKB α ^{-/-} DKO mice were analyzed by flow cytometry using antibodies against CD4 and CD8 cell-surface markers (Fig. 7B). In DNA-PKcs^{-/-} thymus, development of T cells was blocked at the CD4⁻CD8⁻ double-negative (DN) stage: about 80 % of DNA-PKcs^{-/-} T cells were CD4⁻CD8⁻ (DN) cells compared with about 2% DN-stage cells in the wild-type (Fig. 5B and Table 2). T cells escaped from this developmental block in tumors from DNA-PKcs^{-/-} mice, where there was a marked increase in

CD4⁺CD8⁺ double-positive (DP) cells (80%). Interestingly, flow cytometric analysis of T cells from the two thymus lobes of a DNA-PKcs^{-/-} animal displayed differing profiles; one being totally blocked at the DN stage and the other closely resembling the profile of an advanced tumor (Fig. 5B). Consistent with this, the latter was significantly enlarged (0.008 g vs. 0.027 g). In contrast, DNA-PKcs^{-/-}PKB α ^{-/-} DKO thymocytes were almost exclusively blocked DN-stage cells (Fig. 5B and Table 2), which suggests that PKB α has a role in neoplastic expansion of T cells in the DNA-PKcs^{-/-} thymus.

Discussion

In this study, we evaluated the *in vivo* role of DNA-PK in basal PKB Ser473 phosphorylation as well as in the responses to various stimuli. We found that DNA-PK is dispensable for insulin- and growth factor-induced PKB activation. Furthermore, DNA-PK is not essential for the maintenance of glucose metabolism. In contrast, our *in vivo* and *ex vivo* results revealed that DNA-PK is required for phosphorylation of PKB Ser473 upon DNA damage induced by γ -irradiation. Taken together, this implies stimulus-specific regulation of PKB Ser473 phosphorylation by specific upstream kinases. The impaired phosphorylation of both PKB Ser473 and Thr308 residues observed in DNA-PKcs^{-/-} cells as well as in PDK1^{-/-} cells (11) treated with γ -irradiation suggest the requirement of phosphorylation of these two sites for full activation of PKB and imply that two phosphorylation steps are tightly connected and interdependent (11,119). Hence, both Thr308 by PDK1 and Ser473 phosphorylation by DNA-PK appear to be essential for activation of PKB in response to DNA damage induction (11). The fact that increased doses of γ -irradiation led to compromised PKB phosphorylation suggest the dependence of PKB activation upon the extent of DNA damage, which is consistent with data obtained with HUVEC cells (11). In addition, the PKB response to γ -irradiation includes phosphorylation of the downstream target FoxO4 (this study) as well as regulation of p21 (11) and GSK3 (11,120) placing PKB as an important mediator of DNA damage signalling. In addition to its response to irradiation-induced double-strand DNA breaks, PKB is activated by various other DNA damage inducers. Numerous studies have demonstrated the importance of functional PKB signaling for survival after DNA

damage. Doxorubicin promotes PKB activation (Ser473 and Thr308 phosphorylation) in mouse embryonic fibroblasts (11). In addition, doxorubicin induced PKB activation was shown in several breast cancer cell lines as well as *in vivo* by which elevated myocardial PKB signaling ameliorates doxorubicin-induced congestive heart failure and promotes heart growth in mice. Further, a recent study showed that doxorubicin lead to phosphorylation of PKB and concominantly to PKB-dependent inactivation and nuclear exclusion of FoxO4 in human colon carcinoma cell line (121). Moreover, PKB activation is promoted by etoposide (122), cisplatin (123-125), and UV (37,126) in several different cell types. Notably, PKB α is important for survival after UV irradiation, as MEFs lacking PKB α undergo irradiation-induced apoptosis to a much larger extent than the wild-type MEFs (37). These studies place PKB as an important mediator of DNA damage signaling.

Our results revealed deregulation of PKB phosphorylation in DNA-PKcs^{-/-} mice in a tissue-specific manner. The marked increase in basal PKB Ser473 phosphorylation in the absence of an upstream kinase is unexpected. Elevated PKB Ser473 phosphorylation in DNA-PK^{-/-} tissues could most likely be mediated by mTORC2 and reminiscent of cross-talk between the two PKB Ser473 kinases, DNA-PK and mTOR. This regulation might take place at multiple levels of the pathway as our results showed that elevated PKB Ser473 phosphorylation could also be observed without the accompanying changes in S6K Thr389 phosphorylation. Therefore, although we cannot completely rule out the contribution of a S6K mediated mechanism; our data suggest the existence of an alternative mechanism of regulation where deletion of DNA-PK could lead to its disruption and result in deregulation of PKB.

It has been suggested that mTORC2 is necessary for modulation of PKC α Ser657 phosphorylation (127), and was shown that overexpression of mTORC2 components leads to enhanced PKC α activity. Knock-down of rictor or mTOR lead to impaired PKC α phosphorylation (127,128). Further, ablation of rictor or mLST8 was shown to severely compromise PKC α phosphorylation and stability (107). We therefore examined whether PKC α phosphorylation was altered in DNA-PKcs^{-/-} thymus. We observed increased phosphorylation of PKC α Ser657 (Supplementary fig 2) supporting our hypothesis that hyperphosphorylation of PKB Ser473 could be mediated by mTORC2 in DNA-PKcs^{-/-} thymus.

Further, a recent study suggested that TSC1-TSC2 complex positively regulates mTORC2 independent of its effects on mTORC1 (129). Interestingly we observed increased total TSC2 levels in DNA-PKcs^{-/-} thymus (data not shown). However, whether this has an effect on increased PKB Ser473 phosphorylation via positively regulating mTORC2 in DNA-PKcs^{-/-} thymus remains to be established.

Recent studies proposed a role for Tel2 to function as a coordinator among PIKKs and suggested existence of crosstalks between different PIKKs where alteration of one PIKK may influence other (130-133). It has been shown that Tel2, which directly interacts with and stabilizes the protein levels of PIKKs, including DNA-PK and mTOR, (131,133), It has also been proposed that Tel2 may serve as a scaffold protein that mediates signal transduction from PIKKs to their target proteins (132). However, further study is required to understand the mechanistic role of Tel2 as a mediator of PIKK functions. It will be intriguing to see whether Tel2 could have a role mediator role affecting the downstream signaling of PIKKs, in particular within the context of mTOR and DNA-PK.

We have found that the PKB/FoxO pathway is deregulated in DNA-PKcs^{-/-} thymus and shown that increased PKB activity in a DNA-PKcs null background contributes to spontaneous formation of thymic tumors by allowing neoplastic expansion of thymocytes. Significantly, these tumors could be prevented by deletion of PKB α that is highly expressed in thymocytes, particularly in DN- and DP-stages (84-86). Furthermore, subsequent deletion of PKB α gene improves the survival of DNA-PKcs^{-/-} mice. However, the survival of DNA-PKcs^{-/-}PKB α ^{-/-} DKO mice was not restored to that of PKB α ^{-/-} mice, which is not surprising given that DNA-PK has a wide variety of functions, some of which are independent of PKB α . Alternatively, the loss of DNA-PK in a PKB α ^{-/-} background could lead to deterioration of DNA-PK-dependent PKB functions in tissues other than the thymus. Moreover, deregulation of other isoforms of PKB in DNA-PK^{-/-} mice could possibly affect overall organismal fitness and survival. Finally, loss of DNA-PK independent PKB α functions could lead to additive affects in DNA-PKcs^{-/-}PKB α ^{-/-} DKO mice. Further studies using tissue specific DNA-PK^{-/-}PKB α ^{-/-} double knock-out mice will be necessary to evaluate these possibilities.

PKB has been shown to play an important role in DN- to DP-stage transition and to be essential for thymocyte survival and differentiation (84-86). Therefore, persistent PKB activity in the DNA-PKcs^{-/-} thymus, where DN thymocytes predominate, could contribute to malignant transition. It was reported recently that conditional and simultaneous disruption of FoxO1, FoxO3 and FoxO4 genes in mice leads to the development of thymic lymphomas and that thymocytes from these mice show increased proliferation (134,135). Moreover, disruption of FoxO function was shown to accelerate Myc-driven lymphomagenesis (136). Therefore, deregulation of

PKB/FoxO downstream events affecting proliferation or apoptosis may play a role in thymic tumor induction in DNA-PKcs^{-/-} mice.

Given the importance of PKB activity in T-cell development and the role of PKB in lymphomagenesis, the signalling components leading to PKB activation in DNA-PK-deficient thymus need to be established. Pre-T cell receptor (pre-TCR), Notch and interleukin-7 (IL-7) signalling provide critical signals for the survival, proliferation and differentiation of T cells (83,86,137-139). Activation of PI3K/PKB pathway is common to all three receptors and point to PI3K/PKB pathway as a central player that translates these signals into multiple functional outcomes. As pre-TCR signalling requires a functionally rearranged TCR β , which is lacking in DNA-PKcs^{-/-} thymocytes as a result of defective V(D)J recombination, it is unlikely that PKB activity increases in response to pre-TCR signalling. Hence, deregulation of Notch and/or IL-7 signalling and their role in PKB activation needs to be investigated in DNA-PKcs^{-/-} thymus. Frequent mutational activation of Notch has been identified in human T-cell acute lymphoblastic leukemia (T-ALL) and subsequently in mouse thymic lymphomas including those from severe combined immunodeficient (SCID) mice, a DNA-PK deficient mouse model (137,140,141). Recent studies provided evidence that Notch-1 upregulates PI3K/PKB pathway in T-ALL cells bearing Notch-1 mutations and showed synergistic suppression of growth in cells treated with small molecule inhibitor of Notch and rapamycin (142,143). In other studies of T-ALL cells, rapamycin inhibited IL-7-mediated cell cycle progression and cellular proliferation (144). The rapamycin derivative, CCI-779, was shown to reduce mTORC2 signalling and inhibit PKB activation in hematopoietic malignancies although another study reported that PKB activation remained unchanged using a different rapamycin

derivative, RAD001 (145,146). Rapamycin treatment was shown to inhibit PKB phosphorylation in a tissue-dependent manner, particularly in thymus (147). It is plausible that PKB hyperactivity in the thymus in the absence of DNA-PK could be mediated by mTORC2 and tissue-specific disruption of the PKB/FoxO pathway could render DNA-PK deficient thymic lymphomas susceptible to rapamycin treatment. A number of small molecule inhibitors of DNA-PK have been developed and the inhibition of DNA-PK sensitizes cells to DNA damaging agents (148,149). However, the use of DNA-PK inhibitors needs to be evaluated cautiously in the treatment of thymic lymphomas as loss of DNA-PK activity could lead to increased PKB signalling. Taken together, these results provide genetic evidence that PKB is deregulated in DNA-PKcs^{-/-} thymi and demonstrate a key role for PKB α in thymic tumor progression in DNA-PKcs^{-/-} mice. In general, the results indicate elaborate regulation of PKB phosphorylation by upstream kinases and its deregulation leading to malignant transformation.

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Figure Legends

Fig. 1. DNA-PK is dispensable for PKB Ser473 phosphorylation upon insulin- and growth factor-stimulation. After overnight fasting and insulin stimulation (1 U/kg body weight) for 20 min, tissues from 3-month-old wild-type (WT) and DNA-PKcs^{-/-} (KO) mice were analyzed for PKB Ser473 phosphorylation via immunoblotting with phospho-specific antibodies: (A) Skeletal muscle, (B) Liver, (C) Heart, (D) Adipose tissue. Wild-type (WT) and DNA-PKcs^{-/-} MEFs (KO) were stimulated with IGF-1 (50 ng/ml) (E) or serum (10%) (F) for various times and PKB Ser473 phosphorylation analyzed by immunoblotting.

Fig. 2. DNA-PK is dispensable for maintenance of glucose metabolism.

For analysis of glucose metabolism, 3-month-old wild-type (WT) and DNA-PKcs^{-/-} (KO) mice after overnight fasting were treated with insulin (1 U/kg body weight) for insulin tolerance tests (A) or glucose (2 g/kg body weight) for glucose tolerance tests (B). The graphs depict arithmetic means \pm SEM of blood glucose concentrations at the indicated time points following the treatments.

Fig. 3. DNA-PK is the physiological PKB Ser473 kinase in the response to γ -irradiation.

PKB and FoxO4 phosphorylation were analyzed by phospho-specific antibodies in wild-type (WT) and DNA-PKcs^{-/-} (KO) MEF lysates 30 min after the indicated doses of γ -irradiation (A), or at indicated time points after 10 Gy irradiation (B). Right panels: Quantification of fold increase in PKB Ser473 phosphorylation after γ -irradiation determined by phospho-PKB (p-PKB)Ser473/PKB ratio with respect to non-irradiated WT and KO controls. Data were tested for significance using one-way analysis of variance (ANOVA) with the Holm-Sidak multiple comparisons procedure. ns: not significant. (C) Analysis of PKB and FoxO4 phosphorylation in wild-type (WT) and DNA-PKcs^{-/-} (KO) brain 30 min after 1 Gy total body γ -irradiation (TBI) or sham irradiation. Right panel: Quantification of fold increase in p-PKB Ser473/actin ratio and phospho-FoxO4 (p-FoxO4) Ser193/actin ratio after γ -irradiation with respect to sham irradiated WT and KO controls.

Fig. 4. Analysis of basal PKB phosphorylation in DNA-PKcs^{-/-} tissues.

Tissues were collected from 2-month-old random-fed WT and DNA-PKcs^{-/-} mice and analyzed by immunoblotting. (A) Top panel: PKB phosphorylation in WT and DNA-PKcs^{-/-} (KO) adipose tissue. Bottom panel: Quantification of PKB Ser473 phosphorylation determined by p-PKB Ser473/PKB ratio relative to wild type ratio. (B) Top panel: PKB phosphorylation in WT and DNA-PKcs^{-/-} (KO) brown fat. Bottom panel: Quantification of PKB Ser473 phosphorylation determined by p-PKB Ser473/PKB ratio relative to wild type ratio. (C) Top panel: PKB Ser473, PKB Thr308, GSK3 β Ser9, FoxO3a Thr32 and S6K Thr389 phosphorylation and total IRS-1 protein levels in WT and DNA-PKcs^{-/-} (KO) thymi. Bottom panel: Quantification of PKB Ser473 phosphorylation determined by p-PKB Ser473/PKB ratio relative to wild-type ratio.

Fig. 5. Deletion of PKB α prevents spontaneous development of thymic tumors.

(A) Incidence of thymic tumors from wild-type (WT), DNA-PKcs^{-/-} (KO) analyzed between 3-7 months age and DNA-PKcs^{-/-}PKB α ^{-/-} double knock-out (DKO) mice analyzed between 2-15 months age. (B) PKB Ser473, FoxO4 Ser193 and FoxO3a Thr32 phosphorylation in WT thymus, DNA-PKcs^{-/-} tumor and DNA-PKcs^{-/-}PKB α ^{-/-} (DKO) thymi.

Fig. 6. Deletion of PKB α improves survival of DNA-PKcs^{-/-} mice.

Kaplan-Meier survival curves for wild-type (WT), DNA-PKcs^{-/-}, PKB α ^{-/-} and DNA-

PKcs^{-/-}PKBα^{-/-} double knock-out (DKO) mice analyzed by LogRank analysis and the Holm-Sidak multiple comparisons procedure. The survival of DNA-PKcs^{-/-}PKBα^{-/-}DKO mice significantly increased compared with DNA-PKcs^{-/-} mice ($P < 0.001$). Mice that survived longer than 2 months were included in the analysis. Numbers of mice analyzed: WT 52, DNA-PKcs^{-/-} 21, PKBα^{-/-} 26, DNA-PKcs^{-/-}PKBα^{-/-} (DKO) 13.

Fig. 7. Deletion of PKBα prevents neoplastic expansion of DNA-PKcs^{-/-} thymocytes. (A) Histological analysis of thymi from wild-type (WT), DNA-PKcs^{-/-} (KO), and DNA-PKcs^{-/-}PKBα^{-/-} double knock-out (DKO) mice and thymic tumors from DNA-PKcs^{-/-} mice. Thymi were fixed with 4% paraformaldehyde, processed with paraffin, sectioned and stained with hematoxylin/eosin (×400 magnification; scale bar indicates 50 μm). The insets show ×40 magnification. M: medulla C: cortex. (B) Flow cytometric analysis of thymocytes from WT, DNA-PKcs^{-/-} (KO) and DNA-PKcs^{-/-}PKBα^{-/-} (DKO) mice for CD4 and CD8 markers.

Supplementary Figure Legends

Supplementary Fig. 1. Analysis of components of PKB signalling in DNA-PKcs^{-/-} tissues. (A) PKB Ser473 basal phosphorylation levels in wild-type (WT) and DNA-PKcs^{-/-} (KO) tissues. (B) pS6K Thr389, pGSK3 Ser9 and total IRS-1 levels in WT and KO adipose and brown fat.

Supplementary Fig. 2. (A) Analysis of p-PKC levels in wild-type (WT) and DNA-PKcs^{-/-} (KO) thymus. Right panel: Quantification of p-PKC/PKC levels
(B) Immunoblot analysis of TSC2 levels in wild-type (WT) and DNA-PKcs^{-/-} (KO) thymus. Right panel: Quantification of TSC/actin levels.

Figure 1.

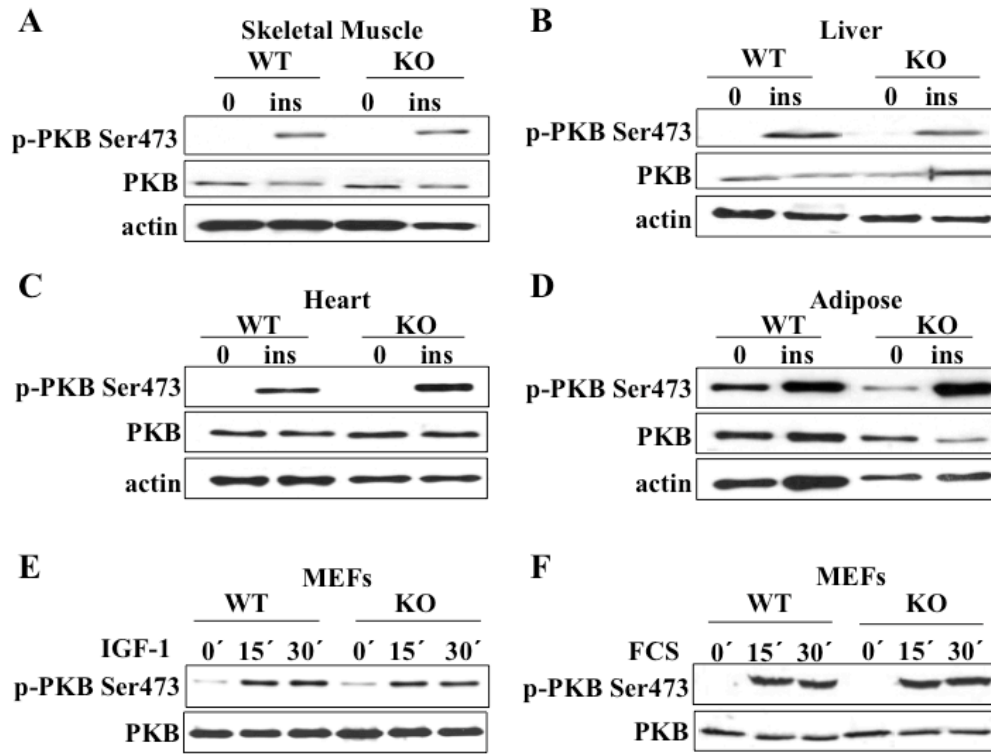


Figure 2.

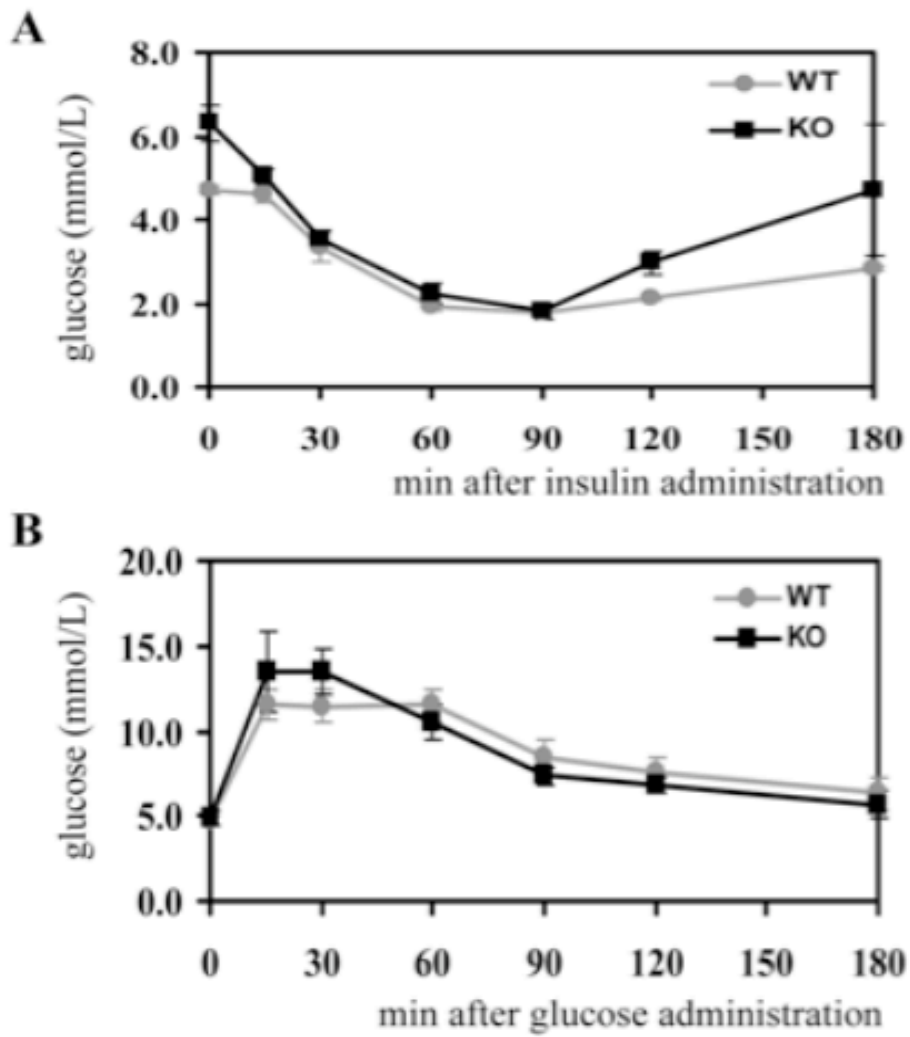


Figure 3.

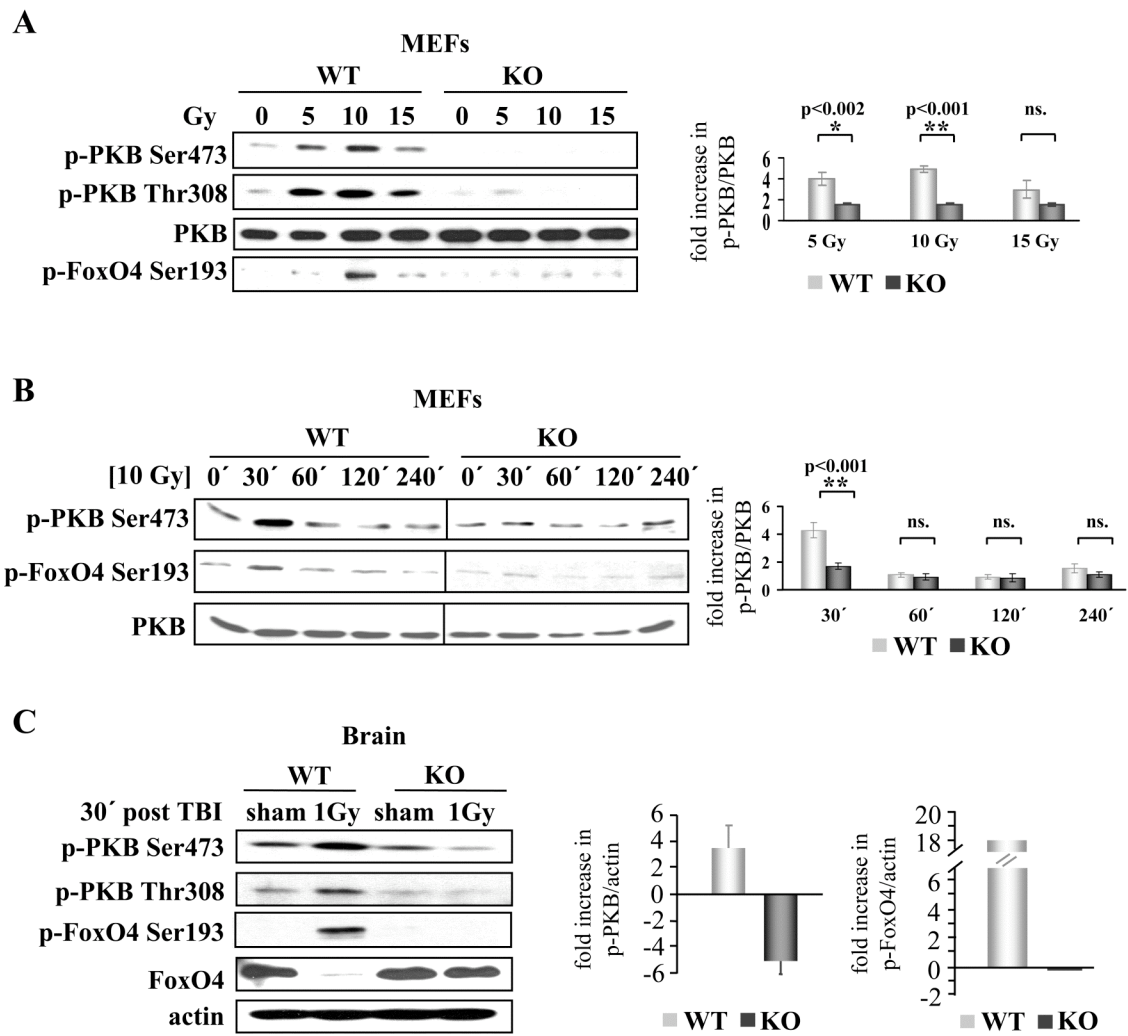


Figure 4.

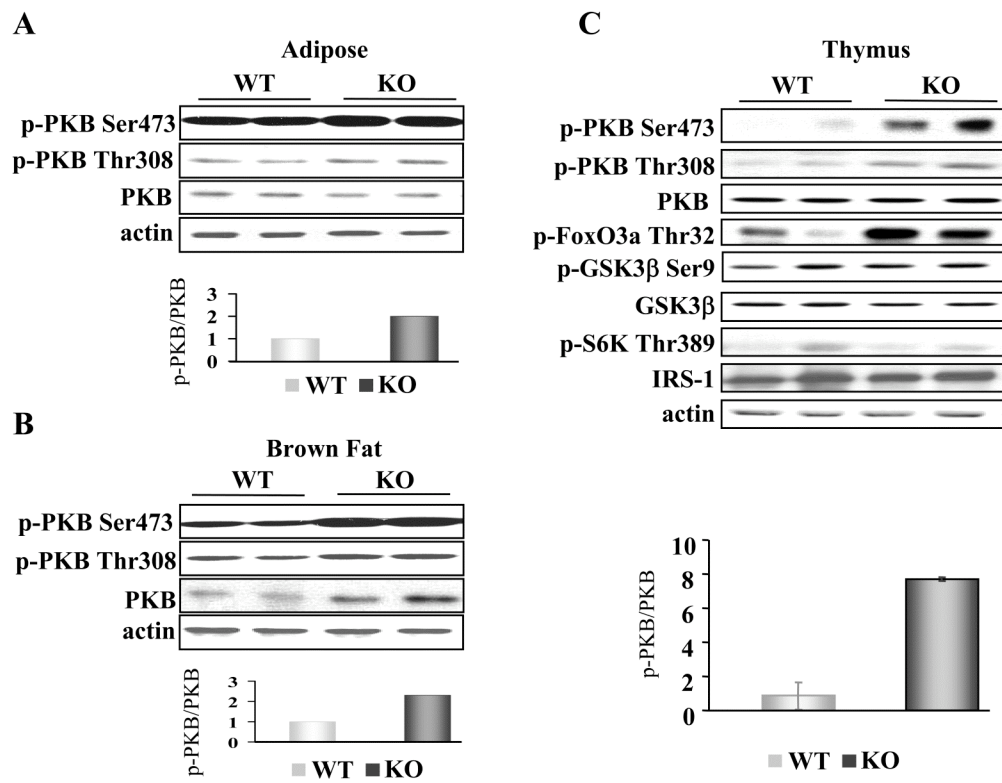


Figure 5.

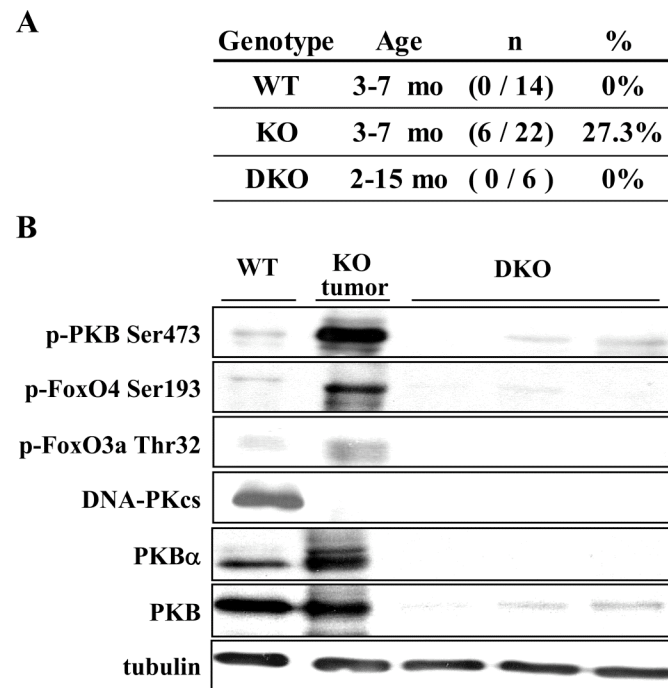


Figure 6.

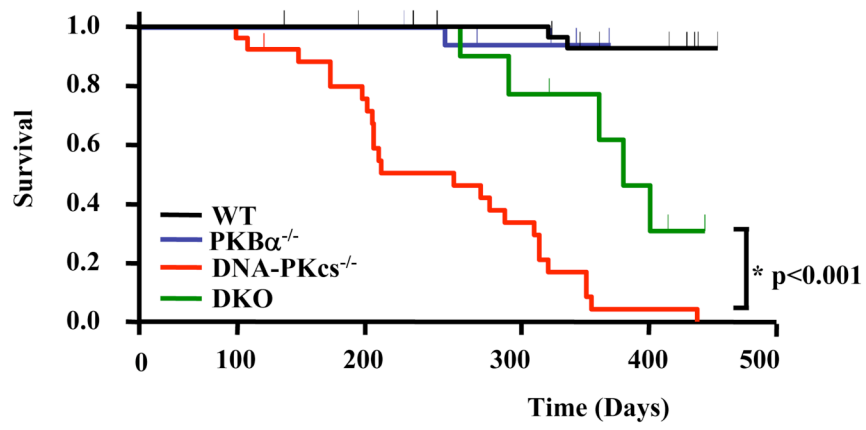
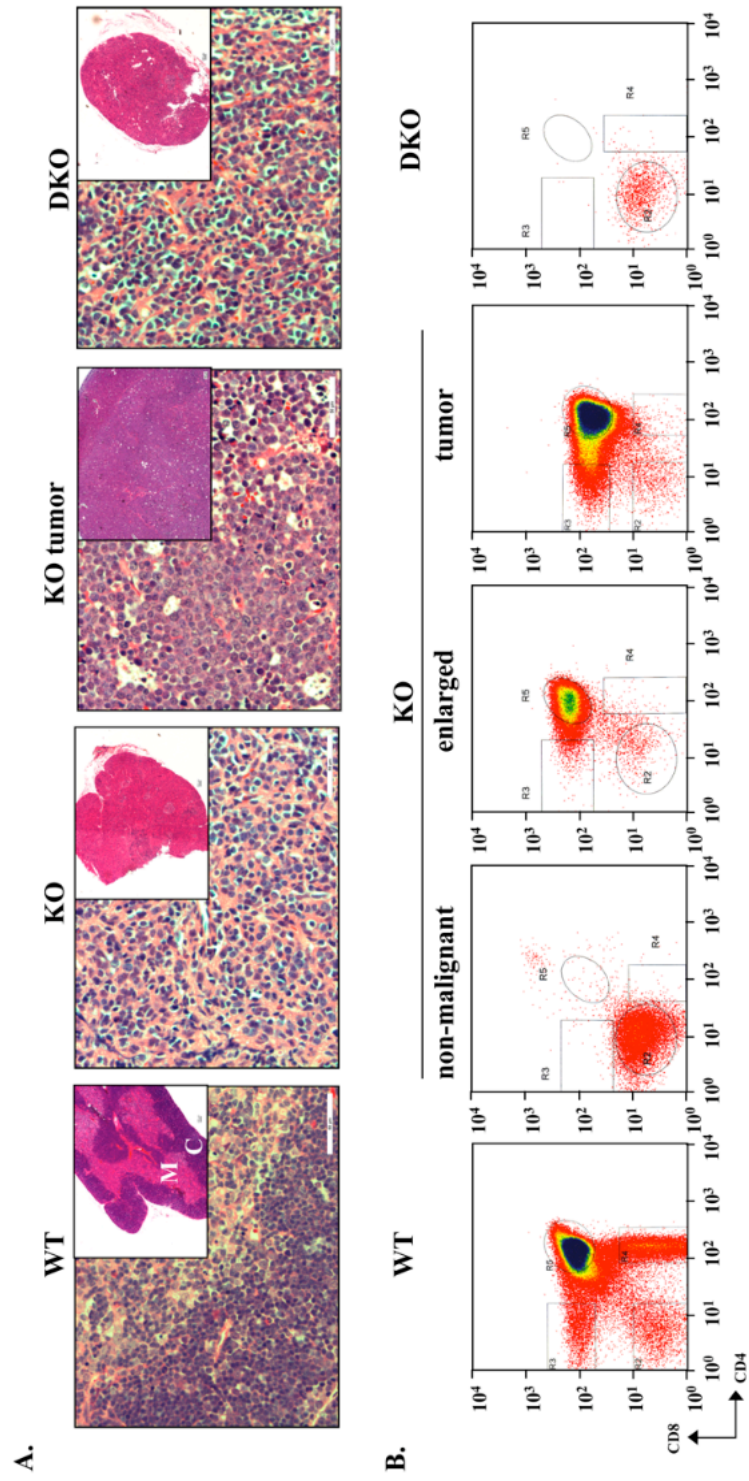
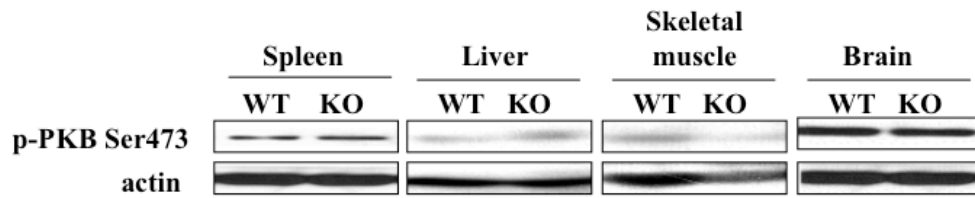
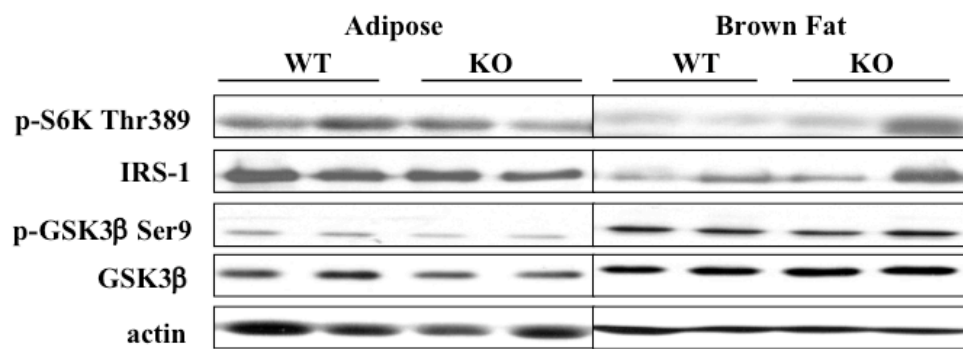


Figure 7.

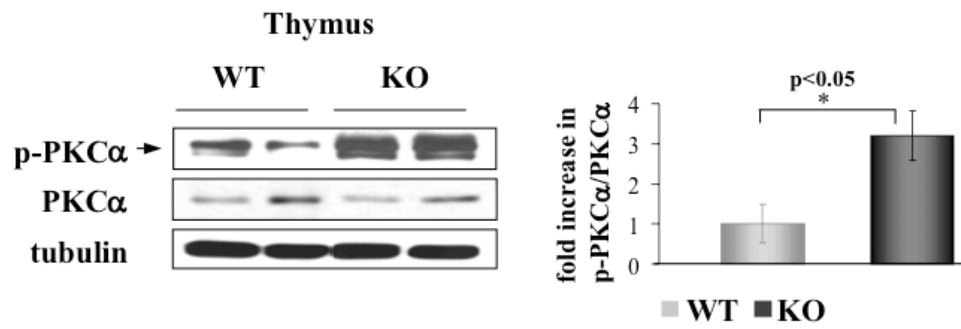


Supplementary Figure 1.

A**B**

Supplementary Figure 2.

A



B

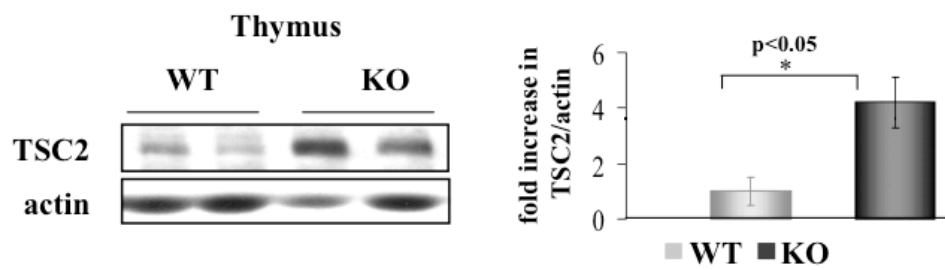


Table 1. Thymic weights of wild-type and DNA-PKcs^{-/-} mice (non-malignant control and tumor)

n: number of mice analyzed, SEM: standard error of mean

Tissue	Thymic weight (± SEM) (g)	Thymic/Body weight (± SEM) (%)	(n)
Wild-type thymus	0.045 (± 0.004)	0.181 (±0.017)	n = 8
DNA-PKcs ^{-/-} non-malignant thymus	0.010 (± 0.001) ***	0.038 (± 0.005) ***	n = 8
DNA-PKcs ^{-/-} thymic tumor	0.313 (± 0.106) **	1.231 (±0.450) *	n = 5

*** $p < 0.001$ DNA-PKcs^{-/-} non malignant thymus vs. wild type thymus** $p = 0.003$ DNA-PKcs^{-/-} thymic tumor vs. DNA-PKcs^{-/-} non malignant thymus* $p = 0.005$ DNA-PKcs^{-/-} thymic tumor vs. DNA-PKcs^{-/-} non malignant thymus

Table 2. Percentages of T cell subsets in wild type, DNA-PKcs^{-/-} and DNA-PKcs^{-/-} PKB α ^{-/-} DKO mice.

T cell subsets that are double negative (DN), double positive (DP), CD4⁺CD8⁻ (CD4⁺), and CD4CD8⁺ (CD8⁺) for CD4 and CD8 cell surface markers. Data provided as % mean (\pm SEM).

T cell subsets	WT	DNA-PKcs ^{-/-} non-malignant	DNA-PKcs ^{-/-} enlarged	DNA-PKcs ^{-/-} tumor	DNA-PKcs ^{-/-} PKB α ^{-/-} DKO
DN	1.66 (\pm 0.14)	78.72 (\pm 2.12)	1.84	0.7	83.71 (\pm 1.18)
DP	79.99 (\pm 1.80)	0.55 (\pm 0.20)	82.47	79.66	0.1 (\pm 0.1)
CD4 ⁺	6.46 (\pm 0.47)	2.50 (\pm 0.62)	0.41	0.27	1.84 (\pm 0)
CD8 ⁺	1.67 (\pm 0.16)	0.64 (\pm 0.24)	2.55	4.41	0.46 (\pm 0.14)

Part 2: Phosphatidylinositol-3-kinase / DNA-dependent protein kinase inhibitor BBD130 blocks PKB phosphorylation induced by DNA damage and inhibits growth.

Banu Surucu, Jianhua Feng, Lana Bozulic, Carlos Garcia-Echeverria, Sauveur-Michel Maira, and Brian A. Hemmings

**Phosphatidylinositol-3-kinase / DNA-dependent protein kinase
inhibitor BBD130 blocks PKB phosphorylation
induced by DNA damage and inhibits growth**

Banu Surucu¹, Jianhua Feng¹, Lana Bozulic¹, Carlos Garcia-Echeverria²,
Sauveur-Michel Maira², and Brian A. Hemmings¹

¹ Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66,
Basel, CH 4058, Switzerland

² Novartis Pharma, Basel, Switzerland

*Corresponding author: Brian A. Hemmings
Tel: +41 61 697 4872
Fax: +41 61 697 3976
E-mail: brian.hemmings@fmi.ch

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Introduction

The proto-oncogene and the serine/ threonine kinase, protein kinase B (PKB)/Akt is a key effector of phosphatidylinositol-3-kinase (PI3K) pathway. PKB is a major regulator of a variety of cellular functions including survival, proliferation, growth, transcription and metabolism (7,8,23). Consistent with its key function in cell homeostasis, PKB is activated by numerous stimuli including hormones, growth factors and DNA damage (8,10). Activation of PKB requires phosphorylation at two key regulatory sites: Thr308 and Ser473 (of PKB α /Akt1). Phosphorylation by PDK1 occurs on Thr308 in the activation loop of PKB. The phosphorylation on Ser473 within a C-terminal hydrophobic motif leads to full activation of PKB and mediated by two members of the PI3K-related kinase (PIKK) family, mTOR/riCTOR complex (mTORC2) (21) or DNA-dependent protein kinase (DNA-PK) (11,19), in a stimulus specific manner. Insulin or growth factor induced PKB Ser473 phosphorylation is regulated by mTORC2 (21,107-109). In contrast, DNA damage-induced phosphorylation of PKB Ser473 is mediated by DNA-PK (11). In addition, a role for DNA-PK in the activation of PKB by CpG-DNA has been established using bone marrow-derived macrophages (22). Once activated, PKB acts on a plethora of substrates to regulate diverse cellular functions (23,103-105,150). As PKB regulated responses could favor tumor initiation and/or progression it is not surprising that PI3K/PKB pathway is one of the most deregulated pathways in human cancers. Several cancer types showed

deregulation of this pathway due mutations, amplifications or overexpression of PKB or PI3K isoforms (52-55). PKB Ser473 phosphorylation is used as a biomarker and strongly correlated with poor prognosis of several cancers (56-58) rendering inhibition of PKB by targeting the components of PI3K/PKB pathway as an effective strategy for cancer treatment (59-62).

Several of the most effective targeted cancer therapies owe their action to synergy through inhibition of several targets (151). The requirement for inhibition of multiple targets likely reflects the complexity of signaling underlying malignant transformation and ability of cancer cells to dynamically adapt to stress. For instance, it has been demonstrated that use of dual inhibitors of PI3K and mTOR shows efficacy in glioma cells and inhibits proliferation (151).

Radiotherapy and DNA damaging drugs are among the most efficient therapies for cancer treatment. Ionizing radiation and the topoisomerase II inhibitors, such as doxorubicin, are commonly used agents in the treatment of a wide range of cancers (152). DNA double-strand breaks (DSBs), which are considered the most lethal type of DNA damage, are the principal genotoxic lesions introduced by these agents. However, effectiveness of genotoxins is limited by treatment-associated toxicity and the emergence of resistance. One of the present efforts in oncology is to increase the potency of genotoxins so that efficacious doses can be lowered. One approach to accomplish this is to inhibit DNA-dependent protein kinase (DNA-PK), which is a part of the non-homologous end-joining (NHEJ) machinery, one of the two major DSB repair

pathways in mammalian cells (110,153). DNA-PK holoenzyme consists of catalytic subunit (DNA-PKcs) and the Ku70/80 heterodimer. Cells deficient in DNA-PK are highly sensitive to ionizing radiation and radiomimetic drugs (88,154-160). Overexpression of DNA-PKcs can accelerate the repair of ionizing radiation-induced, etoposide-induced, and doxorubicin-induced DNA DSBs and confer resistance to these agents (161,162). A number of DNA-PK inhibitors were developed to enhance radio- and chemosensitivity and presented promising outcome in combination with radiation or chemotherapy in preclinical studies (148,149). Another possible role for DNA-PK in chemo- and radio-resistance emerged with the recent findings that DNA-PK phosphorylates and activates PKB in response to DNA double strand breaks and promote survival (11).

Imidazo[4,5-c]quinoline derivatives have been discovered and developed as potent and effective modulators of the PI3K/PKB pathway (163). They can adopt different binding modes in the ATP binding cleft, to mimic the H-bond interactions of the adenine moiety of ATP. BBD130 is a imidazo[4,5-c]quinoline derivative that inhibits PI3K and control PKB activation in cellular and in vivo settings (163).

In this study, we aimed to determine the ability of BBD130 to inhibit PKB activation upon different stimuli to dissect the mode of action of BBD130 and whether simultaneous inhibition of PI3K/DNA-PK would confer efficacy in combination with ionizing radiation.

Results & Discussion

The small molecule inhibitor BBD130, originally identified as a PI3K inhibitor, is a potent inhibitor of DNA-PK in vitro (IC_{50} BBD130=58 nM) (Figure 1). To determine whether DNA-PK dependent PKB phosphorylation induced by DNA DSB-inducing agents such as γ -irradiation is as well could be inhibited by BBD130 in cells, wild type and DNA-PKcs deficient mouse embryonic fibroblasts (MEFs) were pre-treated with varying concentrations of BBD130 prior to 10 Gy γ -irradiation (Fig 2). As expected, PKB was phosphorylated in wild type MEFs but not in DNA-PKcs deficient MEFs. Significantly, BBD130 effectively inhibited DNA damage induced PKB phosphorylation further supporting the inhibition of DNA-PK by BBD130.

Insulin and growth factor induced PKB Ser473 phosphorylation is mediated by mTORC2 (86). To test whether insulin induced PKB phosphorylation could be inhibited by BBD130, the MEFs were treated with BBD130 prior to insulin stimulation (Fig 3A). In line with the previous reports, the DNA-PKcs deficient MEFs did not show impaired PKB phosphorylation in response to insulin treatment. Remarkably, the pretreatment of MEFs with BBD130 showed inhibition of insulin-induced PKB phosphorylation in both wild type and DNA-PKcs deficient MEFs. Moreover pretreatment of HEK293 cells with BBD130 showed efficient inhibition of insulin-induced PKB phosphorylation (Fig 3B). Further, inhibition of S6K phosphorylation on Thr389 confirmed downregulation of mTOR signaling (Fig 3C).

To further investigate the mode of action of BBD130, we made use of a chimeric PKB construct, which contains the C1 domain of Protein Kinase C at the N terminus instead of the PH domain (164). This chimeric protein termed C1-PKB α - Δ PH produces a kinase that can be activated *in vivo* by a second messenger other than phosphatidylinositol 3,4,5-trisphosphate (PIP3), namely phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA). HEK293 cells transfected with C1-PKB α - Δ PH were pretreated with BBD130 prior to stimulation with TPA. The partial inhibition of PKB Ser473 phosphorylation with BBD130 pretreatment indicated that the PKB inhibition is partly due to PI3K inhibition (Fig 3D). Taken together, these results indicate that BBD130 is a potent inhibitor of PKB phosphorylation by acting on multiple levels of PI3K/PKB pathway and that BBD130 is able to downregulate PKB activation by inhibition of PI3K and DNA-PK.

DNA-PK inhibitors were shown to sensitize cells to DNA damaging agents (30,44). We aimed to analyze if DNA-PK inhibitory effect of BBD130 result in sensitization of DNA-PK proficient cancer cells to DNA damage induced by γ -irradiation. For this, we utilized MO59K (DNA-PK proficient) and MO59J (DNA-PK deficient) human glioblastoma cell lines of which MO59J cells are hypersensitive to ionizing radiation. Analysis of growth inhibition following 3 day-treatment with the inhibitor alone showed considerable sensitivity to increasing concentrations of BBD130 with a dose dependent decline in cell viability for both cells lines. However, in the absence of γ -irradiation there was no significant difference in the IC₅₀s for growth in DNA-PK proficient and

deficient cells IC_{50} MO59K: ~32 nM IC_{50} MO59J: ~27 nM (Fig 4A). In order to assess the sensitization of DNA-PK proficient cells to irradiation, MO59K cells were subjected to 10 Gy irradiation alone, 30 nM BBD130 alone or 30 nM BBD130 prior to 10 Gy irradiation and cell viability was determined after 1 or 3 days (Fig 4B). The viability of MO59K cells subjected to 10 Gy irradiation was not significantly affected after 1 day and were about 72 % (72±2.5 %) after 3 days. Likewise, the viability of cells that are treated with BBD130 alone was not affected at day 1 and reduced to 52 % (52±3 %) after 3 days. In contrast when the cells are subjected to 10 Gy γ -irradiation in addition to treatment with BBD130, the viability of DNA-PK proficient MO59K glioblastoma cells were drastically reduced after 24 hours of treatment (75% ± 7.5). Notably, the viability of the DNA-PK proficient cells was comparable to its DNA-PK deficient counterparts, which are subjected to irradiation only after 3 days. Percent viability of MO59J cells subjected to irradiation alone was 40% (40%±3.4) (data not shown), and percent viability of MO59K cells treated with BBD130 and γ -irradiation was 41 % (41%±3.6) after 3 days. These results suggested that there is a synergistic affect of BBD130 and γ -irradiation on growth inhibition of glioblastoma cells possibly through inhibition of DNA-PK. It has become increasingly apparent that the most effective targeted therapeutic agents in clinical trials are inhibitors that block multiple target kinases. Compounds that target major signaling nodes involved in tumor progression could have significant success alone or in combination with current treatments of cancer. We made use of differential activation of PKB by

upstream kinases in response to specific stimuli as a tool to dissect the mode of action of the BBD130. mTORC2 dependent PKB activation was assessed by insulin stimulation and DNA-PK dependent PKB activation was assessed by γ -irradiation. The mechanistic rationale for the efficacy of this combination therapy could be described by cooperative inhibition of PI3K and DNA-PK. Several of the PI3K inhibitors inhibit DNA-PK (60) due to their structural similarity in the kinase domain. Significantly, inhibition of DNA-PK could play dual role in radiosensitization: by inhibition of the DNA repair as well as by inhibition of PKB activation induced by γ -irradiation.

Materials and Methods

In vitro kinase assays. DNA-PKcs and Ku were kindly provided by Dr. O. Hammarsten (Gothenburg University, Gothenburg, Sweden). Each 2.5 $\mu\text{g/ml}$ of DNA-PKcs and Ku, 1mg/ml FSYtide, and 50 μM ATP were used for the assay.

Cell culture, transfections and treatments. HEK293, MO59J, MO59K cells and mouse embryonic fibroblasts (MEFs) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. BBD130 was synthesized by Novartis Pharma Ag, Basel, Switzerland. Compound was initially dissolved in DMSO to make 10 mM solutions, and then was serially diluted to obtain final concentrations for cell treatments. The construction of C1-PKBa-dPH expression vector was described previously (164). HEK293 cells seeded at 0.5×10^6 per 6-cm-dish were transfected the following day, by a modified calcium phosphate method (165), with plasmid DNA (0.5 mg/ml). The transfection mixture was removed after 16-hour incubation, and cells were serum starved overnight before stimulation with 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 100 ng/ml; Sigma). For insulin treatments, cells were starved overnight prior to 1-hour treatment with various concentrations of BBD130. Cells were stimulated with 100 nM insulin (Sigma) for indicated times. For γ -irradiation treatments, cells were starved overnight prior to 1-hour

treatment with various concentrations of BBD130 and subjected to a single exposure of 10 Gy using X-rays [Asteophysics Research Corp, TORREX 120D X-Ray system]. Lysates were collected after 30 minutes.

Western blot analysis. Protein lysates were collected in lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 1% NP-40, 40 mM β -glycerophosphate, 10% glycerol, 0.05 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 50 mM NaF, 1 mM Na_3VO_4 , 1 μM Microcystin LR). Homogenates were centrifuged (13,000 rpm for 20 min at 4°C) to remove cell debris. Protein concentrations were determined using the Bradford assay. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Antibodies against phospho-PKB (Ser473), phospho-PKB (Thr308), phospho-S6K (Thr389) were purchased from Cell Signaling. A rat monoclonal anti- α -tubulin (YL1/2)-producing hybridoma cell line was obtained from American Type Culture Collection. Quantification was performed using ImageQuant TL (Amersham Biosciences) software.

Growth inhibition assays. The cells were seeded in triplicates and treated with indicated concentrations of BBD130 or subjected to 10 Gy γ -irradiation. For combination studies, the cells were pretreated with indicated concentrations of BBD130 for 1 hour prior to 10 Gy γ -irradiation. The cell viability was determined after 1- and 3-day incubation using Vi-CELL Series Cell Viability

Analyzer (Beckman Coulter, Inc.) according to manufacturer's instructions. Data were normalized to growth of untreated control cells and presented as mean \pm SEM. IC₅₀ calculation for growth inhibition was performed using Sigma Plot (SPSS Inc., IL, USA), using a standard four-parameter dose-response equation.

Figure legends

Figure 1. BBD130 is a potent inhibitor of DNA-PK activity in vitro. Each 2.5 mg/ml of DNA-PKcs and Ku, 1mg/ml FSYtide, and 50 μ M ATP were used for the assay.

Figure 2. BBD130 inhibits DNA-PK dependent PKB phosphorylation induced by γ -irradiation. MEFs were pretreated with indicated concentrations of BBD130 for 1 hour prior to treatment with 10Gy irradiation and lysed after 30 minutes.

Figure 3. Inhibition of mTORC2 dependent insulin-induced PKB phosphorylation and mTORC1 dependent S6K phosphorylation by BBD130 is partly due to PI3K inhibition. A. MEFs were pretreated with BBD130 for 1 hour prior to stimulation with 100nM insulin and the cells were lysed after 30 min. B. HEK293 cells were pretreated with indicated concentrations of BBD130 for 1 hour prior to 100 nM insulin stimulation for 15 minutes. C. HEK293 cells were pretreated with BBD130 [1 μ M] for 1 hour and stimulated with 100 nM insulin for the indicated times. D. HEK293 cells transfected with HA-C1- Δ PH PKB α were pretreated with BBD130 [1 μ M] for 1 hour. The cells were stimulated with TPA for the indicated times.

Figure 4. BBD130 sensitizes DNA-PK proficient cancer cells to ionizing radiation. A. DNA-PK proficient and deficient cells were treated with indicated concentrations of BBD130. The viable cells were determined after 3 days. B. For combination studies, the cells were pretreated with 30 nM BBD130 for 1 hour prior to 10 Gy γ -irradiation. The cell viability was determined after 1- and 3-day incubation. Data were normalized to growth of untreated control cells and presented as mean \pm SEM.

Figure 1.

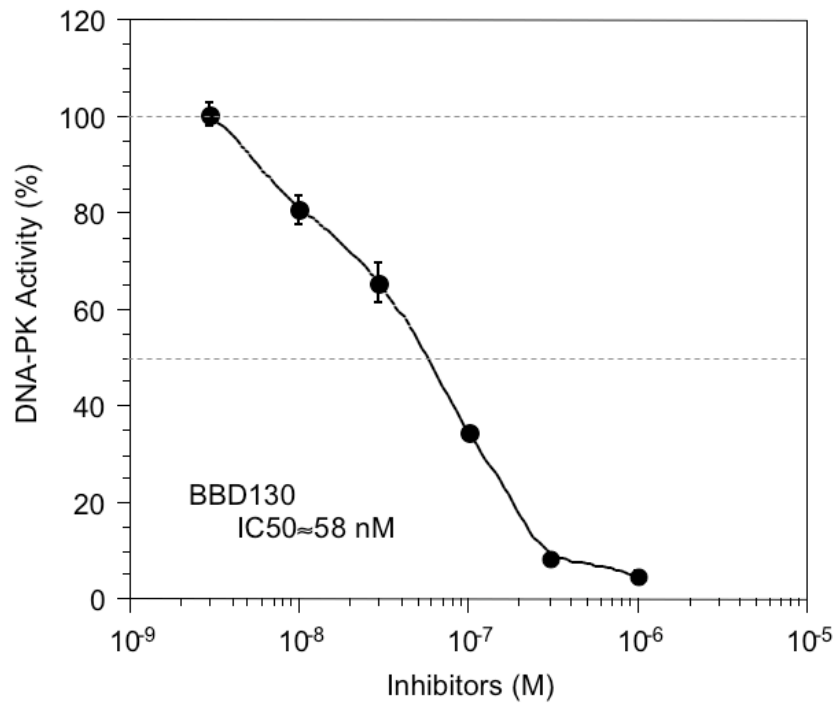


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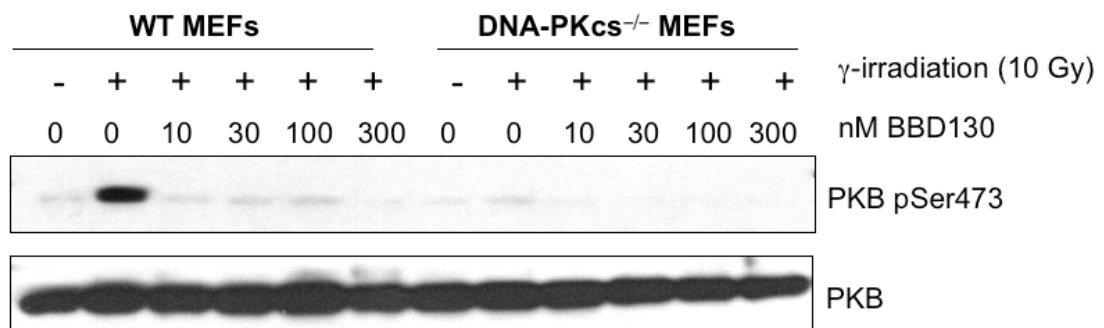


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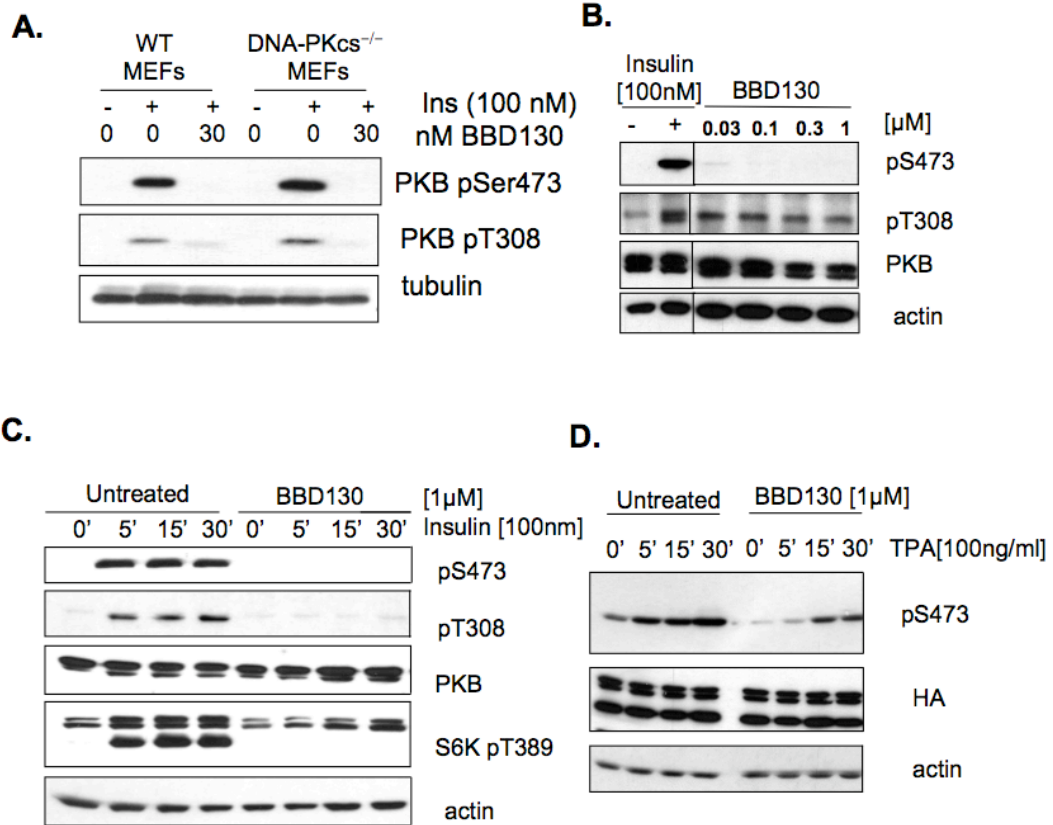
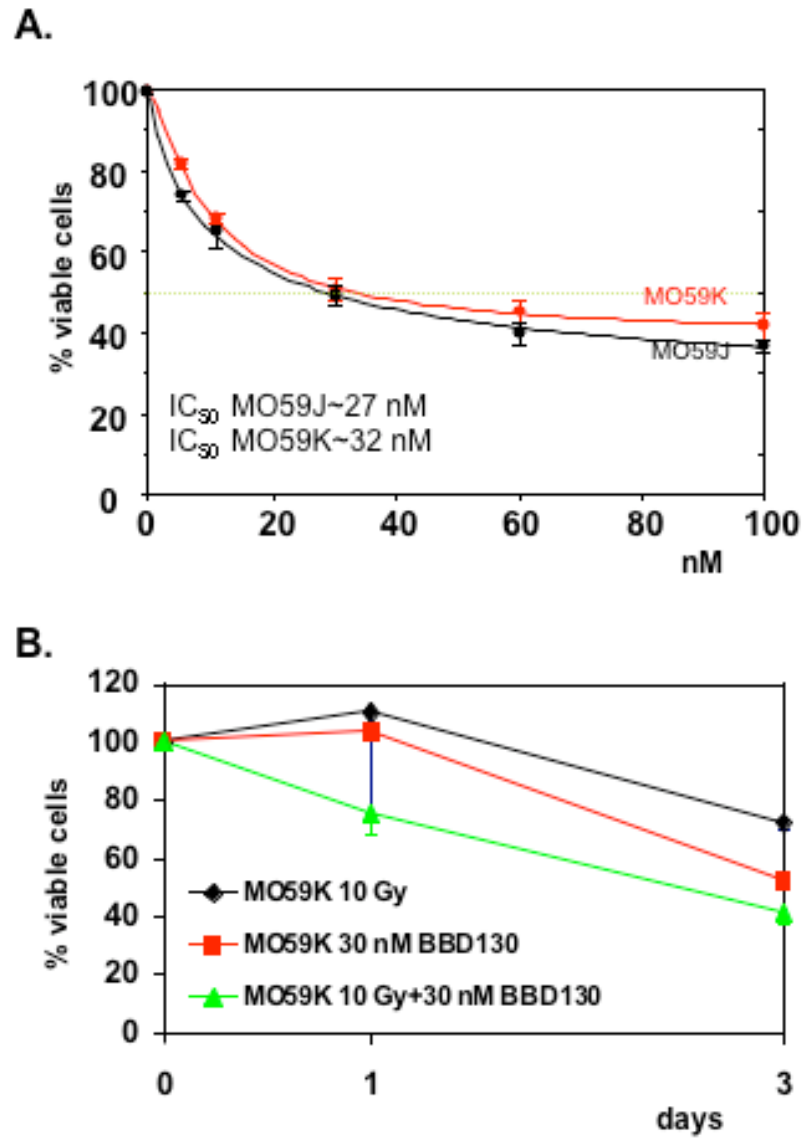


Figure 4.



III. GENERAL DISCUSSION

III. GENERAL DISCUSSION

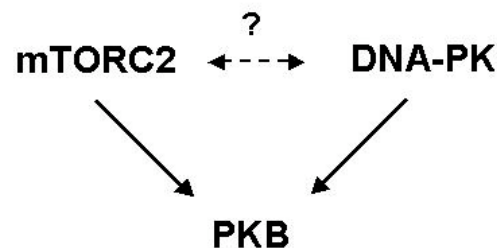
Several lines of evidence indicate the crucial role of PI3K/PKB pathway in survival, proliferation, transcription, and metabolism (7,8,103). PKB exerts its functions by acting on a plethora of substrates upon activation by numerous stimuli (8). Full activation of PKB requires phosphorylation on two sites, Thr308 and Ser473, of which Thr308 phosphorylation is mediated by PDK1. However, the identification of the kinase responsible for the Ser473 phosphorylation remained elusive until recently and studies have shown that Ser473 phosphorylation is not mediated by a single kinase and that both DNA-PK (11,19) and mTORC2 (21) phosphorylate PKB Ser473 depending on the stimuli. Given that a wide range of stimuli including growth factors, cytokines and DNA damage activate PKB, distinct Ser473 kinases activated under distinct conditions relay these signals to downstream effectors.

In the present study, *in vivo* role of DNA-PK in PKB regulation was investigated under different conditions.

DNA-PK is dispensable for insulin and growth factor induced PKB Ser473 phosphorylation whereas DNA-PK is required for PKB Ser473 phosphorylation in response to DNA damage induced by ionizing radiation. Insulin is a well-established stimulus for PKB activation. Our *in vivo* studies showed that DNA-PK is not the physiological Ser473 kinase in response to insulin stimulation. Further DNA-PK is dispensable for growth factor induced PKB Ser473 phosphorylation. On the other hand we showed

that DNA-PK, which is a DNA damage activated kinase, is required for DNA damage induced PKB Ser473 phosphorylation. Given that PKB activation is promoted and that DNA-PK is required for phosphorylation of PKB Ser473 in response to DNA damage (11) places PKB as an important mediator of DNA damage signalling (166). Taken together, these results indicate that PKB activation is mediated by DNA-PK and mTORC2 in a stimulus dependent manner under physiological conditions.

PKB is deregulated in DNA-PKcs^{-/-} mice: evidence of crosstalk between two upstream kinases, mTORC2 and DNA-PK.



Analysis of tissues from DNA-PKcs-null mice revealed that PKB is hyperactivated in a tissue-specific manner. Hyperactivation of PKB in the absence of an upstream kinase is unexpected and molecular mechanism underlying this phenomenon still need to be resolved. Elevated PKB Ser473 phosphorylation in DNA-PKcs^{-/-} tissues could most likely be mediated by mTORC2 and suggest a crosstalk between two upstream kinases of PKB, mTORC2 and DNA-PK. This regulation might take place at multiple levels of the pathway as our results showed that elevated PKB Ser473 phosphorylation

could also be observed without the accompanying changes in S6K Thr389 phosphorylation. Therefore, although we cannot completely rule out the contribution of a S6K mediated mechanism; our data suggest the existence of an alternative mechanism of regulation. For this, further analysis of mice lacking both DNA-PK and mTORC2 will be required.

Recent studies proposed a role for Tel2⁵ to function as a coordinator among PIKKs and suggested existence of crosstalks between different PIKKs where alteration of one PIKK may influence other (130-133,173). It has been shown that Tel2, which directly interacts with phosphatidylinositol-3-kinase related kinase family members (PIKKs) including DNA-PK and mTOR, could act as a stabilizer of PIKKs (131,133) or may serve as a scaffold protein that mediates signal transduction from PIKKs to their target proteins (132). However, further

⁵ Tel2 was discovered in mutant strains of budding yeast *Saccharomyces cerevisiae* that had abnormally short telomeres (167). Lustig, A. J., and Petes, T. D. (1986) *Proc Natl Acad Sci U S A* **83**, 1398-1402 (reviewed in 131). Chang, M., and Lingner, J. (2008) *Science* **320**, 60-61). In addition to maintaining telomere length in *S. cerevisiae*, it functions in DNA replication checkpoint in the fission yeast *Schizosaccharomyces pombe* and in humans (168). Shikata, M., Ishikawa, F., and Kanoh, J. (2007) *J Biol Chem* **282**, 5346-5355, 169. Collis, S. J., Barber, L. J., Clark, A. J., Martin, J. S., Ward, J. D., and Boulton, S. J. (2007) *Nat Cell Biol* **9**, 391-401, 170. Jiang, N., Benard, C. Y., Kebir, H., Shoubridge, E. A., and Hekimi, S. (2003) *J Biol Chem* **278**, 21678-21684). In the nematode *Caenorhabditis elegans*, however, hypomorphic alleles (forms of a gene in which the encoded protein has reduced function compared with the wildtype allele) of *clk-2/rad-5* (the *TEL2* ortholog in *C. elegans*) cause stress during DNA replication and hypersensitivity to DNA double-strand breaks and reduce the rate of several physiological processes, including embryonic and postembryonic development, and reproduction (133). Takai, H., Wang, R. C., Takai, K. K., Yang, H., and de Lange, T. (2007) *Cell* **131**, 1248-1259, 171. Benard, C., McCright, B., Zhang, Y., Felkai, S., Lakowski, B., and Hekimi, S. (2001) *Development* **128**, 4045-4055, 172. Garcia-Muse, T., and Boulton, S. J. (2005) *EMBO J* **24**, 4345-4355). Analysis of the phenotype of *Tel2*-null mouse cells showed that *TEL2* directly interacts with all of the mammalian PIKKs (133). Takai, H., Wang, R. C., Takai, K. K., Yang, H., and de Lange, T. (2007) *Cell* **131**, 1248-1259). Absence of *TEL2* substantially reduced the expression level of all PIKKs in mouse cells. A direct physical association between *Tel2* and PIKKs was also recently observed in fission yeast (173). Hayashi, T., Hatanaka, M., Nagao, K., Nakaseko, Y., Kanoh, J., Kokubu, A., Ebe, M., and Yanagida, M. (2007) *Genes Cells* **12**, 1357-1370).

study is required to understand the mechanistic role of Tel2 as a mediator of PIKK functions. It will be intriguing to study whether Tel2 could have a role mediator role affecting the downstream signaling of PIKKs, in particular within the context of mTOR and DNA-PK.

The hypothesis of existence of crosstalk between DNA-PK and mTOR raises the question whether increased phosphorylation of PKB could also be observed in mTORC2 knock-out mice. The mice generated by disruption of mTORC2 by homozygous deletion of rictor (107,108), mLST8 (107) or mSin1 (174) are embryonic lethal. Recently, a conditional knock-out of mTORC2 complex has been reported (175). Interestingly, muscle-specific deletion of rictor resulted in increase of basal PKB Thr308 as well as FoxO1, GSK3 and S6K phosphorylation (175). On the other hand, the basal Ser473 phosphorylation was reported to remain unchanged with deletion of rictor in the muscle (175). This could suggest that deletion of rictor may not result in an increase in PKB Ser473 phosphorylation albeit an increase in downstream events through possible alternative mechanisms. Further, it could also suggest the tissue specificity of the crosstalk between DNA-PK and mTORC2, as our results also showed that deletion of DNA-PK did not result in a change in PKB Ser473 phosphorylation in skeletal muscle under either fasting or random-fed conditions. On the other hand, we observed a concomitant increase in phosphorylation of both Ser473 and Thr308 in the DNA-PKcs^{-/-} tissues including thymus, adipose and brown fat. Further studies by use of

tissue specific double knock-out mice lacking DNA-PK and mTORC2 components will be required to fully investigate these hypotheses.

PKB hyperactivity in the thymus apparently contributed to spontaneous tumorigenesis in DNA-PKcs^{-/-} mice. Significantly, these tumors could be prevented by deletion of PKB α , which is highly expressed in thymocytes, particularly in DN- and DP-stages (84-86). PKB has been shown to play an important role in DN- to DP-stage transition and to be essential for thymocyte survival and differentiation (84-86). Therefore, persistent PKB activity in the DNA-PKcs^{-/-} thymus, where DN thymocytes predominate, could contribute to malignant transition. Furthermore, subsequent deletion of PKB α gene improves the survival of DNA-PKcs^{-/-} mice. However, the survival of DNA-PKcs^{-/-}PKB α ^{-/-} double knock-out (DKO) mice was not restored to that of PKB α ^{-/-} mice, which is not surprising given that DNA-PK has a wide variety of functions, some of which are independent of PKB α . Alternatively, the loss of DNA-PK in a PKB α ^{-/-} background could lead to deterioration of DNA-PK-dependent PKB functions in tissues other than the thymus. Moreover, deregulation of other isoforms of PKB in DNA-PK^{-/-} mice could possibly affect overall organismal fitness and survival. Finally, loss of DNA-PK independent PKB α functions could lead to additive affects in DNA-PKcs^{-/-}PKB α ^{-/-} DKO mice. Further studies using tissue specific DNA-PK^{-/-}PKB α ^{-/-} DKO mice will be necessary to evaluate these possibilities.

Taken together, these results indicate that PKB phosphorylation is not only elaborately regulated by mTORC2 and DNA-PK but also suggest existence of cross-talk between the two upstream kinases. The delineation of the mechanism how these two kinases could talk to each other will be crucial.

PI3K-DNA-PK inhibitor BBD130 blocks PKB phosphorylation induced by DNA damage and inhibits growth. As PKB mediated signaling events could favor tumor growth and/or progression, is not surprising that PI3K/PKB pathway is one of the most deregulated pathways in human malignancies (52-55). Radiotherapy and DNA damaging drugs are among the most efficient therapies for cancer treatment (152). A number of DNA-PK inhibitors were developed to enhance radio- and chemosensitivity and presented promising outcome in combination with radiation or chemotherapy in preclinical studies (148,149). Another possible role for DNA-PK in chemo- and radio-resistance emerged with the recent findings that DNA-PK phosphorylates and activates PKB in response to DNA damage (11). We made use of differential activation of PKB by upstream kinases in response to specific stimuli as a tool to dissect the mode of action of the BBD130. mTORC2 dependent PKB activation was assessed by insulin stimulation and DNA-PK dependent PKB activation was assessed by γ -irradiation. Combinatorial use of BBD130 and γ -irradiation indicated that there is a synergistic affect of BBD130 and γ -irradiation on growth inhibition of glioblastoma cells possibly through inhibition of DNA-PK. The mechanistic rationale for the efficacy of this combination treatment could

be described by cooperative inhibition of PI3K and DNA-PK. Several of the PI3K inhibitors inhibit DNA-PK as well due to their structural similarity in the kinase domain. Significantly, inhibition of DNA-PK could play dual role in radiosensitization: by inhibition of the DNA repair as well as by inhibition of PKB activation induced by γ -irradiation.

IV. REFERENCES

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V. CURRICULUM VITAE

BANU SURUCU

LANDSKRONSTRASSE 7, 4056, BASEL TEL: +41 76 - 567 0159

E-MAIL: banu.surucu@fmi.ch

DATE OF BIRTH: May 21, 1976

NATIONALITY: Turkish

MARITAL STATUS: Married, 1 child

LANGUAGES: Turkish, English,
German (B1)

EDUCATION

University Attended

Degree Obtained

Dates

Friedrich Miescher Institute for Biomedical
Research / University of Basel, Switzerland

PhD Biochemistry

Jan 2004– June 2008

Bilkent University, Ankara, Turkey

MSc Molecular
Biology and Genetics

Sep 2000 – Sep 2003

Middle East Technical University,
Ankara, Turkey

BSc Biology
Minor in Chemistry,
(*high honors*)

Sep. 1995 – Jun. 2000

RESEARCH EXPERIENCE

PhD Project

Jan 2004– Sep 2008

**Friedrich Miescher Institute for Biomedical Research,
Growth Control Group, Switzerland**

PKB regulation by DNA-PK under physiological conditions

MSc Project

Sep 2000 – Sep 2003

Bilkent University, Ankara, Turkey

Identification of Genes involved in Hepatocellular
Carcinoma

Internship

July 1999-Aug 1999

**Scientific and Technical Research Council of Turkey
(TUBITAK) Marmara Research Center (MAM), Genetic
Engineering and Biotechnology Research Institute**

Development of technology for production, purification
and stabilization of industrial enzymes (*Penicillin acylase*)

Undergraduate Project

May 1999 – Jun. 1999

**Middle East Technical University (METU), Ankara,
Turkey**

Population genetics of forest trees (*Pinus nigra*)

TEACHING EXPERIENCE:

Teaching Assistant
Sep 2000 – Sep 2003

Bilkent University, Ankara, Turkey

SCHOLARSHIPS

Full scholarship for MSc studies (Bilkent University).

2000 – 2003

MEETINGS ATTENDED

2006 May: The CNIO Cancer Conference: PTEN and the AKT route. Madrid, Spain.
(Poster presentation)

Delineating the role of DNA-PK in the activation of PKB by S473 hydrophobic motif phosphorylation.

Banu Surucu, Lana Bozulich, Debby Hynx, Jianhua Feng, Jongsun Park and Brian A. Hemmings

2006 Dec: Targeting the Kinome. Basel, Switzerland.

(Poster presentation)

The role of DNA-PK in the activation of PKB by S473 hydrophobic motif phosphorylation.

Banu Surucu, Lana Bozulich, Debby Hynx and Brian A. Hemmings

PUBLICATIONS:

1. **Surucu B., L. Bozulich, D. Hynx, A. Parcellier, and B. A. Hemmings.** 2008. *In vivo* analysis of PKB/Akt regulation in DNA-PKcs-null mice reveals a role for PKB/Akt in DNA damage response and tumorigenesis. *J Biol Chem* **283**:30025-33.

2. **Bozulich L., B. Surucu, D. Hynx, and B. A. Hemmings.** 2008. PKB α /Akt1 Acts Downstream of DNA-PK in the DNA Double-Strand Break Response and Promotes Survival. *Mol Cell* **30**:203-13.

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VII. APPENDIX

PKB α /Akt1 acts downstream of DNA-PK in the DNA double strand break response and promote survival

Lana Bozulic, Banu Surucu, Debby Hynx, and Brian A. Hemmings
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PKB α /Akt1 Acts Downstream of DNA-PK in the DNA Double-Strand Break Response and Promotes Survival

Lana Bozulich,¹ Banu Surucu,¹ Debby Hynx,¹ and Brian A. Hemmings^{1,*}

¹Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland

*Correspondence: brian.hemmings@fmi.ch

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SUMMARY

Protein kinase B (PKB/Akt) is a well-established regulator of several essential cellular processes. Here, we report a route by which activated PKB promotes survival in response to DNA insults *in vivo*. PKB activation following DNA damage requires 3-phosphoinositide-dependent kinase 1 (PDK1) and DNA-dependent protein kinase (DNA-PK). Active PKB localizes in the nucleus of γ -irradiated cells adjacent to DNA double-strand breaks, where it colocalizes and interacts with DNA-PK. Levels of active PKB inversely correlate with DNA damage-induced apoptosis. A significant portion of p53- and DNA damage-regulated genes are misregulated in cells lacking PKB α . PKB α knock-out mice show impaired DNA damage-dependent induction of p21 and increased tissue apoptosis after single-dose whole-body irradiation. Our findings place PKB downstream of DNA-PK in the DNA damage response signaling cascade, where it provides a prosurvival signal, in particular by affecting transcriptional p21 regulation. Furthermore, this function is apparently restricted to the PKB α isoform.

INTRODUCTION

Induction of DNA strand breaks activates and integrates an extensive network of cellular responses (reviewed in O'Driscoll and Jeggo, 2006). These are critical for sustained viability in the face of a genotoxic insult. The recruitment of DNA damage signaling and repair proteins to sites of genomic damage constitutes a primary event triggered by DNA damage (reviewed in Rouse and Jackson, 2002). DNA-dependent protein kinase (DNA-PKcs), ataxia telangiectasia-mutated kinase (ATM), and Rad3-related kinase (ATR), all members of the PI3-kinase (PI3K)-like family of kinases (PIKKs) are recruited directly to sites of DNA damage in a similar manner (Falck et al., 2005). Consequently activated, they amplify and convey the signal from the damaged DNA to DNA-repair and cell-cycle machineries, such as the p53 pathway (reviewed in Khanna and Jackson, 2001), thus supporting survival. Importantly, DNA-PK, ATM, and ATR were shown to each occupy distinct nuclear subcompartments after DNA damage, namely unprocessed double-strand break

(DSB) ends, DSB-flanking chromatin, and single-strand DNA (ssDNA) microcompartments, respectively. This may to some extent explain their overlapping versus diverse functions and choice of substrates (Bekker-Jensen et al., 2006).

One central regulator of cell metabolism, survival, and proliferation is the AGC family Ser/Thr kinase protein kinase B (PKB/Akt) (reviewed in Brazil et al., 2004). Given the vital roles of the three PKB isoforms PKB α , PKB β , and PKB γ in normal physiology and development, their deregulation has not unexpected consequences in pathology and tumorigenesis (reviewed in Dummler and Hemmings, 2007). Thus regulation of PKB activity is very stringent. After mitogen stimulation, PKB is fully activated following phosphorylation of two key residues. Thr308 in the activation loop is phosphorylated by 3-phosphoinositide-dependent kinase 1 (PDK1) (Alessi et al., 1997). Ser473 in the C-terminal hydrophobic motif (HM) has been shown to be a target of the mammalian target of rapamycin complex 2 (mTORC2) (Sarbasov et al., 2005), another PIKK family member; however, its effects on PKB regulation have been studied so far only in the context of hormone/growth factor signaling or development (Shiota et al., 2006).

PKB Ser473 is also phosphorylated in response to various genotoxic treatments (Tan and Hallahan, 2003). Several components of the PI3K-PKB pathway have been reported to be phosphorylated in the DNA damage response (Matsuoka et al., 2007). Just how the signal from damaged DNA activates PKB remains to be elucidated. We and others have identified DNA-PK as an efficient PKB Ser473 kinase *in vitro* and *in vivo*. DNA-PK-mediated phosphorylation of PKB Ser473 results in \sim 10-fold enhancement of PKB kinase activity (Feng et al., 2004a). Stimulation of bone marrow-derived macrophages (BMDMs) from mice lacking the catalytic subunit of DNA-PK (DNA-PKcs) results in defective phosphorylation and activation of PKB by CpG-DNA (Dragoi et al., 2005).

DNA-PK holoenzyme consists of the catalytic subunit (DNA-PKcs) and the Ku antigen complex (KU70/80) (Suwa et al., 1994). Its activation at the DSBs and its proximal position in the DSB signaling cascade provide a setting for it to function as the PKB upstream kinase in the DNA damage response. With this study, we propose that DNA-PK regulates PKB by hydrophobic motif Ser473 phosphorylation following induction of DSBs in the DNA. PKB in turn provides a prosurvival signal for the cell by affecting DNA damage-induced transcription. These results highlight the role of DNA-PK in defining crucial PKB functions in the DNA damage response.

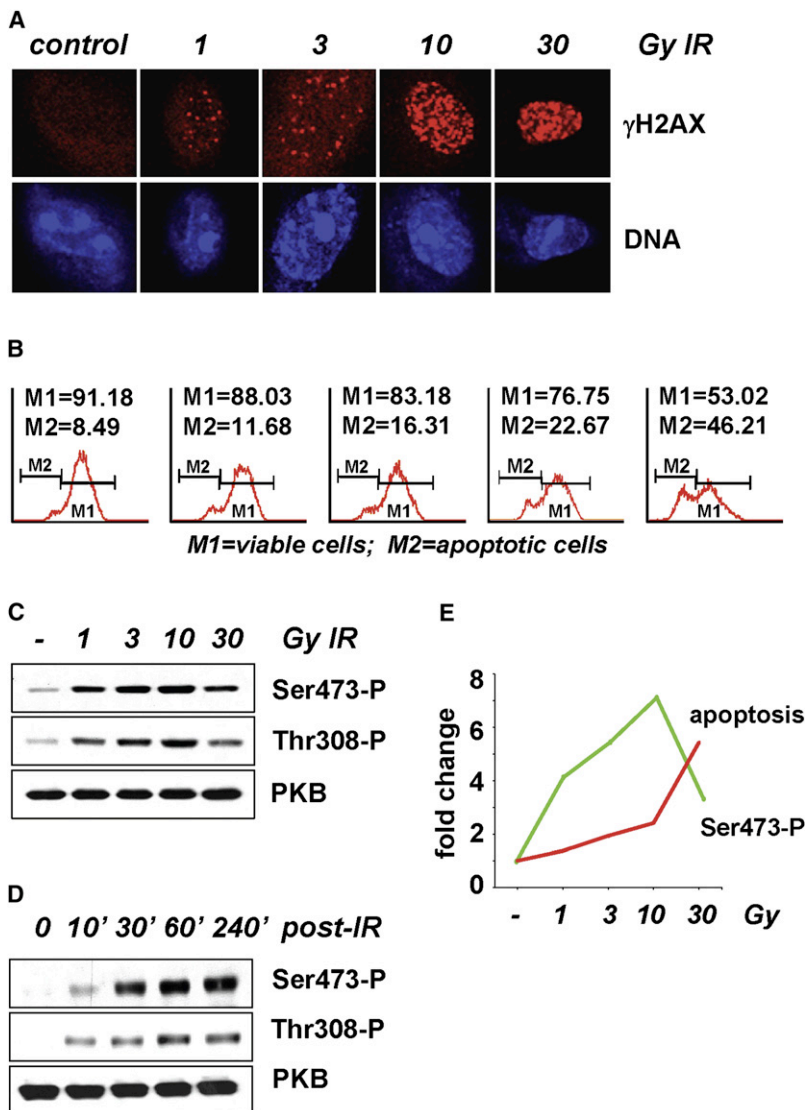


Figure 1. γ -IR-Induced PKB Activation and Apoptosis

HUVEC cells were γ irradiated at indicated doses. After 30 min, cells were analyzed by immunofluorescence for histone H2AX Ser139 phosphorylation (A). After 24 hr, apoptosis was measured by FACS analysis using the specific mitochondrial dye DiIC1₍₅₎. This reagent allows measurement of the mitochondrial membrane potential ($\Delta\Psi$). M1 represents viable cells, and M2 represents apoptotic cells (B). PKB Ser473 and Thr308 phosphorylation was analyzed 30 min after γ -IR by western blotting (C). HUVEC cells were γ irradiated with 3 Gy, and PKB Ser473 and Thr308 phosphorylation was analyzed at indicated time points (D). (E) Schematic representation of PKB Ser473-P regulation and DNA damage-induced apoptosis derived from the quantitation of data from (A), (B), and (C).

measuring $\Delta\Psi$. This population increased from 10% to 20% at lower doses of irradiation and to ~50% when cells were irradiated with 30 Gy γ -IR (Figure 1B). PKB Ser473 was phosphorylated 30 min post-IR (3 Gy) and remained phosphorylated 240 min after irradiation (Figure 1D). PKB phosphorylation correlated with increase in number of DSBs at lower doses of γ -IR (1 to 10 Gy). At 30 Gy, however, PKB Ser473 phosphorylation was less than at lower doses (Figure 1C). Combined, these data lead us to consider PKB phosphorylation after γ -IR as an antiapoptotic mark or a molecular switch (Figure 1E), whereby cellular survival is favored over IR-induced apoptosis, depending on the dose applied.

PKB Activation and Ser473 Phosphorylation in Response to DNA Damage Are DNA-PK Dependent

Genotoxic stress response pathways rely on PIKKs as proximal response elements. As DNA-PKs is activated following its recruitment to the

DSBs (Uematsu et al., 2007), it may be responsible for PKB Ser473 phosphorylation in the context of DNA damage. To test this hypothesis, we compared γ -IR- and insulin-induced PKB Ser473 phosphorylation in HUVEC cells. DNA-PKs was specifically inhibited employing a small molecule inhibitor NU7026 (Leahy et al., 2004). NU7026 inhibited in vitro DNA-stimulated phosphorylation of PKB α Ser473 by DNA-PK; however, it had no effect on the in vitro kinase activity of PKB α (see Figures S1A and S1B available online). A general PI3K inhibitor LY294002 was used as a control. LY294002 pretreatment prevented PKB Ser473 phosphorylation in both stimulating situations. In cells pretreated with NU7026, PKB was phosphorylated only in response to insulin stimulation, and its γ -IR-induced phosphorylation was abolished (Figure 2A). These results supported our hypothesis that generation of DSBs is a specific signal for DNA-PKs to phosphorylate and activate PKB. To test this further, we measured the phosphorylation of both activating residues in PKB (Figure 2B) as well as the kinase activity of PKB (Figure 2C) following γ -IR of untreated or of NU7026

RESULTS

Regulation of PKB Phosphorylation on Ser473 and Irradiation-Induced Apoptosis in Response to Induction of DNA DSBs

PKB is activated by various forms of DNA damage and influences cell-cycle and apoptosis parameters, promoting survival. Entrance to a repair or a cell death program is affected by the extent of DNA damage. To determine the range of DNA damage that activates PKB, we titrated doses of γ irradiation (γ -IR) applied to HUVEC cells from 1 to 30 Gy. We observed a proportional increase in DNA DSBs by visualizing the Ser139 phosphorylation of the histone variant H2AX (γ H2AX) (Figure 1A). γ H2AX forms foci over large chromatin domains surrounding a DNA break (reviewed in Thiriet and Hayes, 2005). An early event initiating the IR-induced apoptosis is the loss of the mitochondrial membrane potential, $\Delta\Psi$ (Taneja et al., 2001). Employing a FACS-based assay, we assessed the apoptotic fraction of cells by

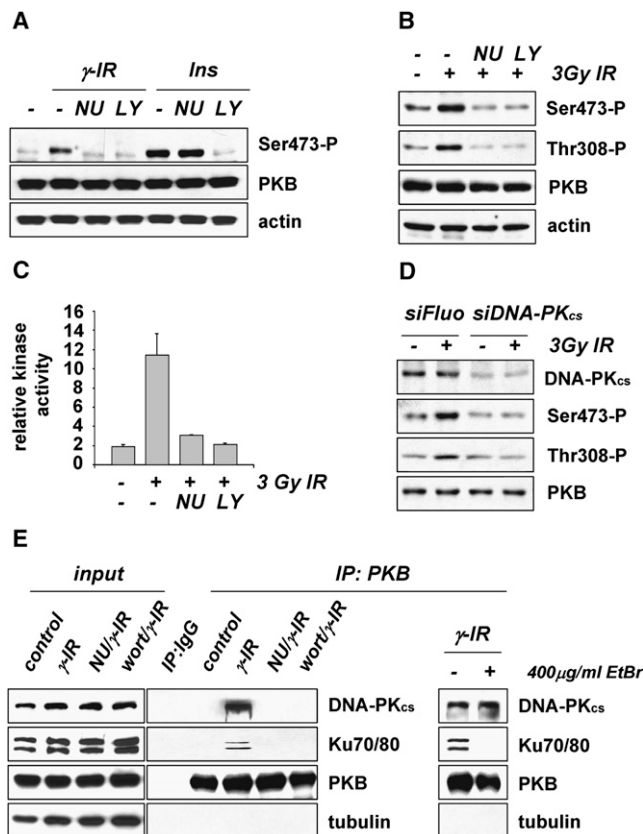


Figure 2. DNA-PKcs-Dependent PKB Activation and Ser473 Phosphorylation in Response to DNA Damage

(A) HUVEC cells were treated with either 3 Gy γ -IR or 100 nM insulin for indicated times following pretreatment with NU7026 (10 μ M, 60 min) or LY294002 (50 μ M, 30 min) as indicated. Whole-cell lysates were analyzed for PKB Ser473 phosphorylation. (B and C) HUVEC cells were treated with 3 Gy γ -IR following pretreatment with the NU7026 or LY294002 inhibitors as in (A). Whole-cell lysates were analyzed for both activating phosphorylation sites on PKB, Ser473, and Thr308 (B). PKB was immunoprecipitated using Ab10, and its kinase activity was measured *in vitro*. Data are represented as means \pm SD of duplicate determinants (C). (D) HUVEC cells were transfected with siRNA against DNA-PKcs, or control nonsilencing siRNA (fluorescein, *siFluo*). At 48 hr after transfection, cells were treated with 3 Gy γ -IR and whole-cell lysates were analyzed 30 min postirradiation. HUVEC cells were treated with 3 Gy γ -IR following pretreatment with NU7026 as in (A) and wortmannin (50 μ M, 30 min). At 30 min postirradiation, whole-cell lysates were analyzed as *Input*, and PKB was immunoprecipitated with Ab10 and the immunoprecipitates were probed for DNA-PKcs and Ku70/80 (E). Immunoprecipitates from irradiated cells were treated with 400 μ g/ml ethidium bromide in the lysis buffer (E), right).

and LY294002 pretreated cells. Appropriately, the γ -IR-induced increase in both PKB kinase activity (6- to 7-fold) and phosphorylation of the PKB Ser473 and Thr308 was inhibited by the NU7026 and LY294002 pretreatments, arguing that the kinase activity of DNA-PKcs is necessary for DNA damage-induced activation of PKB. To test the specificity of the effect of DNA-PKcs inhibition on PKB phosphorylation after γ -IR, we knocked down DNA-PKcs using siRNA. In full support of our data from the NU7026 studies, PKB phosphorylation on both Ser473 and Thr308 was abolished in irradiated cells depleted of DNA-PKcs (Figure 2D).

Ser473-Phosphorylated PKB Forms Nuclear Foci after γ -IR and Interacts and Colocalizes with DNA-PKcs

It may be a common feature of the PIKK family members that these kinases select their substrates based on induced proximity (reviewed in Abraham, 2004). As DNA-PKcs recruitment to the DSBs is crucial for its subsequent autophosphorylation and activation, we investigated whether endogenous PKB localizes in the vicinity of DSBs and active DNA-PK. By immunoprecipitation and western blotting, we detected increased endogenous DNA-PKcs-PKB complex formation after irradiation, but not in irradiated cells that were pretreated with NU7026 or wortmannin. Ethidium-bromide treatment of immunoprecipitates from irradiated cells did not disturb the DNA-PKcs-PKB interaction; however, it caused the release of Ku70/80 that was found to coimmunoprecipitate with DNA-PKcs-PKB. This indicates PKB complexes with the DNA-PK holoenzyme rather than free, inactive DNA-PKcs (Figure 2E). We then visualized active PKB (phosphorylated on Ser473) together with DNA-PKcs in γ -irradiated HUVEC cells as well as HUVEC cells treated with NU7026 prior to irradiation. Strikingly, Ser473-P immunofluorescent staining revealed distinct foci restricted solely to the nucleus of irradiated cells. Moreover, the foci colocalized with DNA-PKcs immunofluorescent staining (see enlargement and colocalization quantification, Figure 3A). NU7026 pretreatment completely abolished PKB Ser473 phosphorylation and therefore the formation of active PKB foci, but had no effect on the nuclear localization of DNA-PKcs (Figure 3A). As the Ser473-P foci were reminiscent of the γ H2AX foci that mark DSBs, we performed a further set of localization experiments visualizing the Ser473-P of PKB together with γ H2AX, again in γ -irradiated HUVEC cells or cells treated with NU7026 prior to irradiation. The γ -IR-induced nuclear Ser473-P foci were found to colocalize with the DSBs (see enlargement and colocalization quantification, Figure 3B). The DNA-PKcs inhibitor pretreatment, as in our previous experiments, prevented PKB phosphorylation following irradiation, but had no effect on the proper γ H2AX foci formation (Figure 3B). Biochemical fractionation experiments confirmed the increase in nuclear Ser473-P after irradiation in untreated but not NU7026-treated cells, while the total PKB levels remained unchanged (Figure 3C). Furthermore, perinuclear and nuclear PDK1 were detected in irradiated HUVEC cells (Figure S3). Overall, our biochemical and localization studies revealed the existence of an exclusively nuclear, active pool of PKB after γ -IR. This Ser473-phosphorylated PKB is concentrated in regions surrounding the DSBs, where it colocalizes and interacts with DNA-PK.

PDK1 and DNA-PK Are the Physiological PKB Kinases in the DNA Damage Response

PDK1 plays a central role in activating AGC kinases, but its role in the regulation of PKB in the DNA damage response by Thr308 phosphorylation is not yet documented. To address this issue unambiguously, we made use of ES cells in which both copies of the PDK1 gene were disrupted. In these cells, there is no detectable IGF1-induced PKB Thr308 phosphorylation (Williams et al., 2000). Phosphorylation of PKB Thr308 and Ser473 in PDK1 proficient (*PDK1^{+/+}*) or deficient (*PDK1^{-/-}*) cells was measured 30 min after an IR dose of 3 Gy of γ -IR. In the *PDK1^{+/+}* cells,

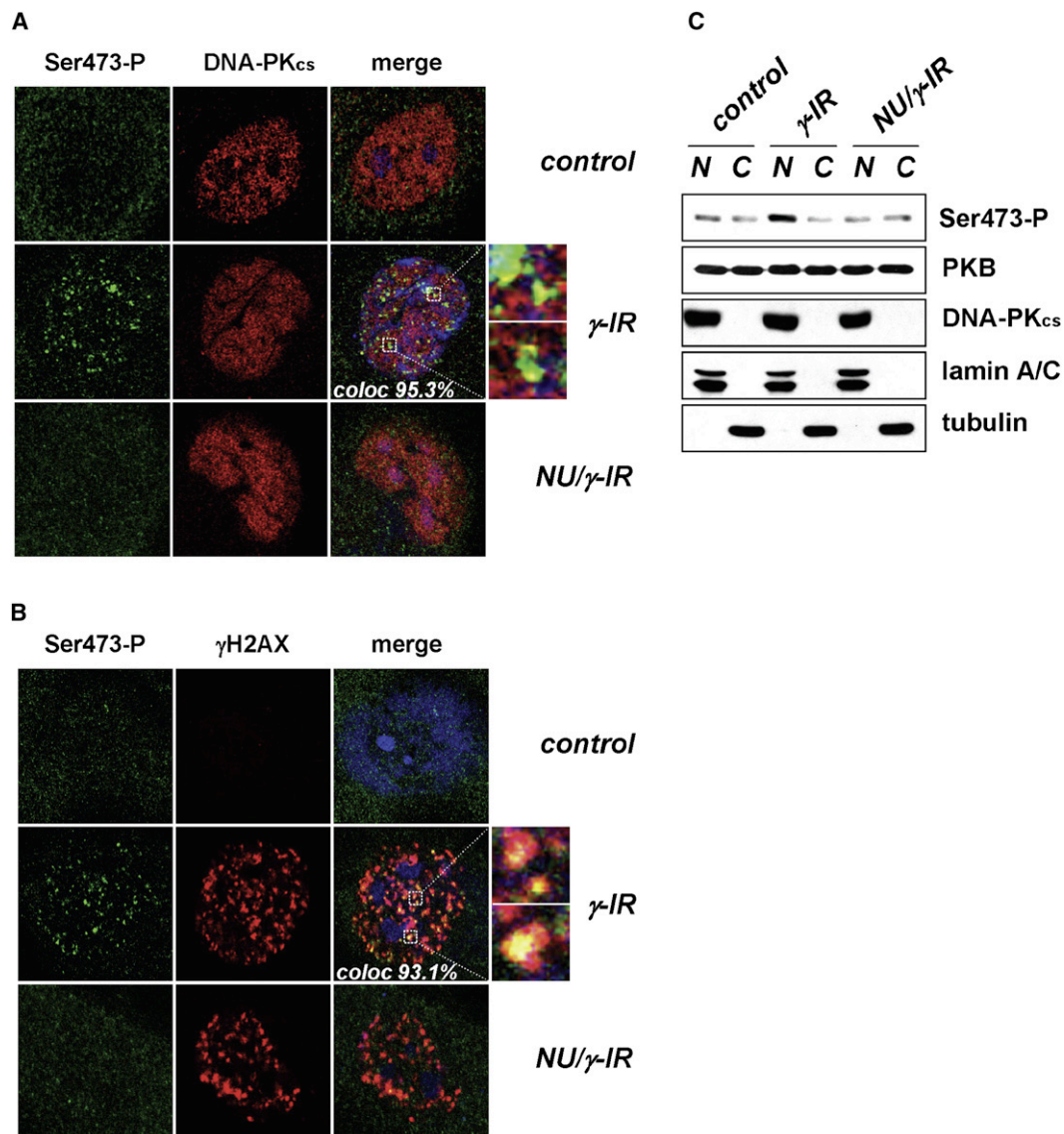


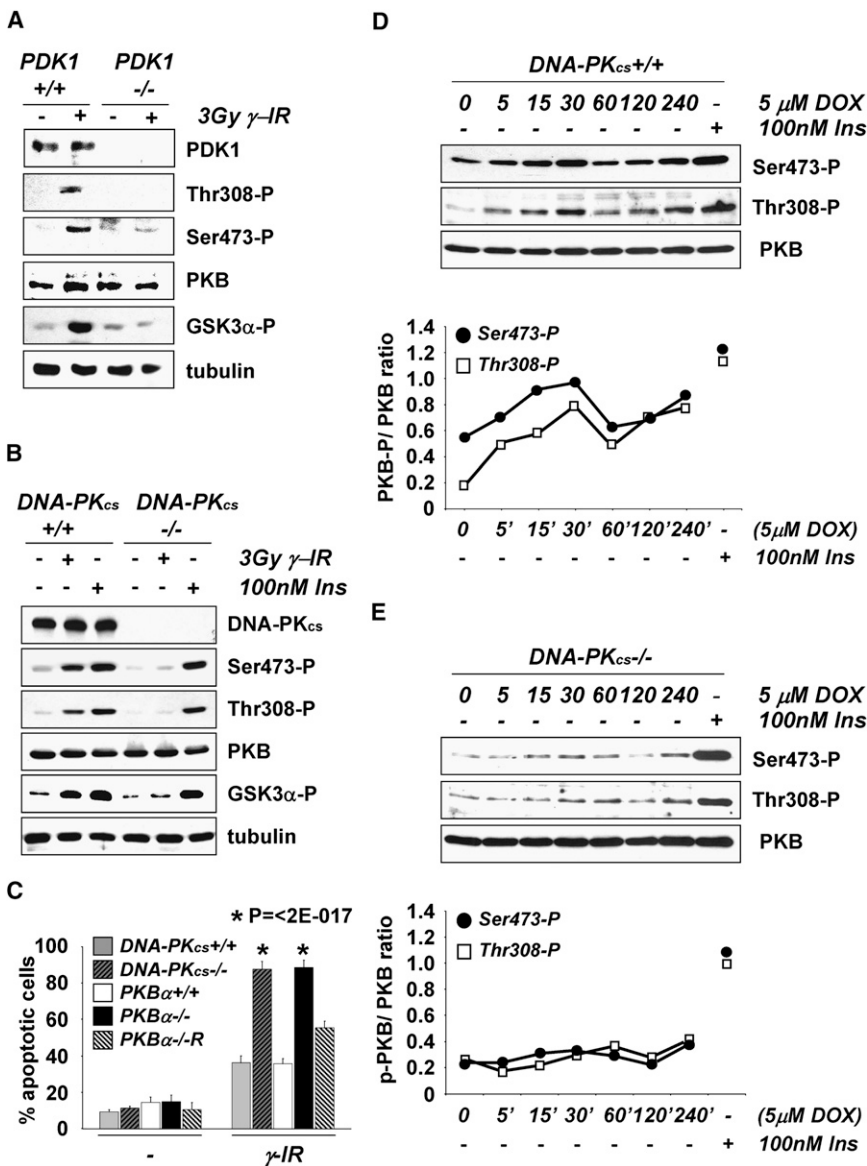
Figure 3. Ser473-Phosphorylated PKB Colocalizes with DNA-PKcs and Double-Strand DNA Breaks after γ -IR

Immunofluorescence staining for Ser473-P of PKB and DNA-PKcs (Ser473-P, green; DNA-PKcs, red; DNA in the merged images, blue; and colocalization of Ser473-P and DNA-PKcs, yellow) (A), or Ser473-P and γ H2AX (Ser473-P, green; γ H2AX, red; DNA in the merged images, blue; and colocalization of Ser473-P and γ H2AX, yellow) (B) in HUVEC cells 30 min after 3 Gy γ -IR treatment. The images are representative of the nuclear stainings observed in the experiments. The colocalization quantification was obtained using Imaris Coloc software with the intensity threshold for analysis set at a 150 cutoff for both channels (green channel, PKB and red channel, DNA-PKcs in [A]; green channel, PKB and red channel, γ H2AX in [B]). The numbers represent percent of region of interest (ROI) material of the PKB channel above threshold colocalized with the DNA-PK or H2AX channels in the ROI, respectively. (C) HUVEC cell lysates 30 min after 3 Gy γ -IR treatment were fractionated to nuclear (N) and cytosolic (C) fractions and subcellular distribution of Ser473-P was analyzed by western blotting.

both residues were efficiently phosphorylated. This corresponded to an increase in phosphorylation of GSK3 α at Ser21, which we took to be a measure of PKB activity. In the *PDK1*^{-/-} cells, however, phosphorylation of Thr308 after γ -IR was not detected. Although DNA-PKcs was active in these cells, as measured by p53 phosphorylation (data not shown), phosphorylation of Ser473 was lower than in wild-type cells. This was further reflected in the lack of phosphorylation of GSK3 α (Figure 4A).

Having found immediate negative effects of DNA-PKcs inhibition or knockdown on PKB regulation after DNA damage, we

sought evidence of a role for DNA-PK as a bona fide PKB activator in DNA-PKcs knockout cells. We isolated and cultured primary prostate epithelial cells from DNA-PKcs knockout mice harboring a mutation in the DNA-PK catalytic subunit gene (Gao et al., 1998). DNA-PKcs proficient (*DNA-PKcs*^{+/+}) or deficient (*DNA-PKcs*^{-/-}) cells were irradiated with 3 Gy γ -IR or as a control stimulated with insulin, and the phosphorylation status of PKB and GSK3 α was examined 30 min later. In irradiated or insulin-stimulated *DNA-PKcs*^{+/+} cells, both Ser473 and Thr308 of PKB were phosphorylated and PKB active in terms of



GSK3 α phosphorylation. In clear contrast, no increases in phosphorylation of the PKB activating residues or GSK3 α were observed after irradiation of $DNA-PKcs^{-/-}$ cells. Insulin stimulation promoted PKB activation and GSK3 phosphorylation in $DNA-PKcs^{-/-}$ cells, indicating they retain an intact insulin pathway (Figure 4B). Altogether, irradiation-induced phosphorylation and activation of PKB were completely abolished in the context of either PDK1 or DNA-PKcs knockout. Thus, the integrity of PDK1 and DNA-PK kinases appears crucial for successful PKB activation in response to DNA damage.

Cells Lacking PKB α but Not PKB β and PKB γ Resemble the DNA-PK-Deficiency Radiosensitive Phenotype

DNA-PKcs knockout MEFs are radiosensitive (Lieber et al., 2003). If the radiosensitivity of these cells is in part due to inefficient activation of PKB, we hypothesized that PKB knockout MEFs would phenocopy DNA-PKcs knockout. To test this, we

compared irradiation-induced apoptosis, in terms of mitochondrial intermembrane potential $\Delta\Psi$, in wild-type, PKB α knockout ($PKB\alpha^{-/-}$), and PKB β/γ knockout ($PKB\beta/\gamma^{-/-}$, double knockout) MEFs. The number of apoptotic cells increased by 35%–40% in $PKB\alpha^{-/-}$ MEFs, as opposed to 15%–20% in wild-type and $PKB\beta/\gamma^{-/-}$ MEFs 24 hr after irradiation (Figure S2E). This prompted us to test whether PKB α confers resistance to irradiation. The expression of PKB α in $PKB\alpha^{-/-}$ MEFs was reconstituted by stable transfection ($PKB\alpha^{-/-R}$) (Figures S2A and S2B). Reintroduction of PKB α rescued the radiosensitivity phenotype, reducing the increase in IR-induced apoptosis 24 hr after irradiation from 35% in $PKB\alpha^{-/-}$ cells to 15% in $PKB\alpha^{-/-R}$ cells (Figure S2D). Finally, we directly compared the sensitivity to γ -IR-induced apoptosis of $DNA-PKcs^{+/+}$, $DNA-PKcs^{-/-}$, $PKB\alpha^{+/+}$, $PKB\alpha^{-/-}$, and $PKB\alpha^{-/-R}$ MEFs. At 36 hr following irradiation, almost 90% of $DNA-PKcs^{-/-}$ and $PKB\alpha^{-/-}$ cells were apoptotic. In contrast, wild-type MEFs and reconstituted PKB α MEFs exhibited 35% and 55% apoptotic cells, respectively (Figure 4C). Overall, the apoptosis data suggest that PKB α communicates the prosurvival signal emanating from DNA-PK at the DSBs. Of note, PKB β and PKB γ were expressed

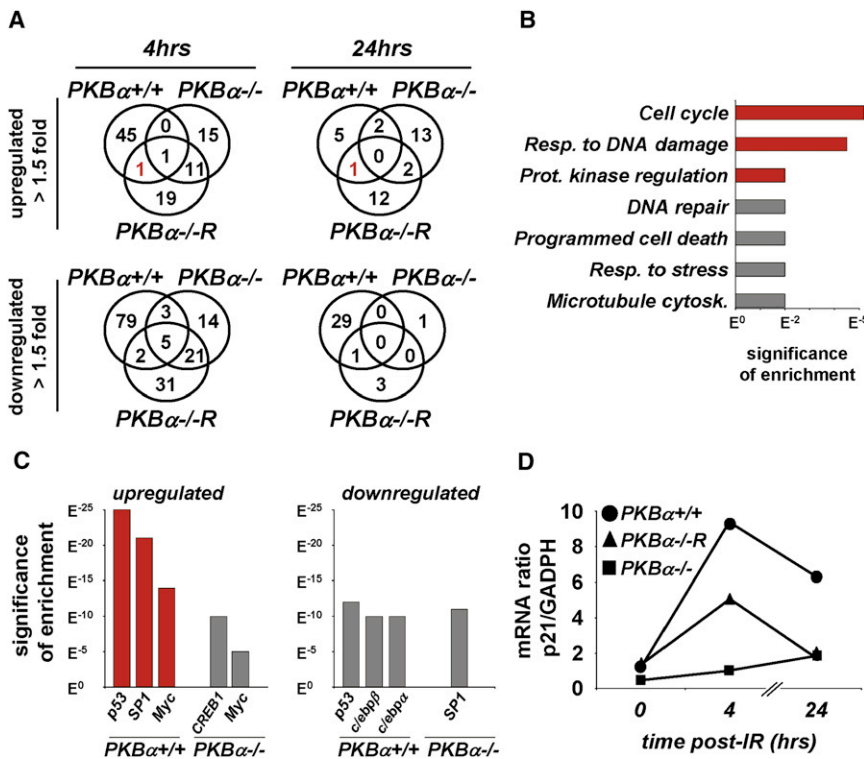


Figure 5. Aberrant DNA Damage-Induced Transcription in Cells Lacking PKB α , Including Transcriptional Misregulation of p21

MEFs were treated with 10 Gy γ -IR, and RNA was prepared from three independent irradiation experiments and subjected to microarray analysis. (A) Venn diagrams showing the numbers of genes differentially expressed in PKB α wild-type (PKB $\alpha^{+/+}$), knockout (PKB $\alpha^{-/-}$), and PKB-reconstituted (PKB $\alpha^{-/-R}$) MEFs 4 and 24 hr after γ -IR. The numbers of genes reproducibly regulated more than 1.5-fold relative to untreated control cells are shown. Top, upregulated genes; bottom, downregulated genes. (B) Genes deregulated in PKB α -deficient cells (upregulated in the wild-type 4 hr after treatment, but not in knockout cells) were compared to biological process Gene Ontology categories; significantly represented categories are shown. Ontology terms are on the y axis; p values for the significance of enrichment are graphed along the x axis. The bars shown in red are categories that include p21. (C) Genes upregulated and downregulated 4 hr following irradiation in cells of indicated genotypes were grouped according to transcription factor regulation. Most significantly represented transcription factor regulons are graphed along the x axis; P values for the significance of enrichment in transcription factor groups are graphed along the y axis. The red bars are those categories that include p21. PKB α wild-type (PKB $\alpha^{+/+}$), knockout (PKB $\alpha^{-/-}$), and

PKB α -reconstituted (PKB $\alpha^{-/-R}$) MEFs were treated with 10 Gy γ -IR. RNA was isolated at the indicated time points to perform real-time PCR analysis. The panel represents p21 mRNA expression levels in cells of all three genotypes at the indicated times normalized to internal GADPH controls (D).

in the MEFs studied (Figure S2A) and a Ser473-P signal was detected after DNA damage in cells lacking PKB α (Figure S2A). Moreover, Ser473-P signal detected by immunofluorescence in PKB $\alpha^{-/-}$ MEFs revealed uniform Ser473-P distribution throughout the cell, failing to form nuclear foci observed in both PKB $\alpha^{+/+}$ and PKB $\beta/\gamma^{-/-}$ MEFs (Figure S2F). This suggests that PKB α is not selectively phosphorylated and activated after DNA damage but rather that the PKB α isoform acts antiapoptotically in the DNA damage response.

PKB was activated in a DNA-PKcs-dependent manner following DNA damage and influenced cell survival (Figures 2 and 4). We compared DNA damage-induced PKB activation to that following hormone stimulation in DNA-PKcs wild-type and knockout MEFs. DNA-PKcs $^{+/+}$ and DNA-PKcs $^{-/-}$ MEFs were treated with doxorubicin (Dox), a topoisomerase II inhibitor widely used as an anticancer drug, or insulin. Doxorubicin induced PKB phosphorylation on both Thr308 and Ser473 in a manner similar to γ -IR. Interestingly, two peaks of PKB phosphorylation were observed, a more prominent peak after 30 min of doxorubicin treatment and a smaller peak at 240 min. Insulin induced a more robust PKB phosphorylation 15 min after stimulation than doxorubicin at any time point in the experiment (Figure 4D). Doxorubicin-induced PKB activation was abolished in DNA-PKcs $^{-/-}$ MEFs throughout the time course, arguing for a loss of PKB phosphorylation and not a delay. DNA-PKcs $^{-/-}$ MEF response to insulin stimulation was, however, comparable to that of wild-type cells (Figure 4E). Notably, in the ATM knock-

out (ATM $^{-/-}$) MEFs, and ATM/ATR double knockout (ATM $^{-/-}$; ATR $^{-/-}$) MEFs, there was no defect in PKB Ser473 or Thr308 phosphorylation (Figure S4A). This underlines the specificity of DNA-PK, among the DNA damage-responsive PIKKs, for PKB regulation. We further tested MEFs proficient (*ric* $^{+/+}$) or deficient (*ric* $^{-/-}$) in rictor, a defining component of the mTORC2, for PKB Ser473 and Thr308 phosphorylation. Both Ser473 and Thr308 were phosphorylated in *ric* $^{+/+}$ and *ric* $^{-/-}$ cells after genotoxic treatment. Somewhat reduced Ser473-P and Thr308-P signal was detected in *ric* $^{-/-}$ MEFs, presumably as a consequence of already compromised steady-state PKB phosphorylation in these cells. As a control, no PKB phosphorylation was detected in insulin-treated *ric* $^{-/-}$ cells (Figure S4B).

Cells Lacking PKB α Display Aberrant DNA Damage-Induced Transcription, Including Transcriptional Misregulation of p21

Numerous studies have implicated PKB in transcriptional regulation. A very important component of the DNA damage response is represented by transcriptional regulation. We, therefore, examined the transcriptional changes following γ -IR of radiosensitive MEFs lacking PKB α . Expression profiles from PKB $\alpha^{+/+}$, PKB $\alpha^{-/-}$, and PKB $\alpha^{-/-R}$ cells 4 and 24 hr after γ -IR were compared to control nonirradiated cells of respective genotypes. At 4 hr after γ -IR, 46 genes were upregulated more than 1.5-fold in PKB $\alpha^{+/+}$ cells. One of these genes was upregulated in the PKB $\alpha^{-/-}$ cells, and upregulation of only one gene, p21^{WAF1/cip1}

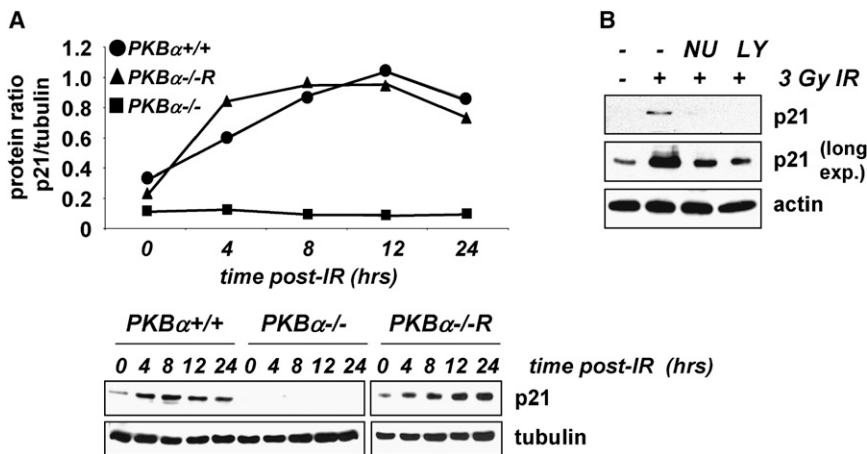


Figure 6. Loss of PKB α and Inhibition of DNA-PKcs Cause Misregulation of p21 Protein Levels

PKB α wild-type (PKB $\alpha^{+/+}$), knockout (PKB $\alpha^{-/-}$), and PKB α -reconstituted (PKB $\alpha^{-/-R}$) MEFs were treated with 10 Gy γ -IR. Whole-cell lysates were analyzed for p21 protein levels by western blotting. The graph presents p21 protein levels in cells of all three genotypes at the indicated times normalized to internal tubulin controls (A). HUVEC cells were treated with 3 Gy γ -IR following pretreatment with the NU7026 (10 μ M, 60 min) or LY294002 (50 μ M, 30 min) inhibitors. Whole-cell lysates were analyzed for p21 protein levels by western blotting (B).

(p21), was restored in PKB $\alpha^{-/-R}$ cells. Importantly, upregulation of exclusively p21 was also restored 24 hr after irradiation (Figure 5A, p21 is indicated in red). At 4 hr after γ -IR, 79 genes were downregulated more than 1.5-fold in PKB $\alpha^{+/+}$ cells, of which 5 were also downregulated in PKB $\alpha^{-/-}$ and PKB $\alpha^{-/-R}$ cells (Figure 5A). Thus, changes in gene expression were always more prominent in PKB $\alpha^{+/+}$ cells. Reconstitution of PKB α in knockout cells, however, did not entirely revert the phenotype observed in PKB $\alpha^{-/-}$ cells. Analysis revealed that the spectrum of genes upregulated in PKB $\alpha^{+/+}$, but not in the PKB $\alpha^{-/-}$ cells, was remarkably enriched for genes controlling responses to DNA damage (Figure 5B). Furthermore, grouping of those genes according to transcription factor regulation indicated a prominent enrichment of p53-regulated genes (Figure 5C). No significant changes were noted in Myc or p53 mRNA levels in these experiments (Figure S4). As the misregulation of p21 seemed to be a recurrent motif in several types of analysis performed, we analyzed and validated the changes in p21 mRNA in an independent real-time PCR assay (Figure 5D). In addition, the protein levels of p21 were found to change correspondingly, i.e., there was a 4- to 5-fold increase in p21 protein levels 4–24 hr after IR in both PKB $\alpha^{+/+}$ and PKB $\alpha^{-/-R}$ cells, while p21 protein was barely detectable in PKB $\alpha^{-/-}$ cells throughout the whole time course (Figure 6A). Unlike PKB $\alpha^{+/+}$ and PKB $\alpha^{-/-R}$, PKB $\alpha^{-/-}$ cells showed no decrease in the S phase of the cell cycle after irradiation (as expected given the loss of the p21 response [Figure S2C]). Moreover, transient transfection of untagged p21 into PKB α knockout MEFs reduced irradiation-induced apoptosis, as measured by decrease in the mitochondrial intermembrane potential $\Delta\Psi$ in wild-type, PKB α knockout p21 transfected (PKB $\alpha^{-/-}$ p21), and PKB α knockout (PKB $\alpha^{-/-}$) MEFs. At 36 hr after irradiation, wild-type MEFs were shown to have 30% apoptotic cells and PKB $\alpha^{-/-}$ p21 was shown to have 65%; in contrast, 90% of PKB $\alpha^{-/-}$ cells were apoptotic. Reintroduction of p21 to PKB $\alpha^{-/-}$ MEFs therefore partially restores viability after irradiation. This reinforces our hypothesis that the increased irradiation-induced apoptosis observed in PKB $\alpha^{-/-}$ MEFs results from an aberrant p21 response to DNA damage. To test whether DNA-PKcs-dependent activation of PKB after IR affects p21 regulation, we pretreated HUVEC cells with NU7026 and LY294002 before irradiation (see Figure 2). In both NU7026 and LY294002

pretreated cells, p21 protein remained at basal levels, in contrast to irradiated cells, where there was an increase in p21 protein (Figure 6B). As was previously reported (Kachnic et al., 1999), we also detected increased p21 protein 4–24 hr after IR in DNA-PKcs $^{+/+}$ MEFs. DNA-PKcs $^{-/-}$ cells were low in p21 with minor changes during the period of irradiation (Figure S6A), similar to PKB α knockout MEFs (see Figure 6A). Overall, these results indicate that PKB α participates in the p53-regulated gene expression program and has a direct effect on p21 regulation after DNA damage. This may explain, at least in part, why PKB α is important for survival after DNA damage.

Mice Deficient in PKB α Show an Attenuated p21 Response to γ -IR and Suffer from Increased DNA Damage-Induced Apoptosis

In cell culture, PKB α is important for the regulation of p21 and for survival after γ -IR (Figures 4, 5, and 6 and Figure S2). To determine the in vivo contribution of PKB α to the transduction of DNA damage signal to p21, we studied p21 and apoptotic responses in PKB α knockout mice. mRNA levels of p21 were higher in PKB $\alpha^{+/+}$ mice kidney and spleen 8 hr after a single dose of whole-body irradiation and reverted to near basal levels 24 hr after treatment. In PKB $\alpha^{-/-}$ mice, there was a very minor increase in p21 mRNA, peaking only at 24 hr after irradiation and only slightly above basal (Figure 7A and Figure S7A). In wild-type mice, p21 protein levels had increased about 2.5-fold by 8 hr post-IR and remained elevated at 24 hr. There were no significant changes in the initially low levels of p21 in knockout mice tissues. Importantly, we also observed a decrease in p53 protein in irradiated PKB $\alpha^{-/-}$ mice tissues compared with PKB $\alpha^{+/+}$ (Figure 7C and Figure S7C). Protein levels of cleaved PARP, as a mark of apoptosis induced by γ -IR, were higher in PKB $\alpha^{-/-}$ mice tissues than in the wild-type (Figure 7D). Finally, we validated the immunoblotting results by visualizing apoptotic cells in mouse tissues employing a TUNEL assay. TUNEL-stained cells were recorded in particular areas of the spleen, with a significantly higher frequency in PKB $\alpha^{-/-}$ than in PKB $\alpha^{+/+}$ mice. Quantitation of TUNEL staining showed an \sim 3-fold increase in the mean number of apoptotic cells per selected area in knockout mice, and an \sim 1.3-fold increase in the wild-type 24 hr after irradiation (Figure 7E). Less striking differences

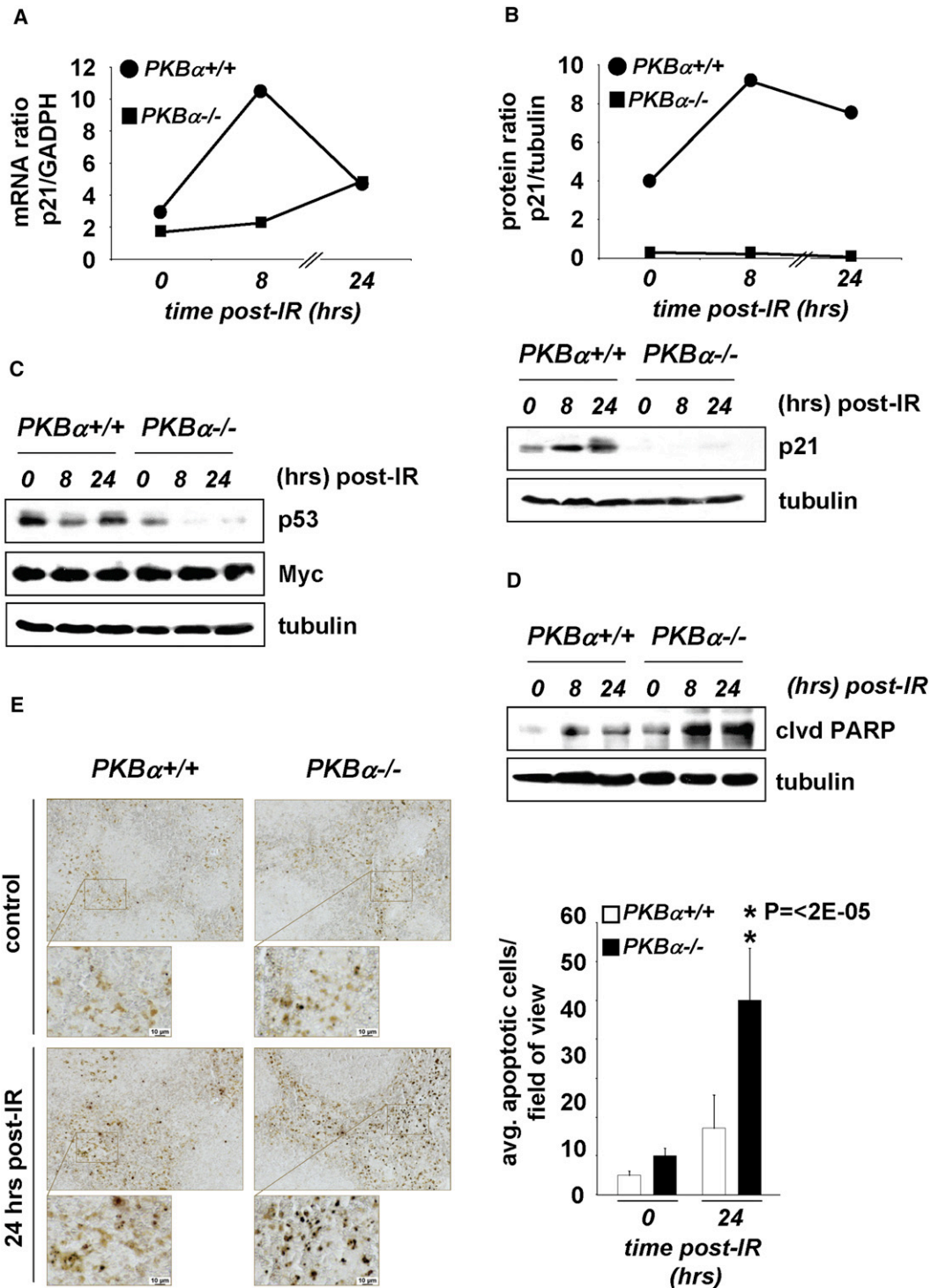


Figure 7. Attenuated p21 Response to γ -IR and Increased DNA Damage-Induced Apoptosis in Mice Deficient in PKB α

PKB α wild-type (PKB $\alpha^{+/+}$) and knockout (PKB $\alpha^{-/-}$) female mice were exposed to a single dose of 3 Gy γ -IR or sham irradiated (control), and sacrificed 8 or 24 hr after irradiation. (A) Total RNA was isolated from several organs for real-time PCR analysis. The panel presents spleen p21 mRNA expression levels normalized to internal GADPH control. (B–D) Proteins were prepared from organs and analyzed by western blotting. (B) The graph (upper panel) represents quantified spleen p21 protein expression levels normalized to internal tubulin control. Lower panel shows western blot measurement of p21 expression levels. (C) The panel shows western blots measuring p53 and Myc expression levels. (D) Western blots showing the protein levels of PARP. (E) Irradiation induced apoptosis (TUNEL assay) in the spleen of PKB α wild-type (PKB $\alpha^{+/+}$, left column) and knockout (PKB $\alpha^{-/-}$, right column) mice 24 hr postirradiation. The graph presents quantitation of

were distinguished at 8 hr after irradiation (Figure S8). Altogether, these *in vivo* results support our observation in cell culture that PKB α has a marked impact on survival after DNA damage due to an effect on transcriptional regulation of p21 and irradiation-induced apoptosis.

DISCUSSION

In the present study we provide a detailed analysis of the modulation of PKB activity and its impact on the DNA damage response in mammalian systems. We report a regulatory network in which DNA-PKs specifically activates PKB by Ser473 phosphorylation after induction of DSBs. Activated PKB α apparently regulates crosstalk to the p53-dependent DNA damage-induced gene expression program.

Two different PKB states can be distinguished (phosphorylated versus dephosphorylated) in the DNA damage response, dependent upon the amount of DNA damage (Figure 1). These PKB states probably reflect the destiny of the cell population with respect to survival after DNA damage. Besides using the DNA-repair program and halting cell-cycle progression, cells also respond to DNA insults by undergoing programmed cell death. Our results suggest that PKB is part of this regulatory mechanism. This is further supported by the demonstration that PKB is activated directly by DNA-PKs, a DSB responsive enzyme directly recruited to the damaged DNA and involved in DNA repair and DNA damage-induced signaling (Figures 2, 3, and 4).

We have confirmed that PDK1 is responsible for PKB Thr308 phosphorylation in the DNA damage response using a knockout ES cell model (Figure 4). DNA-PK phosphorylates HM Ser473 of PKB. However, we also noted similar patterns in T loop Thr308 phosphorylation after γ -IR (DNA-PKs-dependent induction following irradiation although PDK1 activity was unaffected by inhibition of DNA-PKs [data not shown]) (Figures 2 and 4). This indicates that the two phosphorylation steps are tightly connected and interdependent. This may be due to conformational changes in PKB induced by phosphorylation (Yang et al., 2002). It was also described previously that Ser473 phosphorylation precedes Thr308 phosphorylation, and proposed that this is important for the phosphorylation of PKB by PDK1 (Scheid et al., 2002).

A preactivation complex of PKB and PDK1 was reported recently (Calleja et al., 2007). PDK1 was shown to shuttle to the nucleus following mitogen stimulation (Scheid et al., 2005). We have detected perinuclear and nuclear PDK1 in irradiated HUVEC cells (Figure S3). It is therefore plausible that a nuclear PDK1 pool also exists after DNA damage, as there is one of PKB and DNA-PKs. This provides a setting with an increased local concentration of all three proteins, which is clearly a prerequisite and a stimulus for phosphorylation events to take place, especially as 3'-phosphorylated inositides and class I PI3K have been reported to be present in the nucleus (reviewed in Irvine, 2003).

Feng et al. (2004a) isolated DNA-PKs from the membrane fraction of HEK293 cells and showed *in vitro* kinase activity of

the purified active kinase toward PKB Ser473. The authors found impaired PKB phosphorylation after starvation and insulin stimulation in cells lacking DNA-PKs. The cells used by Feng and colleagues were serum starved (quiescent), while in our current experiments they were serum sufficient (cycling). Thus the pathways operating under these two cell states appear to be different. In terms of the classical insulin response we now consider, with all the new data since 2004, that DNA-PK does not play a role in insulin-promoted activation of PKB in serum-sufficient cells. It is therefore unclear whether DNA-PKs present in the lipid rafts (Lucero et al., 2003) plays a role outside of the DNA damage response in facilitating rapid PKB Ser473 phosphorylation following nutrient starvation.

γ H2AX foci are a cytological manifestation of DSB induction that marks the site of the break, and many components of the DNA damage response form IR-induced foci colocalizing with γ H2AX. Although accumulation of DNA-PKs at the DSBs was not obvious, the Ser473-P PKB foci we discovered were colocalized with the γ H2AX staining (Figure 3). This finding is important for several reasons. First, it places active PKB at the site of DNA damage, which of course has implications for its regulation by DNA-PKs. Second, this may help to explain mechanistically why PKB is no longer phosphorylated at a very high γ -IR dose because PKB recruitment to the DSB and to DNA-PKs may be affected in some way by the vastly dense and complex DNA damage generated by a high dose of irradiation.

p21 is a vital regulator of cell-cycle progression after DNA damage. The deregulation in p21 expression we observed in PKB α knockout systems (both cell culture and mouse model) following γ -IR (Figures 5, 6, and 7) was likely due to the effects of PKB α expression and activation on the p53-regulated gene expression program (Figure 5). This possibly occurs at two levels. As p53 does not physically engage with DSBs, PKB may be feeding into p53 as a "messenger" from the DNA lesions. On the other hand, we detected diminished p53 basal protein levels, but no changes in its mRNA levels in PKB α knockout cells and mice (Figure 7 and Figure S5). As a consequence, a whole array of genes was misregulated in the DNA damage response in cells lacking PKB α (Figure 5). p53 is linked in an autoregulatory negative feedback loop with its cellular antagonist Mdm2, to stringently control p53 levels (reviewed in Levine et al., 2006). Mdm2 is a phosphorylation target of PKB (Feng et al., 2004b; Mayo and Donner, 2001) but also a reported target of GSK3 in γ -IR response (Kulikov et al., 2005). We have demonstrated GSK3 phosphorylation dependent on DNA-PK- and PDK1-mediated activation of PKB (Figure 4). Misregulation of Mdm2 downstream of PKB was therefore likely responsible for the impaired p53 response to irradiation in the absence of PKB α . Furthermore, we found that upregulation of p21 following DNA damage was dependent on PKB α and DNA-PK (Figures 5, 6, and 7 and Figure S6). Several conflicting reports regarding the role for DNA-PK in p53-dependent pathways in the DNA damage response exist (Burma et al., 1999; Kachnic et al., 1999). Our data contribute to the current knowledge on this important issue.

the TUNEL assay with the ImageAccess software. Apoptotic cells were counted in three areas per slide at 20 \times magnification and expressed relative to the analyzed surface area. The differences in mean values between the treatment groups were statistically significant as determined by 1-way ANOVA test. The asterisk marks the group with the indicated P value determined by the Holm-Sidak method comparing untreated and γ -irradiated groups of the same genotype.

We focused on p21 as its regulation after γ -IR was normalized in PKB α knockout MEFs by reintroducing PKB α (Figures 5 and 6), arguing for a direct effect. Finally, the regulation of p21 by PKB in the DNA damage response described here reflects the IGF1-promoted muscle cell survival pathway through PKB-mediated induction of p21 (Lawlor and Rotwein, 2000). PKB therefore represents a signaling node at which a prosurvival signal is created following input from both the plasma membrane and the nucleus. Furthermore, as PKB isoforms have already been shown to have various distinct functions (reviewed in Dummler and Hemmings, 2007) it is conceivable that PKB α has a specific role in promoting survival after DNA insult.

Our *in vivo* experiments provide a link between PKB α signaling and survival in the face of genotoxic insult. Recent studies found early tumorigenic abnormalities to induce DNA damage signaling activation (Bartkova et al., 2005; Gorgoulis et al., 2005). Moreover, doxorubicin induces PKB activation and PKB-regulated gene expression in mice (Ichiyama et al., 2007). p21 knockout (p21^{-/-}) mice develop significantly more metastatic tumors after γ -IR than p21 heterozygous (p21^{+/-}) mice (Engelman et al., 2007). Thus PKB remains an attractive target for intervention in the development and treatment of malignancies, although with caution, as cancer therapies involving DNA damage have potential to promote PKB activation and ultimately a survival signal. It will, therefore, be necessary to identify further PKB functions in the nucleus of irradiated cells. These would definitely provide directions for studies of the mechanism of PKB action after induction of DSBs, as well as opportunities for pharmacological interference.

In sum, the results presented here demonstrate that PKB α plays a role in the DNA damage response, a function that may help advance our understanding of how the signal from the damaged DNA is translated to a prosurvival signal at the nuclear level. DNA-PK plays an indispensable role in activating PKB following induction of DSBs. Further understanding of the mechanism(s) of the DNA damage induction of PKB may also hold therapeutic potential in cancer research, besides improving our knowledge of the DNA damage response at the molecular level.

EXPERIMENTAL PROCEDURES

Materials

Antibodies were obtained from the following sources: Ser473-P Akt, Thr308-P Akt, Ser21/9-P GSK3 α / β , GSK3 β , Ser15-P p53, and human p21 were from Cell Signaling Technologies; Ser139-P H2AX and Myc were from Upstate; Thr229 p70S6K was from Abcam; DNA-PKcs, Ku70, and Ku80 were from NeoMarkers; mouse p21 was from Oncogene; and PDK1 was from BD Biosciences. Anti-HA 12CA5, anti-myc 9E10 and anti- α -tubulin YL1/2 antibodies were used as hybridoma supernatants. PKB isoform-specific antibodies were obtained by immunizing rabbits with isoform-specific peptides, and those were previously described (Yang et al., 2005), as well as PKB antibody Ab10 (Hill and Hemmings, 2002). Doxorubicin, insulin, NU7026, and LY294002 were from Sigma.

γ -IR Treatments

Cells were plated at consistent densities 24 hr prior to treatments. Following single-dose γ -IR treatment in a TORREX 120D (Astrophysics Research Corp.) instrument at 5 mA/120 kV and 0.13 Gy/s, cells were left to recover at 37°C for the indicated times before analysis. Single-dose total-body irradiation of ~10 week-old female mice was carried out in the same instrument, after

which the mice were caged separately before being sacrificed at the indicated times after treatment. The mice were monitored for changes in behavior or appearance during the recovery period.

Quantitation of DNA Damage-Induced Apoptosis, Cell Growth, and Viability Measurements

Apoptosis was assessed by measuring changes in the mitochondrial intermembrane potential ($\Delta\psi$) by a FACS-based method, using a cationic lipophilic dye DiIC1(5) assay kit (Invitrogen). Consistent numbers of cells were plated 24 hr prior to DNA-damaging treatment, and at the indicated times after treatment detached cells from supernatants together with attached cells were labeled and processed according to the manufacturer's protocol, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson). For cell growth/viability experiments, cells were harvested daily and analyzed by trypan blue dye exclusion method with the Vicell CoulterCounter (Beckman).

Microarray Analysis of DNA Damage-Induced Transcriptional Changes

Total RNA was extracted from triplicate experiments using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 2 μ g total RNA using the SuperScript cDNA system (Invitrogen) and used to generate biotin-labeled cRNA with the Enzo BioArray High Yield RNA transcript labeling kit (Enzo Diagnostics, USA). After fragmentation, 10 μ g cRNA was hybridized with the mouse MOE430A 2.0 GeneChips (Affymetrix, Santa Clara, USA) following the protocol recommended by Affymetrix. Gene chips were then scanned in an Affymetrix 2500 scanner, and gene expression was analyzed using the GeneSpring Software 7 (Silicon Genetics). The data were submitted to one-way ANOVA (a value of $p < 0.05$ was considered significant). Microarray data are deposited in GEO (GSE9146).

SUPPLEMENTAL DATA

Supplemental Data include eight figures and Supplemental Experimental Procedures and can be found with this article online at <http://www.molecule.org/cgi/content/full/30/2/203/DC1/>.

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Supplemental Data

PKB α /Akt1 Acts Downstream of DNA-PK

in the DNA Double-Strand Break

Response and Promotes Survival

Lana Bozulic, Banu Surucu, Debby Hynx, and Brian A. Hemmings

Figure S1. Bozulich *et al.*

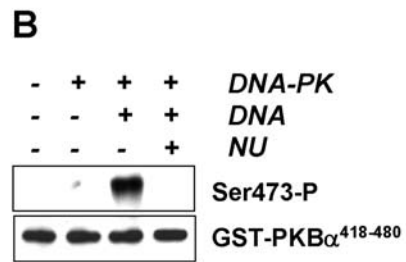
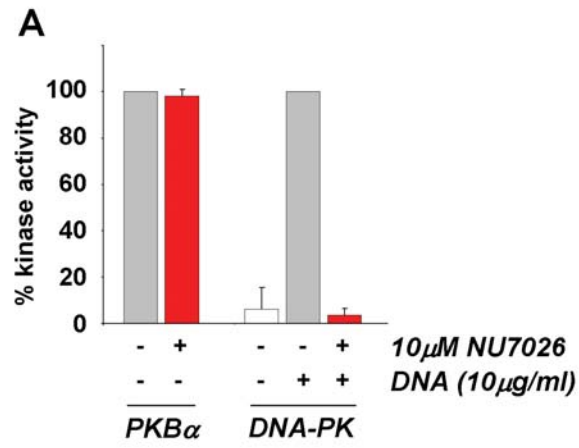


Figure S1. NU7026 Inhibits the DNA-Stimulated In Vitro Kinase Activity of DNA-PK_{cs} toward PKB α , but Not PKB α Itself

(A) Purified active PKB α (Hemmings laboratory) was assayed with 30 μ M of the specific substrate peptide R7Ftide (RPRAATF). Where indicated, NU7026 was added to the kinase reactions. Purified DNA-PK (Promega) was assayed with 0.1 mg/ml FSY peptide containing Ser473 (RRPHFPQFSYSASSTA), corresponding to the hydrophobic motif of PKB α . Calf thymus DNA was added at 10mg/ml in the DNA-PK activation buffer to achieve optimal kinase activity. Where indicated, NU7026 was added to the kinase reactions. Data are represented as means \pm SD of duplicate determinants.

(B) Purified DNA-PK (Promega) was assayed with GST-PKB α ⁴¹⁸⁻⁴⁸⁰ as substrate. Calf thymus DNA was added at 10 μ g/ml in the DNA-PK activation buffer to achieve optimal kinase activity. Where indicated, NU7026 was added to the kinase reactions. Specific phosphorylation was monitored by Western blotting with the Ser473-P antibody.

Figure S2. Bozulich *et al.*

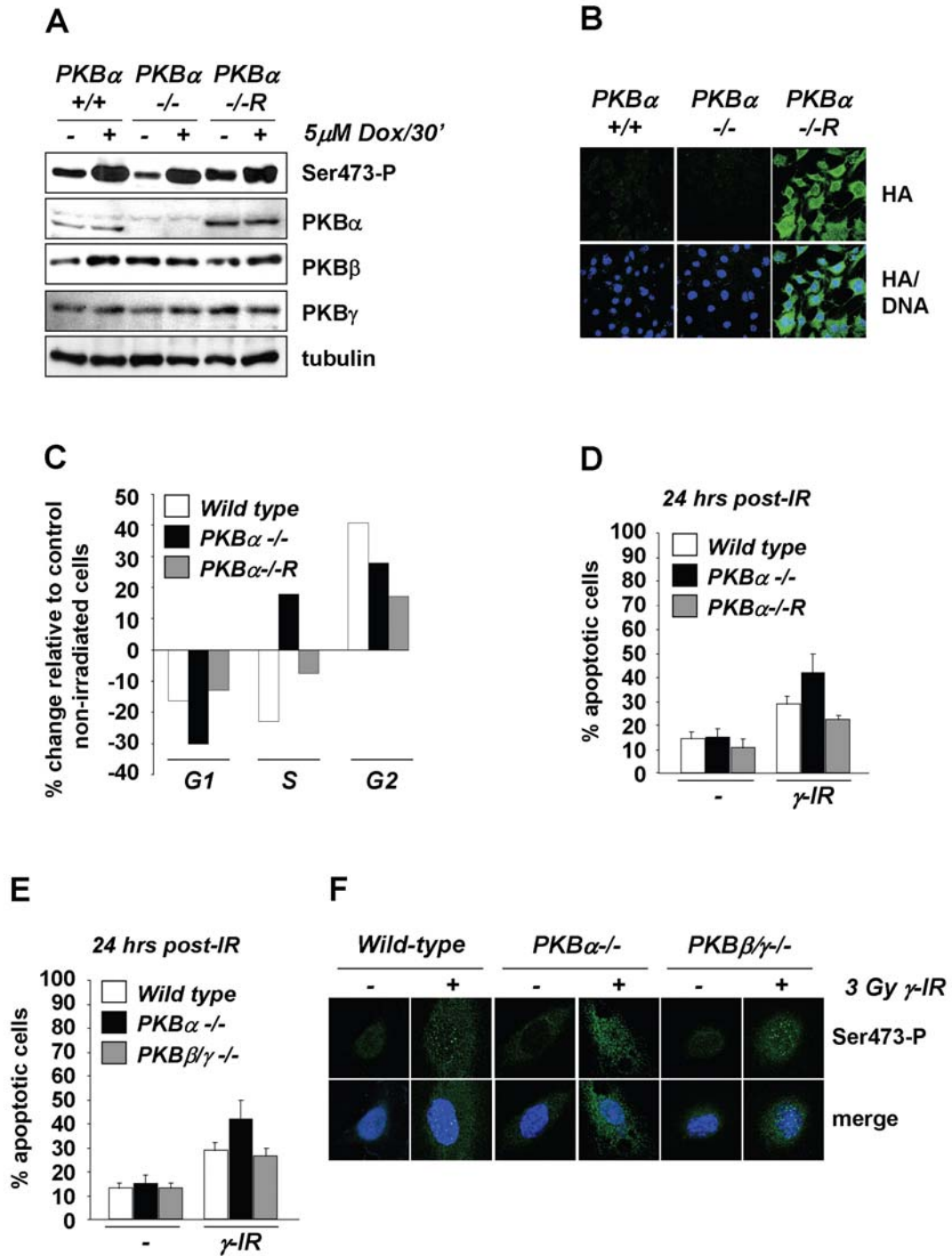


Figure S2. PKB α Isoform Is Important for Survival after DNA Damage

(A) Western blot analysis of PKB isoform protein expression levels and Ser473-P in PKB α ^{+/+}, PKB α ^{-/-} and PKB α ^{-/-}R MEFs treated with 5 μ M doxorubicin for 30 min.

(B) Immunofluorescence staining for HA of re-introduced mouse wild-type HA-PKB α in the PKB α ^{-/-}R cells. Anti-HA staining is shown in green, DNA in blue.

(C) Wild-type mouse embryonic fibroblasts (PKB α ^{+/+}), mouse embryonic fibroblasts deficient in PKB α (PKB α ^{-/-}), or the mouse embryonic fibroblasts deficient in PKB α where mouse wild-type HA-PKB α was re-introduced stably by transfection (PKB α ^{-/-}R) were treated with 10 Gy γ -IR. Cell cycle progression was monitored 8 h after irradiation. Summary panel of the FACScan experiment showing cell-cycle distribution of irradiated cells of the indicated genotypes relative to unirradiated control cells.

(D) At 24 h after irradiation, apoptosis was measured by a FACS based DilC1₍₅₎ assay.

(E) Wild-type mouse embryonic fibroblasts (PKB α ^{+/+}), mouse embryonic fibroblasts deficient in PKB α (PKB α ^{-/-}), or PKB β and PKB γ (double knock-out, PKB β/γ ^{-/-}), were treated with 10 Gy γ -IR and apoptosis measured 24 h after irradiation. In (D) and (E) data are represented as means \pm SD of triplicate determinants.

(F) Immunofluorescence staining of Ser473-P in mouse embryonic fibroblasts as in (E) 30 min after 3Gy γ -IR treatment (Ser473-P green, DNA in the merged images blue).

Figure S3. Bozulich *et al.*

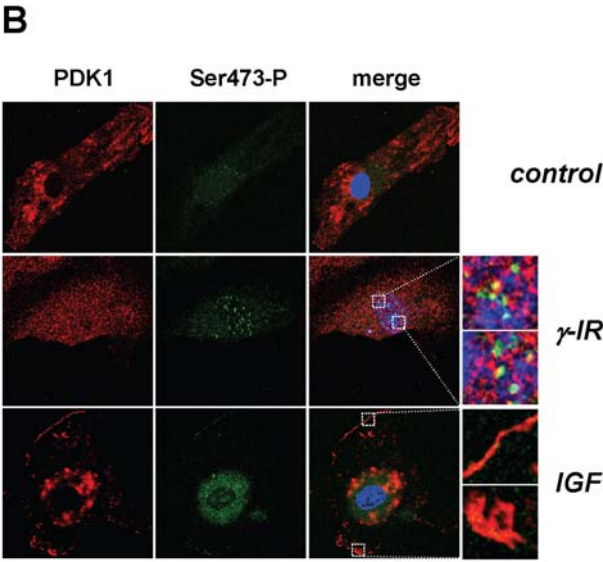
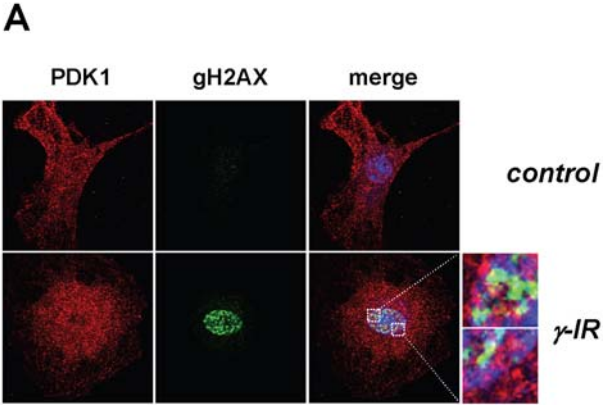


Figure S3. Perinuclear and Nuclear Localization of PDK1 after γ -IR

Immunofluorescence staining for PDK1 and γ H2AX (PDK1 red, γ H2AX green, DNA in the merged images blue, and co-localization of PDK1 and γ H2AX in yellow) (A); or PDK1 and Ser473-P of PKB (PDK1 red, Ser473-P green, DNA in the merged images blue, and co-localization of PDK1 and Ser473-P in yellow) (B) in HUVEC cells 30 min after 3 Gy γ -IR treatment or following 100 ng/ml IGF-1 treatment. The images are representative of the stainings observed in the experiments.

Figure S4. Bozulich *et al.*

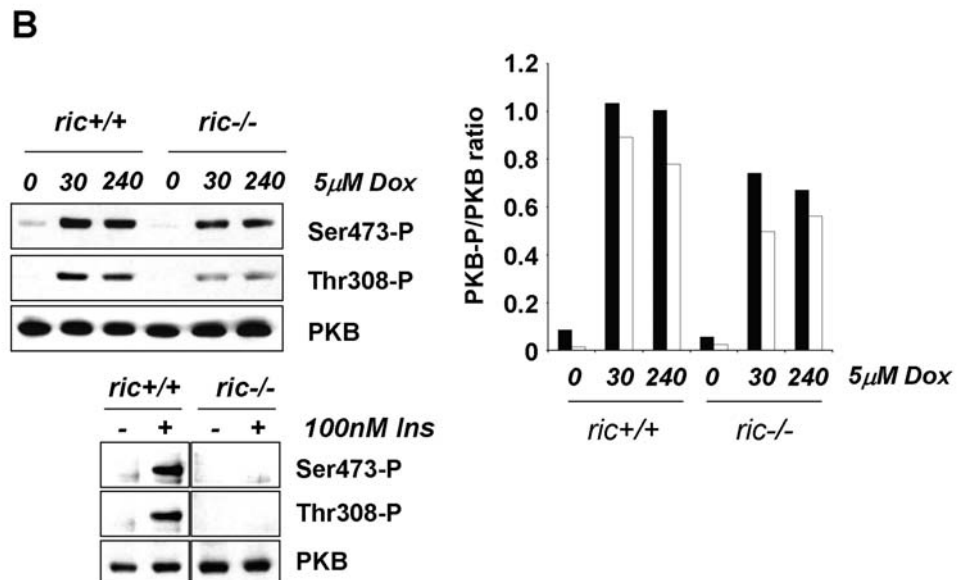
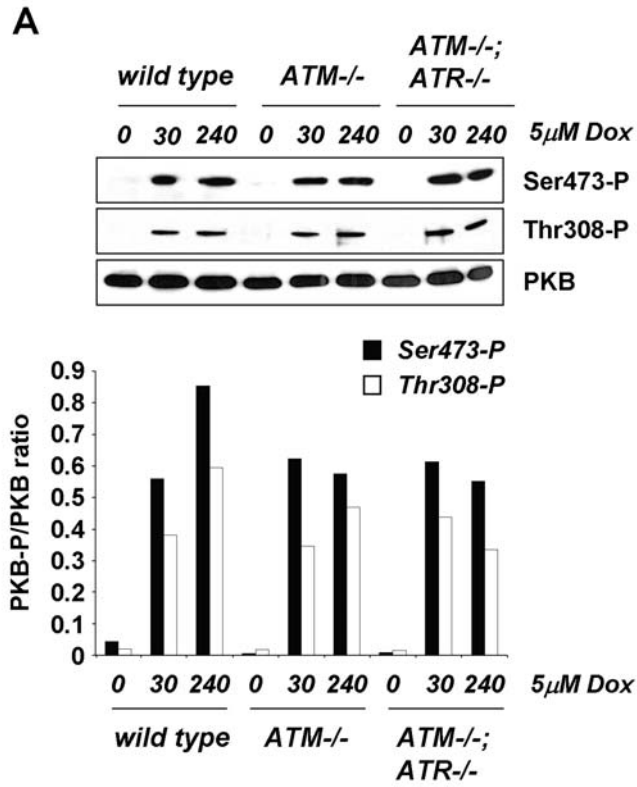


Figure S4. γ -IR-Induced PKB Phosphorylation PIKK-Deficient Cells

(A) Wild-type mouse embryonic fibroblasts (wild type), mouse embryonic fibroblasts deficient in ATM (ATM^{-/-}), or ATM and ATR (double knock-out, ATM^{-/-}; ATR^{-/-}) were treated with 10 Gy γ -IR. Phosphorylation of both activating residues in PKB (Thr308 and Ser473) was measured at the indicated time points by Western blotting. The graph represents Ser473-P-PKB and Thr308-P-PKB levels in cells of all three genotypes at the indicated times normalized to internal PKB controls.

(B) Wild-type mouse embryonic fibroblasts (ric^{+/+}) and mouse embryonic fibroblasts deficient in rictor (ric^{-/-}) were treated with 10 Gy γ -IR. Phosphorylation of both activating residues in PKB (Thr308 and Ser473) was measured at the indicated time points by Western blotting. The graph represents Ser473-P-PKB and Thr308-P-PKB levels in cells of both genotypes at the indicated times normalized to internal PKB controls.

Figure S5. Bozulich *et al.*

A

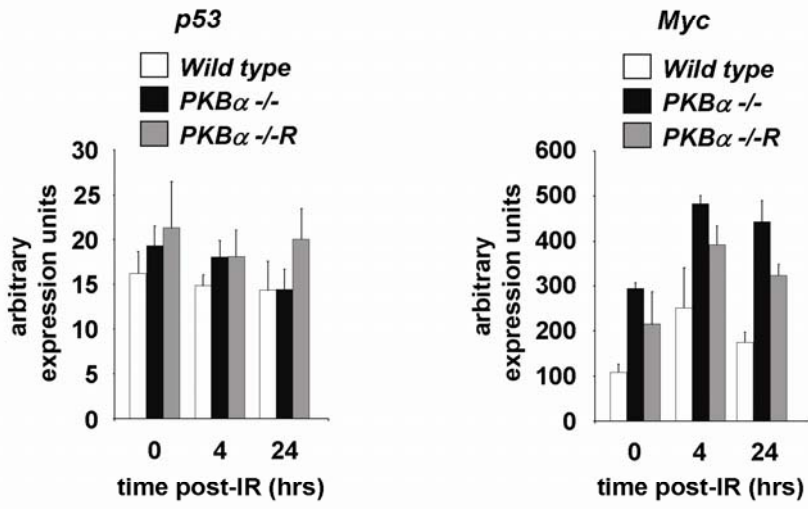
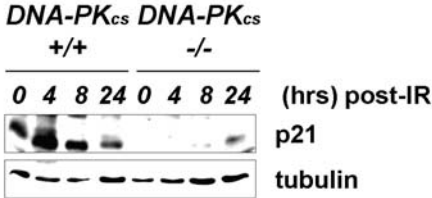


Figure S5. Loss of PKB α Does Not Affect p53 and Myc mRNA Expression

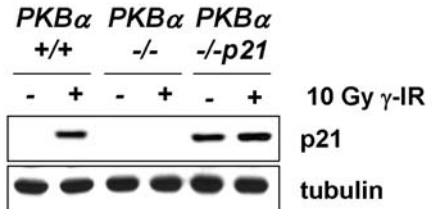
(A) Virtual Northern blots documenting regulation of p53 (left panel) and Myc (right panel). The plots show the regulation of these genes under the indicated conditions as deduced from the microarray experiment shown in Figure 4.

Figure S6. Bozulich *et al.*

A



B



C

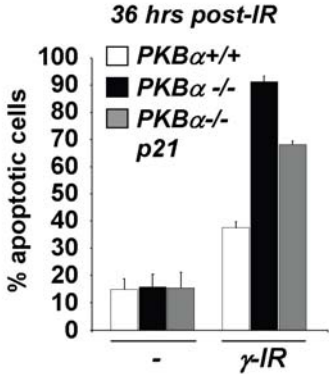


Figure S6. Attenuated p21 Response to γ -Irradiation in DNA-PK-Deficient Mouse Embryonic Fibroblasts

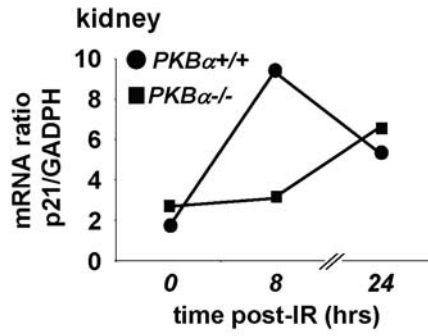
(A) Mouse embryonic fibroblasts proficient (DNA-PK^{+/+}) or deficient (DNA-PK^{-/-}) in DNA-PK were treated with 10 Gy γ -IR. Whole cell lysates were analyzed for p21 protein levels at the indicated time points.

(B) Mouse embryonic fibroblasts deficient in PKB α were transiently transfected with untagged p21 (PKB α ^{-/-}-p21).

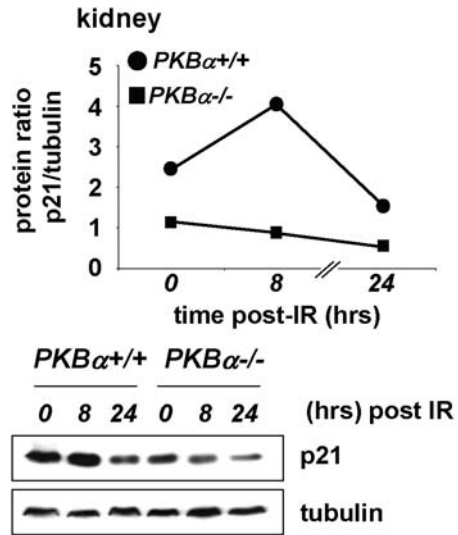
(C) At 12 h after transfection, wild-type mouse embryonic fibroblasts (PKB α ^{+/+}), mouse embryonic fibroblasts deficient in PKB α (PKB α ^{-/-}), or mouse embryonic fibroblasts deficient in PKB α p21 transfected (PKB α ^{-/-}-p21) were treated with 10 Gy γ -IR. At 4 h after irradiation whole cell lysates were analyzed by Western blotting for p21 protein levels (left panel); 36 h after irradiation apoptosis was measured by a FACS based DiIC1₍₅₎ assay (right panel).

Figure S7. Bozulich *et al.*

A



B



C

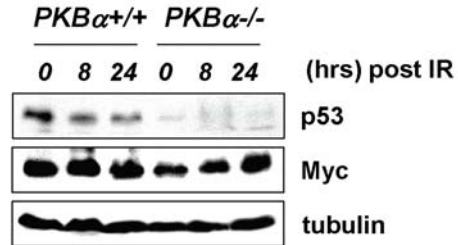


Figure S7. Attenuated p21 Response to γ Irradiation in Mice Deficient in PKB α

Total RNA was isolated from the kidney (in the experiment shown in figure 5A) for realtime PCR analysis. (A) The panel presents p21 mRNA expression levels normalized to internal GAPDH control. (B and C) Proteins were extracted from organs (in the experiment shown in figure 5A) and organ lysates analyzed by Western blotting. The graph (upper panel) presents quantified kidney p21 protein expression levels normalized to internal tubulin control. Lower panel shows Western blot measurement of p21 expression levels. (C) Western blots showing p53 and Myc expression levels.

Figure S8. Bozulic *et al.*

A

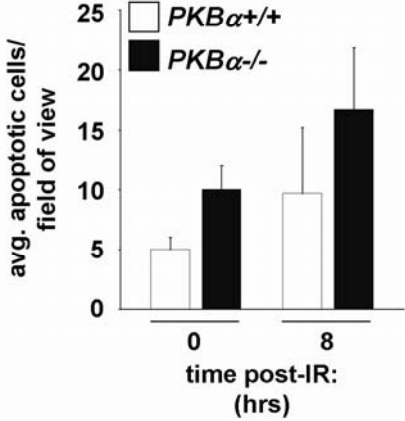
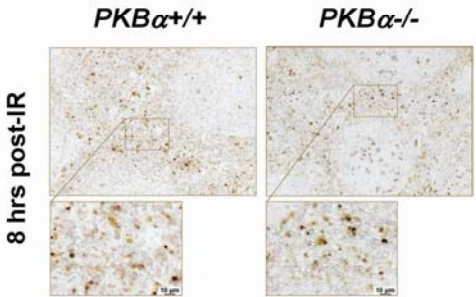


Figure S8. Increased DNA Damage-Induced Apoptosis in Mice Deficient in PKB α

(A) Irradiation induced apoptosis (TUNEL assay) in the spleen (from the experiment shown in figure 5D) of PKB α wild type (PKB α ^{+/+}, left column) and knock-out (PKB α ^{-/-}, right column) mice 8 h post-irradiation. The graph presents quantitation of the TUNEL assay with the ImageAccess software. Apoptotic cells were counted in three areas per slide at 20x magnification and expressed relative to the analyzed surface area. Data are represented as means \pm SD of the triplicate determinants.

Supplemental Experimental Procedures

siRNA-Mediated DNA-PK and PKB Gene Silencing in Mammalian Cells

HUVEC or HeLa cells plated at consistent confluence were transfected using Oligofectamine (Invitrogen) with siRNAs targeting DNA-PK_{cs} (Feng et al., 2004a), PKB [sense: r(GGA CCC CAA GCA GAG GCU U)dTdT, synthesized by Qiagen], or a 21-nucleotide irrelevant RNA duplex (Qiagen) as a control. Cells were analyzed 48 or 72 hrs post-transfection.

PKB Immunoprecipitation and In Vitro PKB Kinase Assay

PKB immunoprecipitation and in vitro PKB kinase assay were as described previously, using monoclonal A4D6 [described in (Maira et al., 2001)] or polyclonal Ab10 anti-PKB antibodies, and the specific peptide RPRAATF (R7Ftide) as PKB substrate (Hill and Hemmings, 2002).

Immunofluorescence Microscopy

Immunofluorescence microscopy was carried out essentially as described previously (Hergovich et al., 2007). In brief, cells were fixed in 3% paraformaldehyde for 10 min at 37°C before being permeabilized using 0.2% Triton X-100 in PBS for 2 min at room temperature and incubated with appropriate antibodies. Confocal images were acquired with a laser scanning microscope (Olympus FV500) with the Fluoview 1000V.1 application software, and processed and quantitated using the Imaris program (Bitplane AG, Zurich, Switzerland) and Photoshop 6.0 (Adobe System Inc).

Cell Lines

Cell lines were obtained from following sources: DNA-PK_{cs} MEF from P. A. Jeggo (University of Sussex, Sussex, UK), ATM^{-/-};ATR_{flox}^{-/-} (Cre was expressed to delete the ATR allele) MEF from E. J. Brown (University of Pennsylvania, Philadelphia, USA), PDK1^{-/-} ES from D. R. Alessi (MRC, Dundee, UK) and rictor^{-/-} MEF from M. A. Magnuson (Vanderbilt University School of Medicine, Nashville, Tennessee, USA).

Generation of Stable MEF Cell Lines by Retroviral Infection

To produce the retroviruses, BOSC retrovirus packaging cells were transiently transfected with the retroviral pBABEpuro empty vector or pBABEpuro HA-PKB α construct by the calcium phosphate method. 48 hours after transfection, viral supernatants were harvested, filtered through a 0.45 μ m membrane and applied to MEFs in 10 cm dishes with 5 μ g/ml polybrene (Sigma). A second infection of MEFs was performed after 8-12 hours. 24 hours after retroviral infection, cells were selected with 3-5 μ g/ml puromycin (Sigma) for 6-8 days and resistant clones were propagated.

Isolation and Culture of Primary Prostate Epithelial Cells from DNA-PK_{cs}

Mice

Dissected prostates were cut into small pieces and digested with 0.8 mg/mL collagenase (GIBCO) in DMEM/10%FCS at 37°C for 90min. Cells were filtered through a nylon mesh, washed twice in DMEM/10%FCS, resuspended, counted and plated. Cells were passaged three times, before plating for experiments at standard numbers.

Real-Time PCR Detection of p21, myc, p53, and GADPH mRNA Levels Following γ -IR

Primer sequences (all for mouse proteins) were: *p21* fw 5'-GCCTTAGCCCTCACTCTGTG, rv 5'-AGGGCCCTACCGTCCTACT; *myc* fw 5'-GCCAGTGAGGATATCTGGA, rv 5'-ATCGCAGATGAAGCTCTGGT; *p53* fw 5'-AGAGACCGCCGTACAGAAGA, rv 5'-CTGTAGCATGGGCATCCTTT. ABI Prism 7000 detection system was used in conjunction with the Primer 3 Software program, the SyBr Green PCR Master Mix, and the ABI Prism 7000 SDS Software (provided with the ABI Prism 7000).

Statistics, Bioinformatics, and Quantitation of Western Blots

GO term enrichment analysis was performed with the GoStat program (Beissbarth and Speed, 2004). MetaCore from GeneGo Inc. was used to formulate transcriptional modules of significantly changed genes. Western blots were quantified using ImageQuant software (Molecular Dynamics).

Quantitation of DNA Damage-Induced Apoptosis in Tissues

Apoptosis was measured by TUNEL staining (TUNEL assay kit, Invitrogen) in paraffin embedded organs as described previously (Yang et al., 2005). Vectastain ABC kit (Vector Laboratories) for color development was used as described by the manufacturer. For quantitation of apoptosis, the numbers of TUNEL-positive cells per three fields of each section were counted with the help of the ImageAccess software.