Protein Kinase B: The beta version
Elucidating Novel Contributions Of Protein Kinase B beta (PKBβ/Akt2) To Endocrine Metabolism, PCOS and Cancer

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(Dekan)

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i. SUMMARY

Protein kinase B (PKB/Akt) is a serine/threonine protein kinase that mediates signaling crucial for normal cellular metabolism, proliferation, survival, and differentiation. PKB/Akt mediates these functions by virtue of its role as the major effector kinase upon which phosphatidylinositol 3,4,5-triphosphate kinase (PI3K) signaling converges. The PI3K signaling pathway transduces extracellular signals from cellular receptors, like the insulin receptor (InsR), the insulin-like growth factor receptor (IGF1R) and the epidermal growth factor (EGF/ErbB) receptors, to co-ordinate cellular responses.

In mammals, PKB/Akt exists as three isoforms, PKBα/Akt1, PKBβ/Akt2, and PKBγ/Akt3. these isofoms share the same domain structure and over 85% sequence similarity, suggesting these isoforms mediate similar and overlapping functions. However, these three isoforms are encoded by genes on distinct chromosomes and have differential tissue expression, supporting a concept that these isofoms have evolved to mediate specific and unique biological signals. Indeed, numerous studies have clearly demonstrated that distinct, isoform specific functions do exist which are often context and cell-specific. To elucidate these functions in a physiological setting, KO mouse models of all PKB/Akt isoforms have been generated confirming that these isoforms do have both redundant and non-redundant, isoform-specific functions. PKBα/Akt1 KO mice are viable but exhibit ~30% perinatal lethality, growth retardation and increased spontaneous apoptosis, strongly implicating PKBα/Akt1 as the major isoform in growth and survival. PKBβ/Akt2 KO mice are viable and of normal size, however they progressively develop a diabetes-like syndrome characterized by insulin resistance and hyperglycemia, illustrating a crucial role for this isoform in transducing signals regulating organism metabolism. PKBγ/Akt3 is viable and normal except for decreased brain and testis size that are the major expression sites of PKBγ/Akt3. This indicates this isoform may have more specialized or subtle functions. This is also supported by compound knockouts that lack PKBγ/Akt3. PKBα/Akt1-PKBγ/Akt3 double knockout mice die at embryonic day12 (E12) with severe growth retardation and developmental defects, whereas PKBβ/Akt2-PKBγ/Akt3 mice are viable but with

a reduction in animal size and an enhancement of the single isoform knockout phenotypes. Together this suggests the PKBγ/Akt3 isoform does contribute to normal function of both PKBα/Akt1 and PKBβ/Akt2. Similarly, loss of PKBβ/Akt2 on the background of PKBα/Akt1 deletion enhances the phenotype of PKBα/Akt1, resulting in 100% perinatal lethality and additionally leading to defects in bone and skin development. These mouse models highlight that all three PKB/Akt isoforms contribute, albeit to different degrees, to control cellular metabolism, growth, proliferation and survival in tissues throughout the organism. Furthermore, they illustrate that in regulating these functions the PKB/Akt isoforms also maintain whole organism metabolism and growth, with deletion of various isoforms in the whole organism or in specific-organs leading to defects in organism metabolism and growth. These observations in mouse models correlate well with human metabolic syndromes and diseases, particularly in insulin resistance/diabetes and cancer that invariably display aberrant PKB/Akt activation. This makes these mouse models excellent tools to explore the contribution of the PKB/ Akt isoforms to such human pathologies and identify isoform specific actions and downstream substrates that could provide targets for therapeutic intervention.

Accordingly, this work utilized PKBβ/Akt2 null mice to explore defects in metabolism in the context of insulin resistance, as well as exploring its contribution to tumour development driven by hyperactivation of the PI3K pathway. We observed that aged PKBβ/Akt2 KO mice, but not wild-type or PKBα/Akt1 KO mice, develop severe ovarian cysts with thecosis and consequent increases in testosterone production. We show that this may reflect an unknown role for PKBβ/Akt2 in regulating testosterone production in the ovary with a potential contribution to the human metabolic disorder Polycystic Ovarian Syndrome (PCOS). PCOS affects 5-10% of women of reproductive age and is the leading cause of infertility. It is characterized by hyperactive leutinizing hormone signaling in ovary, resulting in increased testosterone production and subsequently development of numerous follicular cysts within the ovary. Using a mouse model of PCOS driven by tonic administration of leutinizing hormone, mice lacking PKBβ/Akt2 developed cysts with a threefold increase in size compared to wild-type mice. Furthermore, the contribution of

PKBβ/Akt2 to neoplasia was analyzed by utilizing the Pten heterozygous mouse model. Pten acts as the major negative regulator of PI3K signaling and reduction of Pten in mice results in the development of neoplasia in a broad range of organs due to hyperactivation of PKB/Akt signaling. By deletion of PKBβ/Akt2 on this background, its effect on neoplasia formation in multiple organs was assessed. We observed a variety of effects on neoplasia development in various organs, with the most striking being an almost complete inhibition of adrenal medulla pheochomocytomas formation. Pheochromocytoma formation upon Pten loss in mice activates cellular proliferation and transcriptional changes to drive tumour development and progression. This includes increased proliferative signaling via mTORC1 and stimulation of adrenomedullin expression. $Pten^{+/-}$ mice also reflect the clinical setting with increases in catecholamine production and secretion that is observed in 90% of human pheochromocytoma patients. Analysis of adrenals, illustrated that PKBβ/Akt2 is required for early development of neoplasia and severely hinders growth and progression through attenuating mTORC1 activation and subsequent cellular proliferation. Cellular signaling required for catecholamine production and secretion was also suppressed ands reflected in decreased expression of the rate-limiting enzyme required for catecholamine generation: dopamine β-hydroxylase. Adrenomedullin that can trigger increased cAMP production and growth in various tumour settings, displayed increased expression in $Pten^{+/-}$ adrenals but decreased almost to wild-type levels upon additional deletion of PKBβ/Akt2. These findings indicate that PKBβ/Akt2 exerts an isoform specific role in promoting pheochromocytomas exhibiting hyperactivated PKB/Akt.

The findings from these studies illustrate novel contributions by PKBβ/Akt2 isoform specific signaling to metabolic dysfunction and tumour formation, thereby highlighting the potential of identifying the signaling pathways and targets involved in these actions. Accordingly, these results provide both the basis and a starting point for further studies to elucidate these signaling pathways and PKBβ/Akt2 specific substrates that may represent novel targets for therapeutic intervention.

ii. ABBREVIATIONS

AGC	cAMP-dependent kinase, cGMP-dependent kinase and protein kinase C family of kinases
cAMP	3'-5'-cyclic adenosine monophosphate
CREB	cAMP response element-binding protein
CYP11A	P450 cholesterol side-chain cleavage enzyme
CYP17A	17 α-hydroxylase/17,20-lyase/ cytochrome P450 17A
DHT	double heterozygous mice (PKBβ/Akt2$^{+/-}$Pten$^{+/-}$)
DKO	double knockout mice (PKBβ/Akt2$^{-/-}$Pten$^{+/-}$)
EGFR/ErbB	epidermal growth factor receptor family
ERK	mitogen-activated protein kinase/ extracellular-signal-regulated kinase 1/2
$G_{\alpha s}$	stimulatory G-protein alpha subunit
GnRHAnt	gonadotrophin-releasing hormone antagonist
GSK3α/β	glycogen synthase kinase 3alpha/beta
HT	heterozygous
HPO axis	hypothalamic-pituitary-ovarian axis
IGF1	insulin-like growth factor 1
InsR	insulin receptor
IR	insulin resistance
IRS	insulin receptor substrate
KO	knockout
LDLR	low-density lipoprotein receptor
LH	leutinizing hormone
LHR	leutinizing hormone receptor
mTOR	mammalian target of rapamycin
PCOS	polycystic ovarian syndrome
PDK1	3-phosphoinositide-dependent protein kinase 1
PH	pleckstrin homology
PI3K	phosphoinositide-3-kinase
PIP_2	phosphatidylinositol-4,5-biphosphate (also PI(3,4,5)P2)
PIP_3	phosphatidylinositol-3,4,5-triphosphate (also PI(3,4,5)P3)

PKA	protein kinase A
PKBα/Akt1	protein kinase B alpha/ v-Akt murine thymoma viral oncogene 1
PKBβ/Akt2	protein kinase B beta/v-Akt murine thymoma viral oncogene 2
PKBγ/Akt3	protein kinase B gamma/v-Akt murine thymoma viral oncogene 3
PKC	protein kinase C
Pten	phosphatase and tensin homolog
Raptor	regulatory-associated protein of mTOR
RTK	receptor tyrosine kinase
Rictor	rapamycin-insensitive companion of mTOR
SH2	Src homology 2
SKO	Single knockout mice ($Pten^{+/-}$)
StAR	steroid acute regulatory protein
S6K1/2	p70 ribosomal protein S6 kinase 1/2
TSC	tuberous sclerosis complex
WT	wild-type
4EBP1	eIF4E binding protein 1

Amino acid residues are described in text using standard three-letter nomenclature and single-letter nomenclature in figures.

Less frequently used abbreviations are defined upon their first use in the text.

I. INTRODUCTION

1. Protein Kinases As Master Regulators Of Cellular Signaling And Function

Protein kinases comprise of a family of approximately 500 different proteins that constitutes the largest family of enzymes in the human genome. Protein kinases mediate their action through protein phosphorylation, a mechanism by which a single phosphate moiety is added to a protein amine group. The consequences of this modification on the target protein include control of its activity, interactions and localization. Kinases exert these actions on one-third of all intracellular proteins, impacting on aspects of cell biology from metabolism to transcription, growth, proliferation, migration, survival and differentiation, thereby making kinases master regulators of signaling and function. To ensure correct cellular signaling and function, kinases themselves are tightly regulated temporally, spatially and quantitively to ensure their appropriate activation and downstream signaling.

Stringent control of kinase activation is apparent in the AGC family of protein kinases of which PKB/Akt is a member. The AGC family of protein kinases, was originally named after three early identified members the cAMP-dependent kinase, cGMP-dependent kinase and protein kinase C. The protein kinases of the AGC family share defining structural and regulatory aspects. AGC kinases display structural determinants that control protein localization and a flexible peptide loop, commonly referred to as the activation loop, which is found near the catalytic pocket and upon phosphorylation stimulates kinase activation. Additional structural domains control the amplitude of kinase activation.

2. PKB/Akt Isoforms, Structure and Activation

PKB/Akt is the cellular homologue of the transforming v-Akt oncogene found in a retrovirus termed Akt8. It is conserved with increasing complexity from lower organisms up to mammals, where it exists as three isofoms (Figure 1).

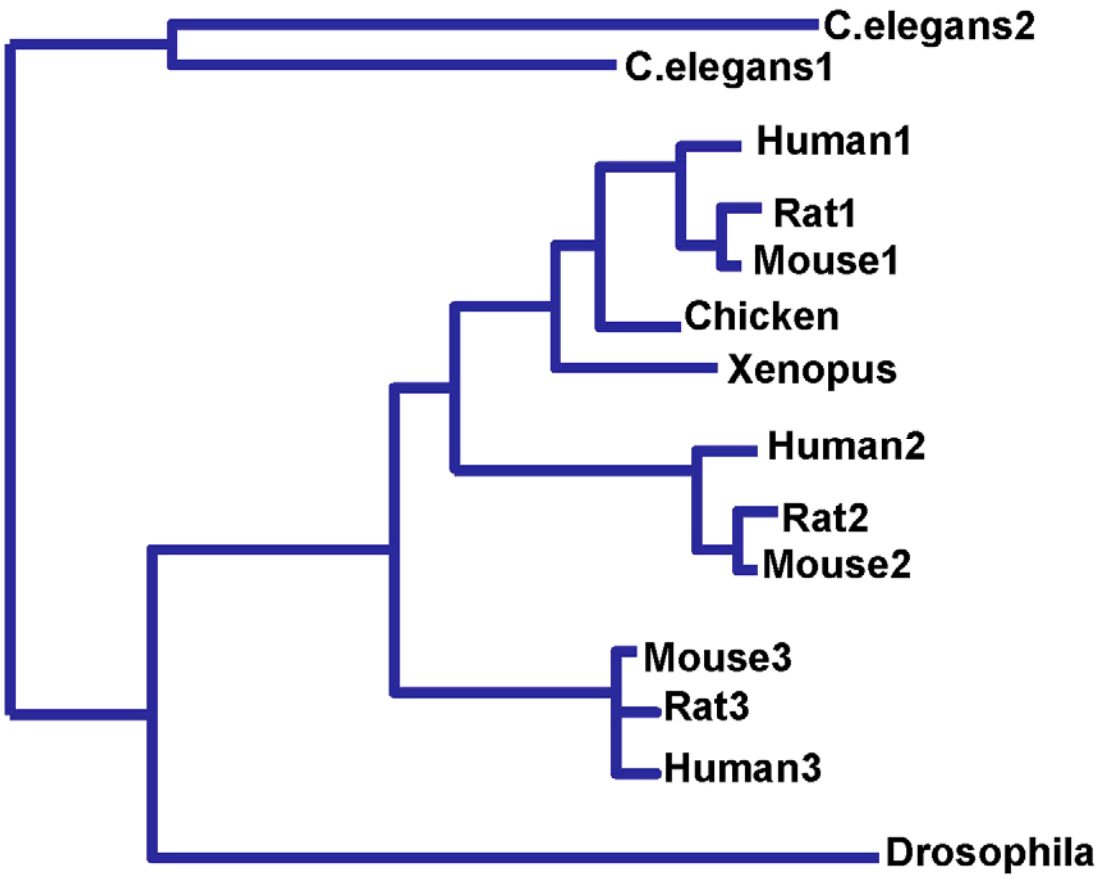

Figure 1. Phylogenetic Tree Of The PKB/Akt Proteins. PKB/Akt is conserved from lower organisms to mammals where all three isoforms are observed. Lower organisms show differential presence of PKB/Akt isoforms suggesting the evolutionary development of multiple PKB/Akt isoforms was a requirement for the regulation of more complex signaling found in higher organisms (Adapted from Riehle *et al*., 2003).

These three isoforms of PKB/Akt, termed PKBα/Akt1, PKBβ/Akt2, and PKBγ/Akt3 are found on distinct genes but exhibit greater than 85% sequence identity and share the same structural organization crucial for regulation of activity (Figure 2).

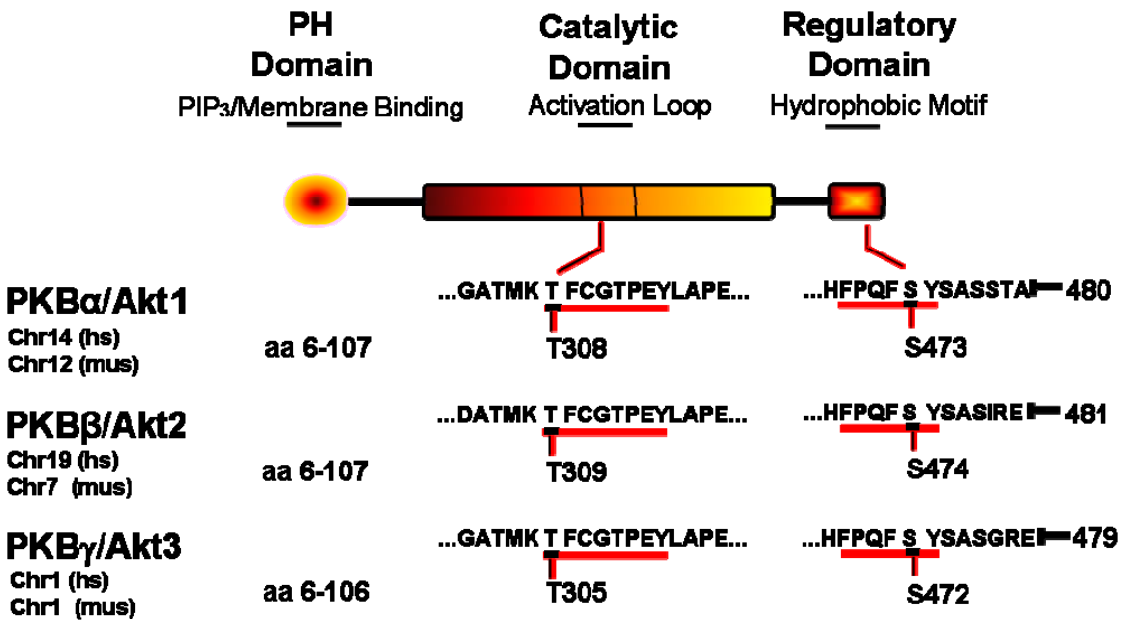

Figure 2. Domain Structures Of The PKB/Akt Isoforms And Roles In PKB/Akt Activation. PKB isoforms display 85% similarity with three highly conserved domains. All isoforms contain a pleckstrin homology (PH) domain responsible for tethering PKB/Akt at the plasma membrane via binding to the phospholipid, PIP_3, a catalytic domain containing the activation loop and PDK-1 threonine phosphorylation site and a C-terminal regulatory domain containing the hydrophobic motif and mTORC2/DNA-PK phosphorylation site required for full activation of PKB/Akt. PH, pleckstrin homology, PIP_3, phosphatidylinositol 3,4,5-triphosphate, Chr, chromosome; aa, amino acid; T, threonine; S, serine. Other amino acids illustrated surrounding the phosphorylation sites are represented by capital letters consistent with standard nomenclature.

The PKB/Akt isoforms possess an amino-terminal pleckstrin homology (PH) domain for binding to membrane 3-phosphoinositides, a central catalytic domain and a carboxy-terminal regulatory domain. The central catalytic domain contains the activation loop with a threonine phosphorylation site that activates the kinase, whilst the regulatory domain contains the hydrophobic motif with the serine phosphorylation site ($FPQFS^{P}Y$). Phosphorylation of the hydrophobic serine stabilizes the active conformation of PKB/Akt and stimulates a ten-fold increase in activity and full activation of the kinase (Figure 3). Activated PKB/Akt isoforms phosphorylate serine/threonine residues on target substrates with the consensus phosphorylation sequence Arg-X-Arg-X-X-Ser/Thr-Hyd, where X is any amino acid and Hyd is a bulky hydrophobic residue.

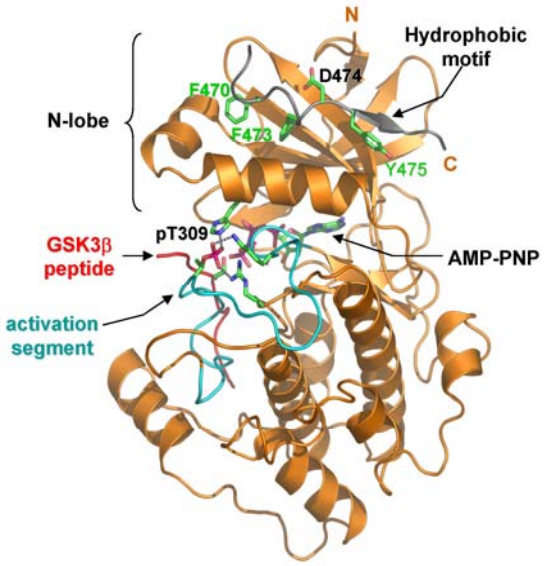

Figure 3. Structure Of Activated PKB/Akt. Activated PKBβ/Akt2 ternary complex with the GSK3-peptide (red) bound in the substrate-binding site and a hydrolysis-resistant ATP analogue (AMP-PNP) in the ATP-binding site. Note the hydrophobic motif of PKB/Akt has been replaced by that of the AGC kinase, PRK2 to allow stable crystal formation. Thr309 in the activation segment is shown in blue. Adapted from (Yang et al., 2002).

3. The PI3K-Pten-PKB/Akt Signaling Pathway, Downstream Substrates And Functions

The PI3K-PTEN-PKB/Akt signaling pathway transduces signals from membrane receptors to its major effector molecule, PKB/Akt (Figure 4).

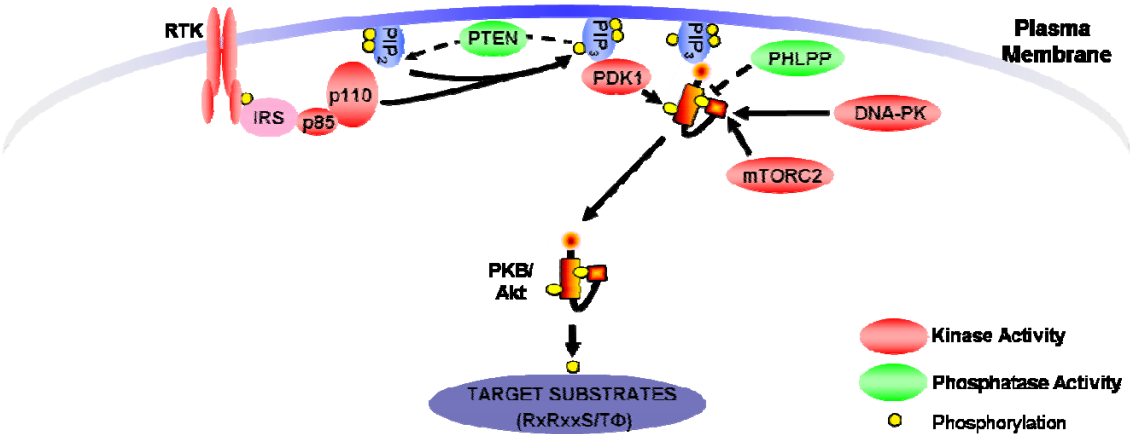

Figure 4. The PI3K Pathway And PKB/Akt Activation. RTK: Receptor Tyrosine Kinases; IRS: Insulin Receptor Substrate; PI3K: class I Phosphoinositide-3-Kinase (p85 subunit, p110 subunit); PIP: Phosphatidylinositol Lipids (PIP2: phosphatidylinositol-4,5-biphosphate, PIP3: phosphatidylinositol-3,4,5-triphosphate); PTEN: Phosphatase and Tensin Homolog; PDK1: 3-Phosphoinositide-Dependent Protein Kinase-1; mTORC2: mammalian target of rapamycin complex 2 (mTOR kinase, rictor, PRR5, mLST8, SIN1); DNA-PK: DNA-Dependant Protein Kinase; PHLPP: PH-domain leucine-rich repeat-containing protein phosphatases; PKB/Akt: Protein Kinase B/v-Akt Murine Thyoma Viral Oncogene; R: Arganine; x: Any Amino Acid; S: Serine; T:Threonine: Φ: Hydrophibic Amino Acid. *Adapted from Fayard et al., JCS 2005*

This pathway is conserved in lower organisms and is ubiquitous in mammalian cells, where it promotes cell growth, proliferation and survival, as well as mediating hormone metabolism, immune responses and angiogenesis (for a review, see Alessi, 2001; Brazil and Hemmings, 2001; Altomare and Testa, 2005; Manning and Cantley, 2007; Bozulic and Hemmings, 2009). Receptor tyrosine kinase stimulation activates PKB/Akt via a tightly controlled multi-step process (Fig. 1). Activated receptors stimulate class 1A PI3K directly or via adapter molecules such as the insulin receptor substrate (IRS) proteins. Class 1A PI3Ks bind via one of their five regulatory subunits (p85α, p85β, p55α, p55γ or p50α), which in turn binds to one of three catalytic subunits [p110α, p110β or p110δ (in leukocytes)], allowing conversion of phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P_2] lipids to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] at the plasma membrane. PKB/Akt binds to PtdIns(3,4,5)P_3 at the plasma membrane, where 3-phosphoinositide-dependent protein kinase 1 (PDK1) can then access the ‘activation loop’ of PKB/Akt to phosphorylate threonine 308 (Thr308), leading to partial PKB/Akt activation (Alessi et al., 1997). This PKB/Akt modification is sufficient to activate mTORC1 by directly phosphorylating and inactivating proline-rich Akt substrate of 40 kDa (PRAS40) and tuberous sclerosis protein 2 (TSC2). These phosphorylation events release the kinase mammalian target of rapamycin (mTOR) that is bound to PRAS40, prevent TSC2 GTPase activity and allow active, GTP-bound Rheb to activate mTORC1. mTORC1 substrates include the eukaryotic translation initiation factor, 4E, binding protein 1 (4EBP1) and the ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (S6K1), which phosphorylates the ribosomal protein S6 (S6; also known as RPS6), to promote protein synthesis and cellular proliferation.

Phosphorylation of PKB/Akt at Ser473 in the C-terminal hydrophobic motif, either by mTOR associated with the mTOR complex 2 (mTORC2) (Sarbassov et al., 2005) or by the DNA-dependent protein kinase (DNA-PK) (Feng et al., 2004) stimulates full PKB/Akt activity. Full activation of PKB/Akt leads to additional substrate-specific phosphorylation events in both the cytoplasm and nucleus, including inhibitory phosphorylation of the pro-apoptotic FOXO proteins. Dephosphorylation of Ser473 by the PH-domain leucine-rich repeat-containing protein phosphatases PHLPP1 and PHLPP2, and the conversion of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 by PTEN, antagonizes PKB/Akt signaling.

PKB/Akt signals to a plethora of substrate to mediate numerous cellular functions including angiogenesis, metabolism, cell growth, proliferation, protein synthesis, transcription and apoptosis (relevant functions are discussed in further detail in later sections) as illustrated in figure 5

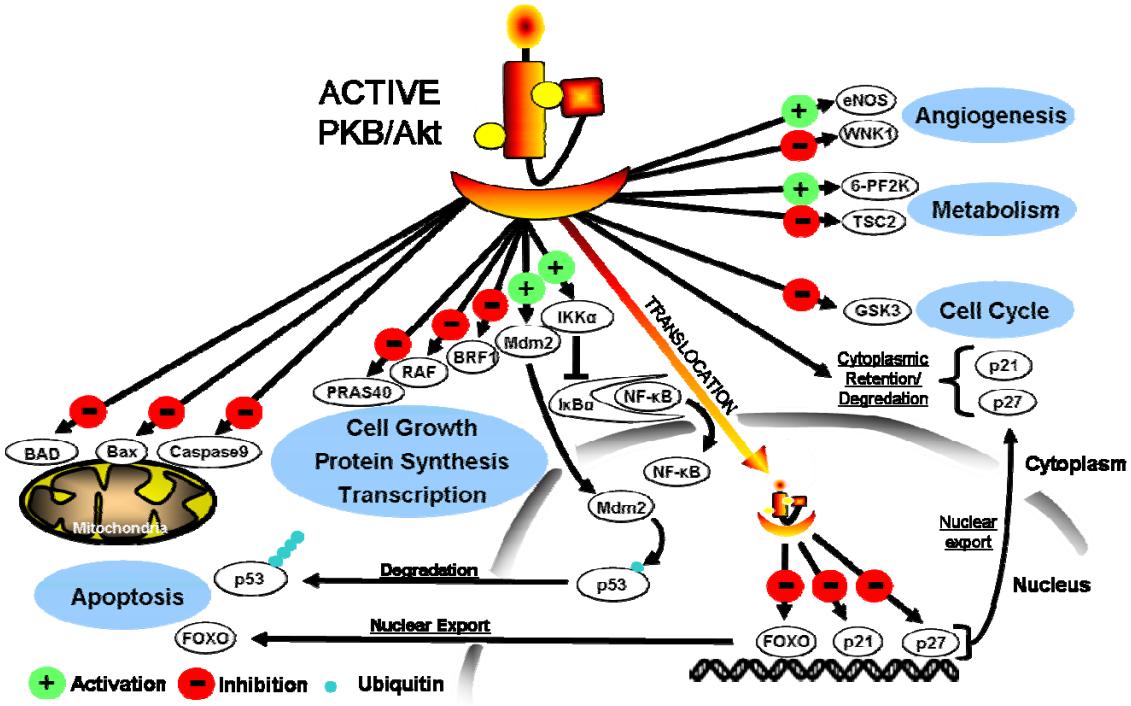

Figure 5. Regulation Of Physiological Functions By Activated PKB/Akt Through Inhibitory And Stimulatory Phosphorylation Of Downstream Target Substrates. Activated PKB/Akt mediated regulation of cellular functions including angiogenesis, metabolism, growth, protein synthesis, transcription and apoptosis via phosphorylation of downstream substrates to inhibit or activate these proteins. Substrates are grouped according to their function and the effect of PKB/Akt phosphorylation is indicated by (+) activation or (-) inhibition. BAD, Bcl-2 antagonist of cell death; BRF1, Butyrate response factor1; eNOS, endothelial cell nitric oxide synthase; FOXO1/3a/4, Forkhead Box O1/3a/4 (FOXO1/FKHR, FOXO3a/FKHRL1, and FOXO4/AFX); GSK3, glycogen synthase kinase; IKK, inhibitor kappa B kinase; Mdm2, mouse double minute 2; Myt1, membrane associated and tyrosine/threonine specific 1; NF-κB, nuclear factor-kappa B; PHLPP, PH domain and leucine rich repeat protein phosphatase; p53, tumour protein p53, 6-PF2K, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Plk1, Polo like kinase1; PP2A, protein phosphatase 2 subunit A; RAF, v-raf-1 murine leukemia viral oncogene homolog 1; TSC2; tuberous sclerosis complex protein 2; WNK1, WNK lysine deficient protein kinase 1. (Adapted from Fayard *et al*., 2005)

4. Genetically-modified PKB/Akt Mice: Providing Insights And Models For Probing PKB/Akt Isoform Specific Functions In Disease And Cancer

The generation of Isoform-specific knockout PKB/Akt mice has illustrated that these isoforms display both redundant and isoform specific functions (Table 1).

PKB/Akt Isoform(s)	Phenotype	References
PKB α/ Akt1	Small, neonatal lethality (~30% females), increased spontaneous apoptosis, placental defects and impaired adipogenesis	(Chen et al., 2001; Cho et al., 2001a; Yang et al., 2003)
PKB β/ Akt2	Diabetes-like phenotype, insulin resistance, fasting hyperglycemia, glucose intolerant, age-dependent loss of adipose tissue	(Cho et al., 2001b; Garofalo et al., 2003)
PKB γ/ Akt3	Reduced brain size with decreased cell size and number	(Easton et al., 2005; Tschopp et al., 2005)
PKB $\alpha\beta$/ Akt1/2	Death following birth with small size, skin development defects, loss of skeletal muscle, impaired skin and bone development, defective adipogenesis	(Peng et al., 2003)
PKB $\alpha\gamma$/ Akt1/3	Embryonic lethal at day 12 with severe growth retardation and developmental defects in cardiovascular and nervous systems.	(Yang et al., 2005)
PKB $\beta\gamma$/ Akt2/3	Viable but with ~25% reduction in size, severely reduced brain and testis and impaired glucose homeostasis.	(Dummler et al., 2006)
PKB $\alpha^{+/-}\beta\gamma$/ Akt1$^{+/-}$/2/3	Viable but with ~40% reduction in size.	(Dummler et al., 2006)

Table 1. PKB/Akt Knockout Mouse Models And Phenotypes. (Chen et al., 2001; Cho et al., 2001; Garofalo et al., 2003; Peng et al., 2003; Yang et al., 2003; Easton et al., 2005; Tschopp et al., 2005; Yang et al., 2005; Baudry et al., 2006; Dummler et al., 2006)

All PKB/Akt isoforms knockout mice are viable illustrating that no single isoform is essential for viability. PKBα/Akt1 mice, whilst viable, do display ~30% perinatal lethality, in addition to growth retardation and increased spontaneous apoptosis, strongly implicating PKBα/Akt1 as the major isoform in growth and survival. This is supported by the generation of tissue-specific mice with increased PKBα/Akt1 activity that exhibit increased cell number and cell size (Bernal-Mizrachi et al., 2001; Chen et al., 2001; Cho et al., 2001; Malstrom et al., 2001; Tuttle et al., 2001; Condorelli et al., 2002; Matsui et al., 2002; Shioi et al., 2002; Yang et al., 2003). PKBβ/Akt2 mice are viable and of normal size, however they progressively develop a diabetes-like syndrome characterized by insulin resistance and hyperglycemia. This illustrates a crucial role of the

PKBβ/Akt2 isoform in transducing signals regulating organism metabolism. PKBγ/Akt3 KO mice are viable and normal except for decreased size of the brain and testis that are the major sites of PKBγ/Akt3 expression. This indicates this isoform may have more specialized or subtle functions in normal development and physiology. This is also supported by compound knockouts that lack PKBγ/Akt3. PKBα/Akt1-PKBγ/Akt3 double knockout mice die at embryonic day 12 (E12) with severe growth retardation and developmental defects, whilst PKBβ/Akt2-PKBγ/Akt3 mice are viable but with a reduction in size and an enhancement of the single isoform knockout phenotypes. Together this suggests the PKBγ/Akt3 isoform does contribute to normal functions primarily mediated by both PKBα/Akt1 and PKBβ/Akt2. Similarly, loss of PKBβ/Akt2 on the background of PKBα/Akt1 deletion enhances the phenotype of PKBα/Akt1, resulting in 100% perinatal lethality and additionally leading to developmental defects in bone and skin. These mouse models highlight that all three PKB/Akt isoforms contribute, albeit to different degrees, to control cellular metabolism, growth, proliferation and survival in tissues throughout the organism. Furthermore, they illustrate that in regulating these functions the PKB/Akt isoforms also maintain whole organism metabolism and growth, with knockout of various isoforms in the whole organism or in specific-organs leading to defects in organism metabolism and growth.

Aberrant PKB/Akt activation is frequently observed in human disease, particularly in metabolic syndromes displaying insulin resistance, like diabetes, as well as in cancer. In diabetes, decreased insulin receptor phosphorylation and tyrosine kinase activity, reduced levels of active intermediates in the insulin signaling pathway, and impairment of GLUT4 translocation have all been illustrated that are consistent with the observed development of insulin resistance and a diabetes-like phenotype in the PKBβ/Akt2 mice (Caro et al., 1987; Olefsky and Nolan, 1995; Petersen and Shulman, 2006). Familial tumour syndromes, like those caused by mutation of PTEN or TSC1/2 directly disrupt PKB/Akt activation or downstream signaling. The presentation of the corresponding human syndrome also has numerous similarities in the mouse phenotypes (Table 2), indicating the suitability of these mice as models to gain insights into these diseases.

Mutated Protein	Human Syndrome	Human Presentation of Syndrome	Mouse Phenotype Upon Gene Deletion
PTEN	Cowden Disease, Bannayan-Riley-Ruvalcaba Syndrome, Proteus Syndrome, Proteus-like Syndrome	Breast, thyroid and uterine neoplasia, lipomas, macrocephaly, hamartomatous polyps of the gastrointestinal tract, mucocutaneous lesions	Homozygous lethal. Conditional deletion in tissues generally results in tumors. Heterozygote develop a range of neoplasms (adrenal, thyroid, uterine, breast, prostate, gastrointestinal tract)
TSC1/2	Tuberous Sclerosis	hamartomata and cysts in multiple organ systems, polycystic renal disease, renal carcinoma	Homozygote lethal. Heterozygote develop renal cystadenomas, liver hemangiomas, lung adenomas

Table 2. Mouse Phenotypes Of Common Human Familial Tumour Syndromes. All data taken from: Online Mendelian Inheritance in Man, OMIM (TM). McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), 2009. World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim/ and http://www.informatics.jax.org/ (Restuccia and Hemmings, 2010)

In addition to the familial syndromes, spontaneous tumours from various tissues displaying hyperactivated PKB/Akt signaling is well documented, particularly as a consequence of loss of PKB/Akt regulation via mutation of Pten and suggests that specific isoforms of PKB/Akt can contribute to tumour formation dependant upon tumour type (Table 3).

Tumour Type	Hyperactive PKB/Akt (%)	Major PKB/ Akt Isoform	PTEN Abnormalities
Prostate Carcinoma	45–55	PKBα	Mutations, LOH
Thyroid Carcinoma	80–100	PKBα	Mutations
Non-Small Cell Lung Carcinoma	30-75	PKBα	Inactivation
Small-Cell Lung Carcinoma	60	PKBα	Inactivation
Acute Myeloid Leukemia	70	PKBα	Mutations
Breast Carcinoma	20–55	PKBαβ	LOH, Silencing
Endometrial Carcinoma	>35	PKBβ	Mutation, Silencing
Glioma	55	PKBβ	Mutation
Gastric Carcinoma	80	PKBβ	Aberrant Transcripts
Pancreatic Carcinoma	30–70	PKBβ	Amplification[1], Mutation, LOH
Ovarian Carcinoma	40–70	PKBβγ	Amplification[1], AI, Mutations
Malignant Melanoma[a, b]	43-67	PKBγ	Mutation, Silencing
Pheochromocytomas	80[3]	PKB α βγ?	LOH[2]

AI= allelic imbalance, LOH = Loss of Heterozygousity,
[a]Altomare *et al.* (2005); [b]Robertson (2005); Bellacosa *et al.* (2005), [c]Chang *et al* (2006), Hyun *et al* (2006); [1]Futreal *et al* (2004); [2]van Nederveen *et al* (2006); [3]Fassnacht *et al* (2005).Adapted from Altomare & Testa (2005) and Vivanco & Sawyers (2002).

Table 3. Incidence Of PKB/Akt Hyperactivation In Human Tumours, The Major Reported PKB/Akt Isoform Involved And Pten Abnormalities. [[a]Altomare *et al.* (2005); [b]Robertson (2005); Bellacosa *et al.* (2005), [c]Chang *et al* (2006), Hyun *et al* (2006); [1]Futreal *et al* (2004); [2]van Nederveen *et al* (2006); [3]Fassnacht *et al* (2005). Adapted from Altomare & Testa (2005) and Vivanco & Sawyers (2002)]

These observations from the clinic indicate that loss of Pten in mice would provide an excellent model to study both the contribution of PKB/Akt hyperactivation and isoform-specific roles of PKB/Akt in tumour development and progression. Based upon this premise, a number of mouse models have been generated that are heterozygous for Pten (Table 4).

Features	Pandolfi	Mak	Parsons	Wu
Deletion	Exons 4-5	Exons 3-5	Exon 5	Exon 5
Background	129/C57	129/CD1 or 129/C57	129/C57	129/BALB/c
Homozygotes die at:	<E7.5	E9.5	E6.5	E9.5
Tumors in heterozygotes				
-Earliest detection (months)	1.5	2	1.5	6
Tumor types and frequencies				
-Gastrointestinal hyperplasia	All?	All?	90% *(lymphoid)*	2% *(inflammation)*
-Lymphoid hyperplasia	100% female 83% male	88% *(T-cell lymphoma)*	100% female 45% male	33% female 20% male
-Adrenal medullary tumor	100%	23%	NR	None
-Endometrial hyperplasia	70%	80%	100%	9% *(45% hemangioma)*
-Breast	NR	49%	NR	37%
-Prostate	50%	44%	75%	90%
-Thyroid	60%	None	30%	None

Table 4. Pten Heterozygous Mouse Models And Phenotypes. *Adapted from (Freeman, Dan et al., 2006)*

These mice develop a broad spectrum of tumours, albeit with varying severity based upon differences in genetic background. Importantly, these tumours show similar signaling and pathology to tumours from the clinic, indicating they do recapitulate crucial elements of hyperactivation of PKB/Akt in the human setting. This indicates these models provide a valuable tool for understanding the contribution of hyperactivated PKB/Akt signaling and the contribution of the PKB/Akt isoforms to this process.

7. Scope of this thesis

The general aim of this thesis was to identify PKBβ/Akt2 isoform specific contributions to pathological settings by the utilization and analysis of genetically-modified mouse models.

This thesis sought to examine this by focusing on two main areas:

(1) metabolic dysfunction and

(2) tumourigenesis.

(1) As loss of PKBβ/Akt2 functions results in insulin resistance through its effects on classical insulin-responsive tissues involved in glucose homeostasis, a particular goal was to determine if PKBβ/Akt2 contributed to other metabolic dysfunction through effects on non-classical insulin responsive tissues.

(2) Whilst PKBβ/Akt2 is deregulated in various human cancers, mouse models leading to hyperactivation of PKB/Akt indicate that in most tissues tumour formation is dependant upon the presence of PKBα/Akt1. Therefore, this section of the thesis aimed to clarify two poorly understood aspects of PKB/Akt tumourigensis. Firstly, if PKBβ/Akt2 is responsible for driving tumourigenesis in organs of mouse tumour models that are not significantly affected by PKBα/Akt1 loss. Secondly, if PKBβ/Akt2 contributes or is redundant to the tumour development and progression in organs where PKBα/Akt1 is know to drive tumour formation.

By identifying contributions of PKBβ/Akt2 to metabolic disorders and tumourigenesis, this thesis aims to provide both a basis and also stimulation for future studies to identifying downstream PKBβ/Akt2 specific targets that can be targeted without potential adverse consequences, like insulin resistance, that currently confounds efforts to target PKBβ/Akt2 dysfunction in human pathologies.

Loss of Protein Kinase B beta (PKBβ/Akt2) Predisposes Mice To Ovarian Cyst Formation And Increases The Severity Of Polycystic Ovary Formation *in vivo*

Introduction

1.1. Endocrine Function Controlling Reproduction Via The Hypothalamic-Pituitary-Gonadotrophin Axis

Endocrine functions in the human regulate whole body homeostasis by mediating communication between organs at distant sites in the body, including insulin signaling to regulate glucose homeostasis and gonadotrophin signaling to control reproductive function. Gonadotrophin signalling is controlled by endocrine signaling between the hypothalamus, the pituitary and the ovary in what is referred to as the hypothalamic-pituitary-ovarian (HPO) axis, as illustrated in Figure 1.1.

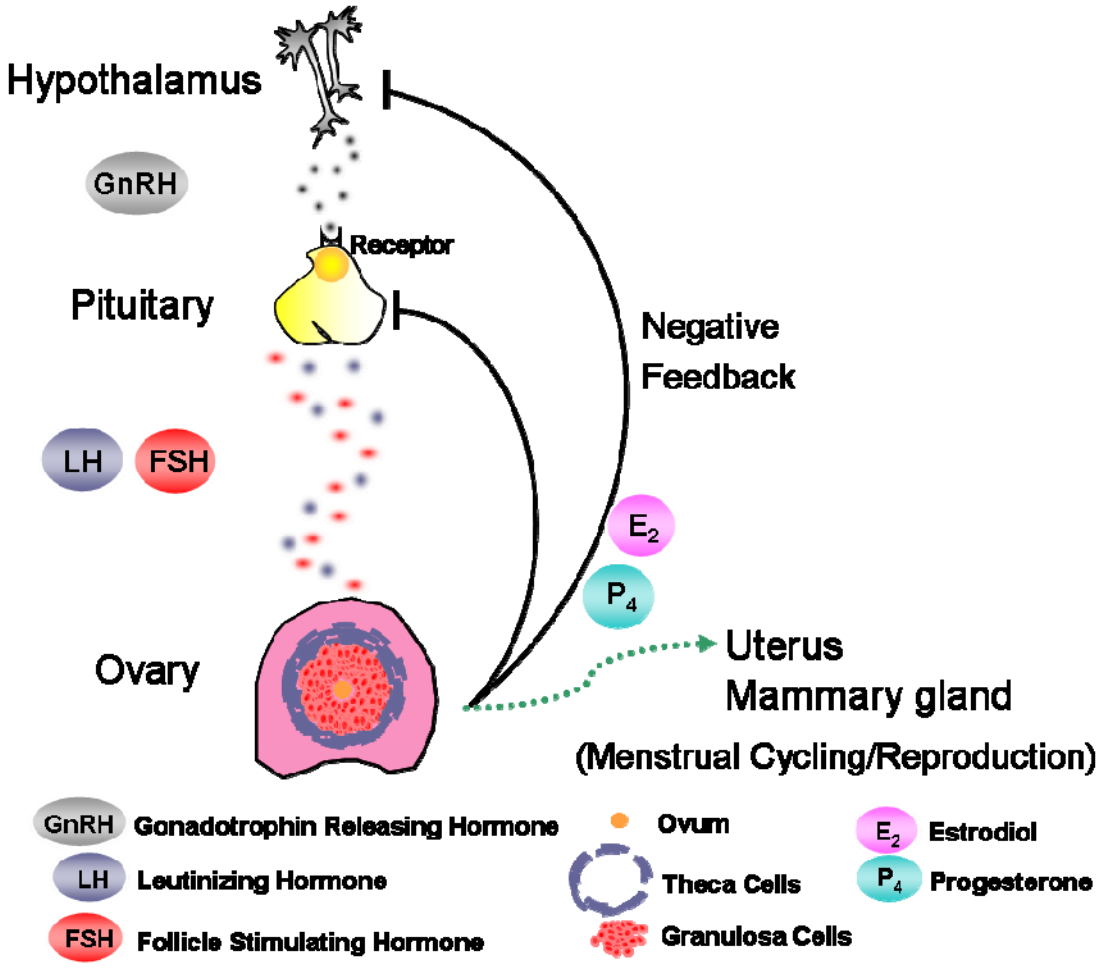

Figure 1.1. The Gonadotrophin-Pituitary-Ovarian Axis In Regulation of Reproductive Function.

Gonadotrophin signaling is triggered by the release of gonadotrophin releasing hormone (GnRH) from the hypothalamus in the brain. GnRH acts upon its

receptor in the pituitary to trigger release of leutinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH then act upon the ovary to stimulate ovarian steroidogenesis, resulting in the production of progesterone and estrogens that act on reproductive targets tissues. These actions support the implantation of successfully fertilized ovum into the uterus and its development into a fetus, or in the case of unsuccessful fertilization the degeneration of the uterine lining and reinitiation of the reproductive cycle. In either scenario, these hormones also signal back to the hypothalamus and pituitary, providing negative and positive cues to maintain appropriate release of GnRH, LH and FSH.

1.2. Ovarian Steroidogenesis

The ovary is the central organ for production of female sex steroids. The ovary is a complex organ that consists of a number of functionally distinct structures, including the follicle, the interstitium and the corpus luteum. Whilst functionally distinct, they must nevertheless communicate with each other to ensure their normal function and that of the ovary. This is regulated by the presence of multiple cell types, of which the granulosa cells and thecal cells are the most crucial to normal ovarian function. Figure 1.2 highlights the ovum (labeled Ov), granulosa cells (stained brown in i and labeled GC) and surrounding thecal cells (bounded by dashed lines and labeled TC).

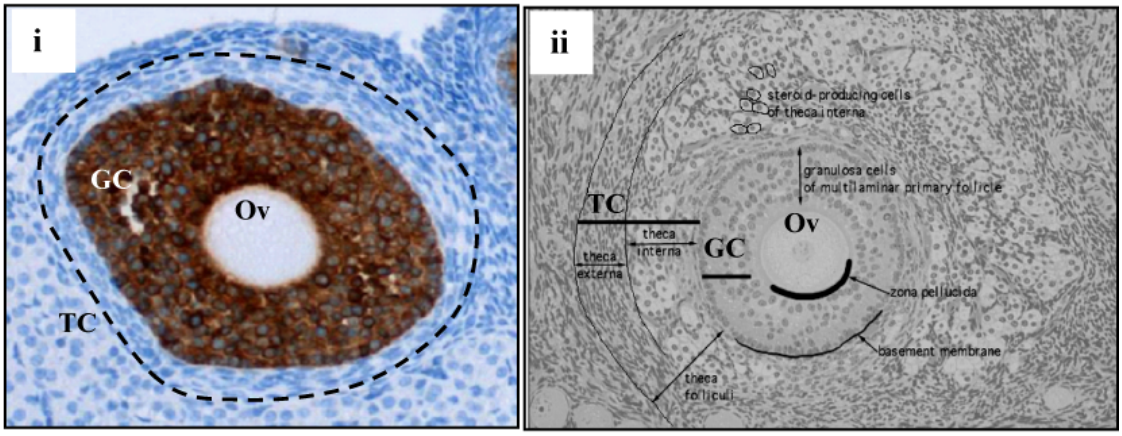

Figure 1.2. Ovarian Follicle Structure. The ovum, Ov (i,ii), is supported by the granulosa cells, GC (ii, i and stained brown in i), which are in turn supported by the surrounding thecal cells, TC (i,ii). *Figure ii taken from http://www.bu.edu/histology/p/14805loa.htm*

Steroidogenesis within the ovary is mediated by the thecal and granulosa cells and directly affects ovum survival, menstruation and fertility, as illustrated in Figure 1.3.

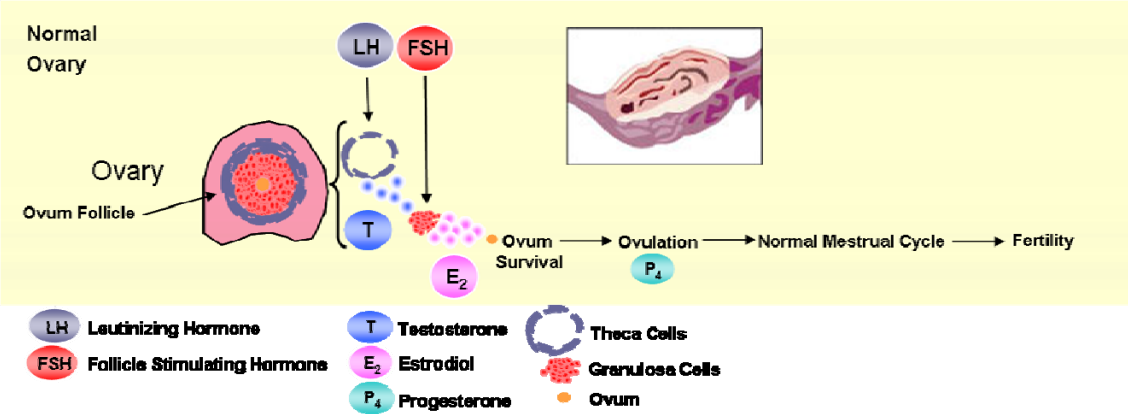

Figure 1.3. Role Of LH And FSH On Thecal And Granulosa Cell Function And Control Of Normal Menstruation And Fertility.

Thecal and granulosa cells are activated respectively by pituitary-released LH and follicle-stimulating hormone (FSH) to allow conversion of cholesterol to the hormones progesterone, testosterone and estrogen. LH stimulation of thecal LH receptors (LHRs) triggers conversion of cholesterol to progesterone, which can be further converted to testosterone. Testosterone then diffuses to neighboring granulosa cells where it is converted to estrogen by FSH-stimulated granulosa cells. In addition to their effects on distal target tissues, these ovarian hormones, along with other factors released by the thecal and follicular cells, provide positive and negative feedback to the pituitary to control the pro-steroidogenic stimuli mediated by pituitary LH and FSH. This tightly regulated HPO axis ensures appropriate temporal and quantitative release of LH and FSH that in turn dictates production of progesterone, testosterone and estrogen to maintain normal menstrual cycling.

1.3. Thecal Cell Signaling In Androgen Production

Stimulation of the thecal G-protein coupled LHR activates the canonical steroidogenic pathway via stimulatory G-protein alpha subunit ($G_{\alpha s}$) - adenylyl-3',5'-cyclic adenosine monophosphate cyclase (cAMP) - protein kinase A (PKA). This pathway triggers both a rapid, acute steroidogenesis response, by

indirectly stimulating mitogen-activated protein kinase/extracellular-signal-regulated kinase 1/2 (ERK) activation to phosphorylate and activate the steroid acute regulatory protein (StAR) (Arakane et al., 1997), and a chronic steroidogenesis response by direct activation of the cAMP response element-binding protein (CREB) transcription factor that initiates expression of steroidogenic proteins to facilitate *de novo* protein synthesis required for sustained steroid production (Johnson and Sen, 1989; Tremblay et al., 2002; Towns et al., 2005; Towns and Menon, 2005). The transcription of proteins involved in steroidogenesis initiated in the chronic response is crucial, as steroids are not stored within the cell and without *de novo* protein synthesis the cell would rapidly exhaust the substrates required for steroid production. Transcription of proteins regulating the chronic response includes proteins involved in uptake of cholesterol into the cell, like the low-density lipoprotein receptor (LDLR), the crucial rate-limiting StAR protein that mediates transport of the cholesterol into the mitochondria (Tremblay et al., 2002), and hydroxylase/reductase enzymes like P450 cholesterol side-chain cleavage enzyme (CYP11A) within the mitochondria or 17α-hydroxylase/17,20-lyase cytochrome P450 A (CYP17A) in the endoplasmic reticulum, both involved in processing cholesterol to bioactive hormone products like testosterone. In addition, but less understood, is the role of the non-classical cAMP-independent signaling downstream of LHR, which involves amongst others, protein kinase C (PKC) and PI3K-PKB/Akt signaling (Figure 1.4).

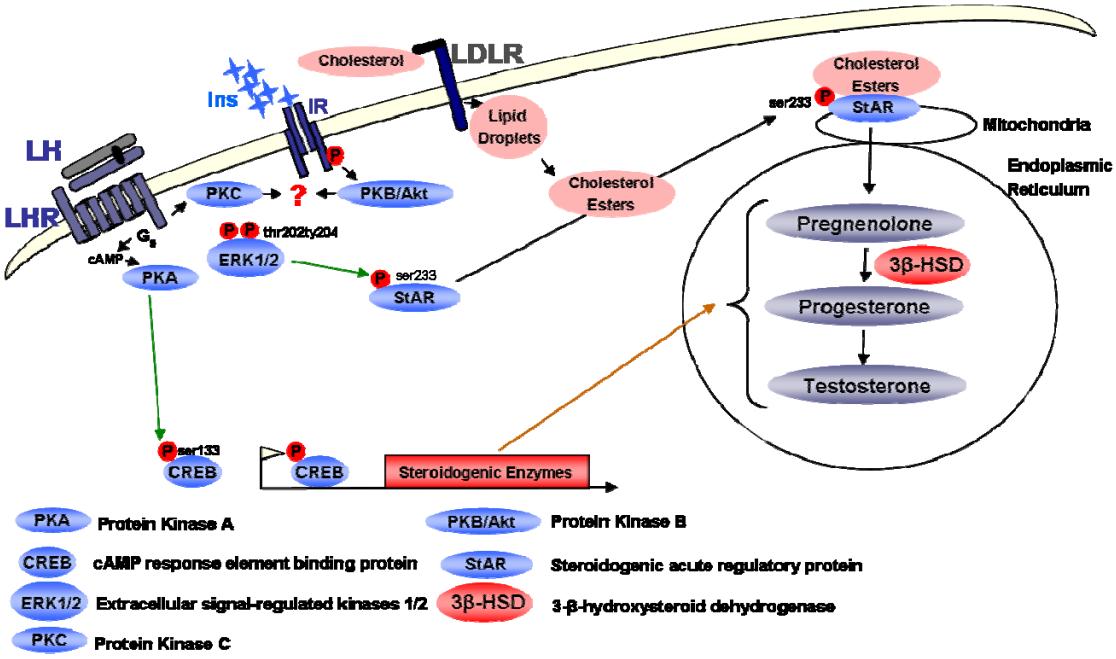

Figure 1.4. Thecal Cell Signaling Pathways In The Production Of Ovarian Androgens.

1.4. Deregulation Of The Hypothalamic-Pituitary-Ovarian Axis And Ovarian Hormone Production In Ovarian Cyst Development And PCOS

Disruption of the HPO system can lead to ovarian and uterine abnormalities including formation of fibroids, tumours and cysts. Ovarian cysts affect women of all ages and are the most common female reproductive abnormality, ensuring ovarian cysts contribute a significant social and economic burden in their management [for review see (Goodarzi et al., 2011)]. Cysts can be divided into large simple cysts and polycystic ovarian syndrome (PCOS). Simple cysts have been poorly studied as they often result in only minor discomfort, can resolve without treatment and are generally slow growing and benign. Large simple cysts are most commonly detected in an older population and as these women are often no longer concerned with maintaining fertility, uni- or bi-lateral oophorectomy surgery (removal of the ovary) is regularly performed. Conversely, PCOS, which is characterized by the formation of multiple small cysts in the ovary is observed from puberty, can result in infertility and affects 5-10% of women of reproductive age. PCOS can be resolved in some cases by lifestyle changes and weight loss, although in other cases treatments range from insulin-sensitizing drugs to hormone supplementation, whilst in particularly

refractory cases as well as when the affected individual is attempting to get pregnant, ovarian wedge resection or in vitro fertilization can be required. PCOS is due to deregulation of LH signaling which can occur at various levels of the HPO axis to stimulate hyperproduction of androgens. Two major means of deregulation are hyperstimulation of the pituitary by the hypothalamus leading to increased LH release and by far the most common form, hypersensitivity of the ovarian thecal cells to LH. In both cases the common feature is abnormal LHR signaling within the LH-responsive ovarian thecal cells, resulting in an increased steroidogenic response and androgen production. As a consequence, granulosa cell and ovum survival is compromised, leading to anovulation, initiation of anovulatory cycling and subsequent infertility, as illustrated in Figure 1.5.

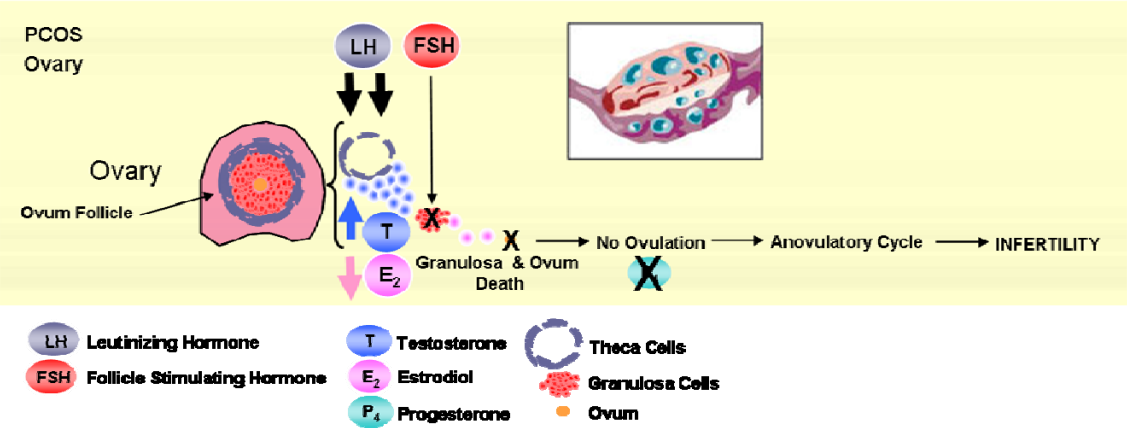

Figure 1.5. Disruption Of The HPO Axis In PCOS And Infertility.

1.5. PKB/Akt In Steroidogenic Signaling

Deregulation of the canonical thecal steroidogenic pathways is central to cyst development, with genetic manipulation of LHR signaling in mice resulting in ovarian cyst development and the hallmark of increased testosterone production. These aspects are observed both in PCOS mice models and patients whom display abnormalities at various steps in thecal steroidogenesis. However, a contribution of the non-classical cAMP-independent signaling in PCOS is unquestionable, as defects in these signaling pathways are highly prevalent in PCOS patients. This is particularly relevant for insulin receptor (InsR) signaling that normally activates PI3K-PKB/Akt signaling, as this is defective in the 50-70% of PCOS patients whom display insulin resistance (IR).

Additionally, up to 60% of PCOS patients are obese, which is the most common factor leading to IR and can result in decreased InsR expression and post-receptor dysfunction in downstream kinase activation. Furthermore, defects in InsR phosphorylation (Dunaif et al., 1995) and genetic lesions in this pathway, including InsR, PKBβ/Akt2 and the PKB/Akt substrate glycogen synthase kinase beta (GSK3β) are associated with PCOS patients (George et al., 2004; Tan et al., 2007; Goodarzi et al., 2008; Mukherjee et al., 2009). This indicates that PKB/Akt and particularly, PKBβ/Akt2, may contribute to development of PCOS and PKBβ/Akt2 KO mice could therefore provide a means to explore this question in a physiological setting.

Results

2.1. Ablation Of The PKBβ/Akt2 Isoform Specifically Leads To Development Of Severe Ovarian Cyst In Aged Mice

We observed aged female mice presenting with distended abdomens and upon examination noted mice lacking PKBβ/Akt2 but not wild type (WT) mice developed severe ovarian cysts (Figure1.6a, b).

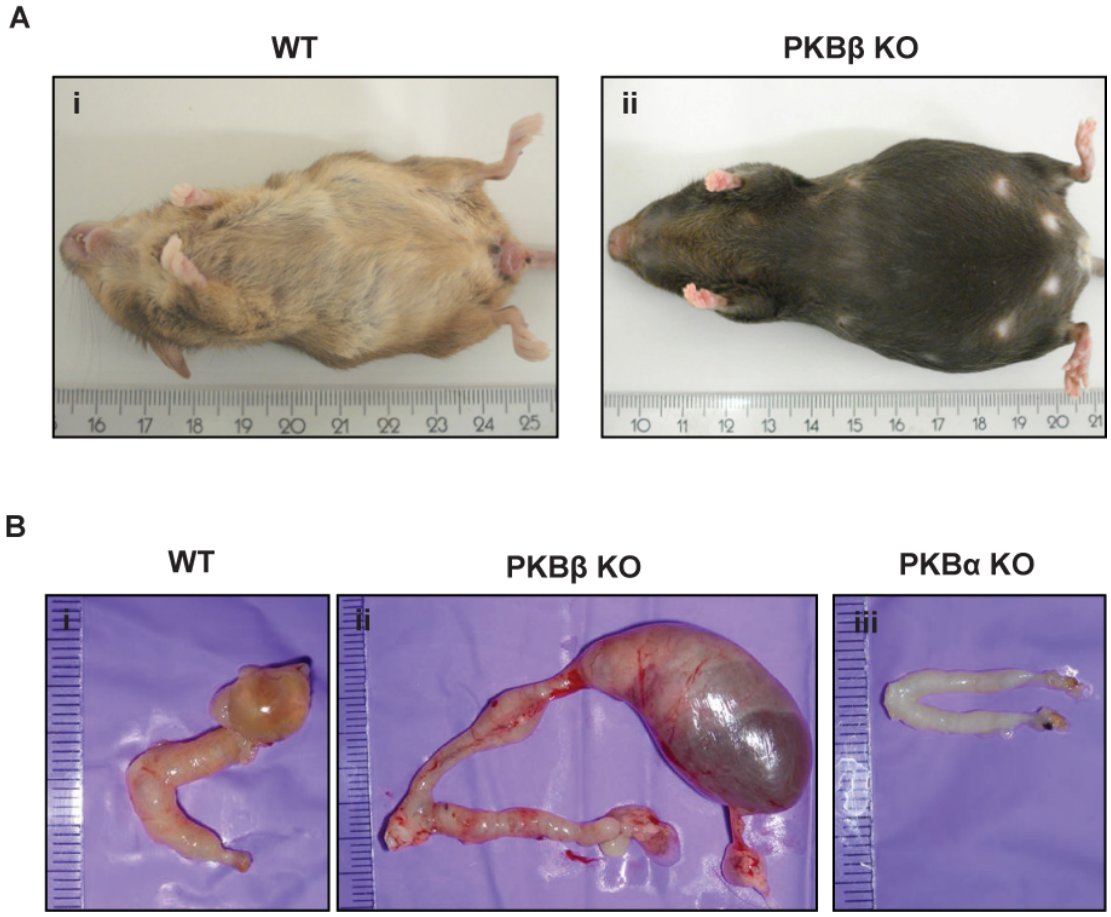

Figure 1.6. Specific Loss Of PKBβ/Akt2 In Aged Mice Results In Development Of Severe Ovarian Cysts. (A) WT (i) and PKBβ/Akt2 KO (ii) mice present with distended abdomens between 91 and 120 weeks of age. (B) Cystic ovaries isolated from WT (i) and PKBα/Akt1 KO (iii) mice fail to show atresia or small ovarian cyst formation, whilst PKBβ/Akt2 KO mice show severe ovarian cyst formation.

Further examination of PKBβ/Akt2 KO mice revealed cysts development in almost 80% of mice, which generally contained serous fluid with a predominantly right-side involvement, although bi-lateral presentation was also common (Table 1.1).

Mouse Line	PKB α/Akt1		PKB β/Akt2	
	WT	KO	WT	KO
No. Of Animals	6	14	15	18
No. Of Animals With Ovarian Cysts	2 (33%)	7 (50%)	7 (47%)	14 (78%)
-Unilateral/Bilateral	2/0	5/2	5/2	7/7
-Maximum Diameter (mm)	5	8	8	40
-Average Diameter (mm)	3.5	4.5	4.3	9.6
-Earliest Detection (wks)	98	91	91	91
Mean Age Of Animals (wks)	~112	~108	~108	~106
Age Range of Animals	98-121	91-128	91-128	91-134

Table 1.1. Overview Of Ovarian Cyst Incidence And Characteristics From Aged Female PKBβ/Akt2 KO And PKBα/Akt1 KO Mice. Analysis of ovarian cyst development in WT, PKBβ/Akt2 KO and PKBα/Akt1 KO mice aged between 90 and 120+ weeks from PKBβ/Akt2 KO and PKBα/Akt1 KO mouse colonies.

Cysts were either absent in WT mice or generally restricted to a small follicular cysts with a uni-lateral involvement (Figure 1.6b). Larger cyst size was also observed in PKBβ/Akt2 KO mice at older ages, suggesting an increase in size with age. To determine if this was due to a reduction of total PKB/Akt levels, aged mice lacking the other major PKB/Akt isoform found in the ovary, PKBα/Akt1, were examined. Ovaries from PKBα/Akt1 mice were similar to WT mice with a small size and predominantly uni-lateral presentation (Figure1.6b and table 1.1), indicating that the severe cyst development was due to specific loss of the PKBβ/Akt2 isoform.

2.2. Ovarian Cysts In Aged Mice Are Characterized By Thecal-Interstitial Hyperplasia

To understand what abnormalities within the ovaries may be driving cyst development, haematoxylin and eosin (H&E) and immunohisotochemistry (IHC) staining was performed on cysts isolated from PKBβ/Akt2 KO mice. H&E staining illustrated that ovaries lacked corpus luteum structures and granulosa cells, indicating cessation of estrous cycling, whilst hyperplasia of spindle-like stromal cells were observed that increased with ovarian cyst size (Figure 1.7a and Supplemental Figure 1.1a,b). Positive staining for vimentin of cysts from

PKBβ/Akt2 KO mice indicated that the hyperplasic cells represented the thecal-interstitial cell population (Figure 1.7c). Granulosa cell staining against anti-mullerian inhibiting substance produced in granulosa cells (Supplemental Figure 1.1a, b) was negative, consistent with H&E staining, suggesting follicular exhaustion in these cysts.

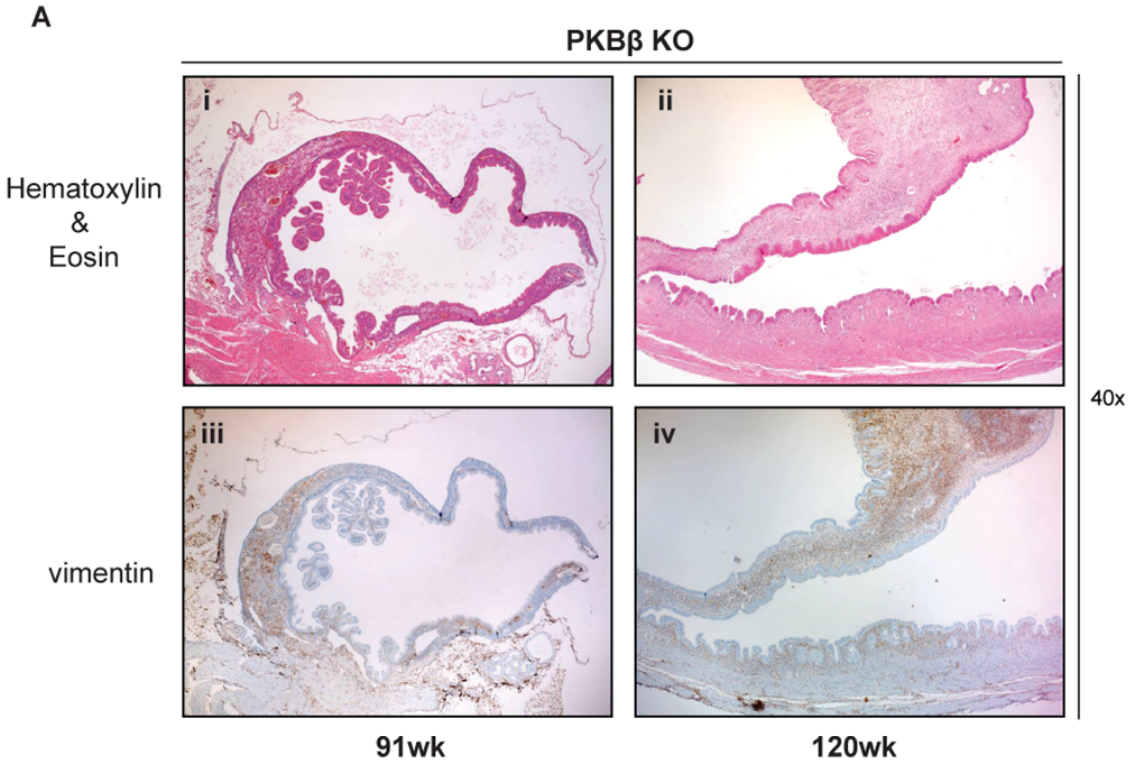

Figure 1.7. Ovarian Cysts In PKBβ/Akt2 KO Aged Mice Show An Increase In the Thecal-Intersitial Cell Populations. **(**A) Early 91wk old (i,iii) and late 120wk old (ii, iv) aged PKBβ/Akt2 KO mice show increasing stromal cells by heamatoxylin and eosin staining (i, ii) reflecting of increased thecal-interstitial hyperplasia, indicated by positive vimentin staining by immunohistochemistry (iii, iv). 40x magnification.

2.3. Ovarian Cysts In Aged Mice Show Increased Steroidogenic Capacity

The observation that cystic ovaries showed hyperplasia of LH-responsive thecal-interstitial cells responsible for ovarian steroidogenesis, coupled with an absence of follicular cells that are important in maintaining LH levels via negative feedback signaling on the pituitary, indicated the potential of active steroidogenic signaling by the thecal-interstitial cells in cystic ovaries. Crucial to steroidogenesis mediated by LHR activation is the phosphorylation of Ser133 of the CREB transcription factor and Thr202 and Tyr204 of the ERK kinase, that together mediate the transcription of enzymes and intracellular signaling

required for the cellular uptake of C-21 cholesterol and its enzymatic conversion to C-19 androgens. Cystic ovaries from PKBβ/Akt2 KO mice displayed activating phosphorylation of CREB and ERK, with strong ERK activation commonly observed in the cells adjacent to the cystic lumen (Figure 1.8a, ii, iii, v, vi).

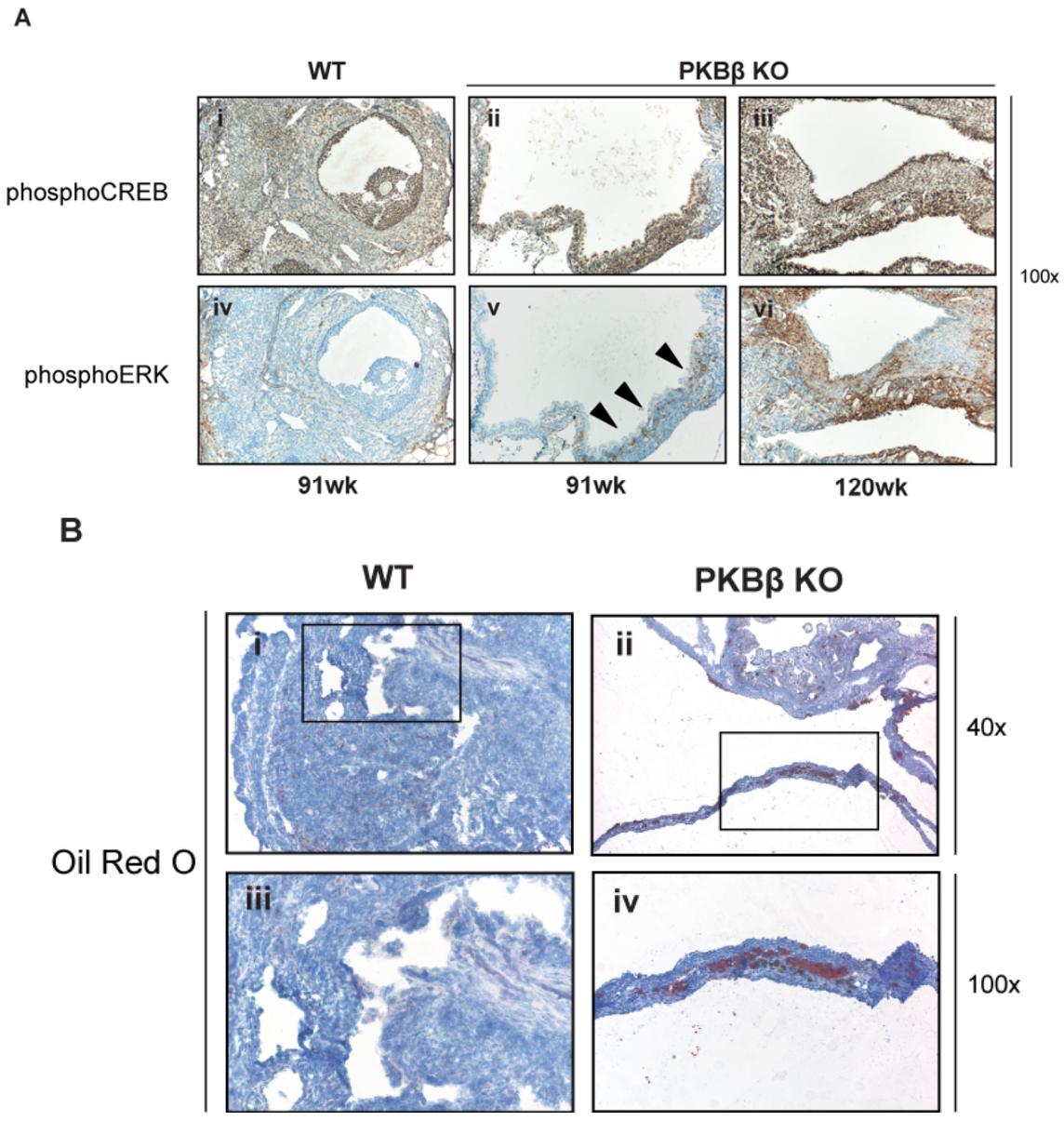

Figure 1.8. Aged PKBβ/Akt2 KO Ovarian Cysts Show Active Steroidogenic Signaling And Lipid Accumulation With Increased Circulating Testosterone Levels Compared To Wild-type Mice. (A) PKBβ/Akt2 KO ovarian cysts display both active CREB (i-iii) and ERK (iv-vi) signaling required for steroidogenesis. ERK is located at the cystic lumen (arrows) and increases with severity of cysts and age of mice (ii,iii & v,vi) but is absent in WT mice (i, iv). Magnification 100x. (B) PKBβ/Akt2 KO ovarian cysts (ii, iv) display increased lipid accumulation adjacent to the cystic lumen, required for conversion to steroids, which is absent in WT mice (i, iii). Magnification 40x and 100x.

In contrast, WT ovaries, whilst displaying CREB activation, were devoid of ERK activation that is essential for activation of the steroidogenic acute regulatory protein (StAR) that mediates transport of cholesterol to the mitochondria for enzymatic processing (Figure 1.8a, i, iii).

Consistent with the activation of ERK in PKBβ/Akt2 KO cysts, lipid staining was observed in cells surrounding the cystic lumen of PKBβ/Akt2 KO mice, but was absent in WT mice, indicating functional uptake of cholesterol for steroidogenesis in PKBβ/Akt2 KO mice (Figure 1.8b).

To determine if this was enzymatically processed to bioactive androgens, serum testosterone levels were measured in WT and PKBβ/Akt2 KO mice. Serum from WT mice showed generally low to negligible testosterone levels, however, aged PKBβ/Akt2 KO mice consistently showed increased testosterone levels with on average an approximate two-fold increase in serum testosterone levels (Figure 1.8c), indicating the hyperplastic thecal-interstitial cell population observed in ovarian cysts from PKBβ/Akt2 KO mice are steroidogenically active and producing testosterone.

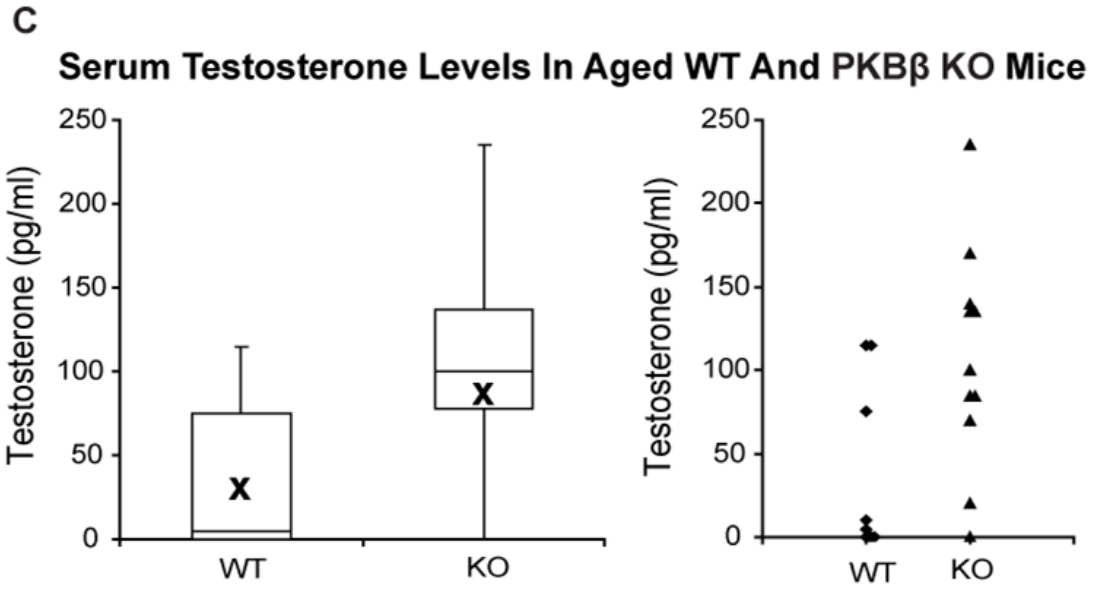

Figure 1.8. Aged PKBβ/Akt2 KO Ovarian Cysts Show Active Steroidogenic Signaling And Lipid Accumulation With Increased Circulating Testosterone Levels Compared To Wild-type Mice. (C) Consistent with increased active steroidogenesis, PKBβ/Akt2 KO mice show increased serum testosterone levels compared to wild type mice.

2.4. Young PKBβ/Akt2 Ablated Mice Display Normal Steroidogenic And Reproductive Function

As cysts from aged PKBβ/Akt2 KO mice displayed abnormal steroidogenesis, young PKBβ/Akt2 KO mice were examined to determine if steroidogenic or reproductive functions might be compromised by loss of PKBβ/Akt2 in these animals. Activation of steroidogenic signaling illustrated by phosphorylation of CREB and ERK showed no differences between WT and PKBβ/Akt2 KO ovaries (Figure 1.9A), with the thecal-interstitial population showing low to moderate activation of both proteins in contrast to that seen in aged cystic ovaries.

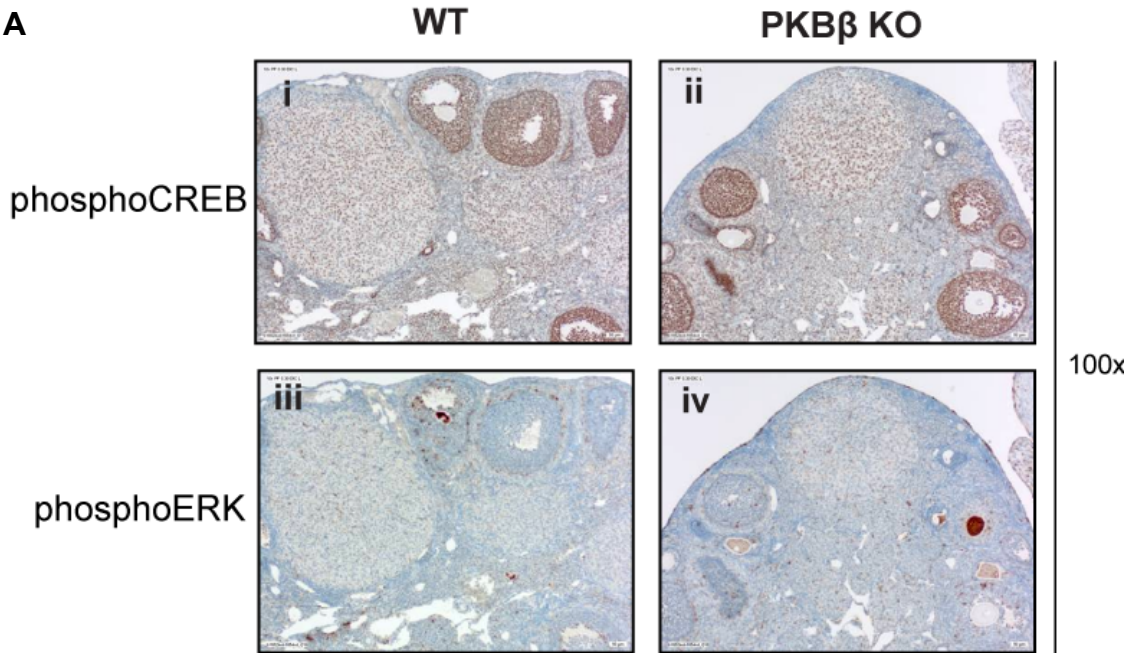

Figure 1.9. Analysis Of Young WT And PKBβ/Akt2 KO Mice Show Loss Of PKBβ/Akt2 Has No Significant Impact On Normal Ovarian Steroiodogenic Signaling Or Reproductive Function. (A) Steroidogenic signaling through CREB (i, ii) and ERK (iii, iv) is normal in ovaries of both WT (i, iii) and PKBβ/Akt2 KO (iii, iv) mice. (B) Circulating serum hormone levels of testosterone (i) and estradiol (ii) are similar in both WT and PKBβ/Akt2 KO animals, indicating steroidogenic production is unaffected by PKBβ/Akt2 loss under normal conditions.

Consistent with this, testosterone and estradiol serum levels were equivalent in both WT and PKBβ/Akt2 knockout animals (Figure 1.9B).

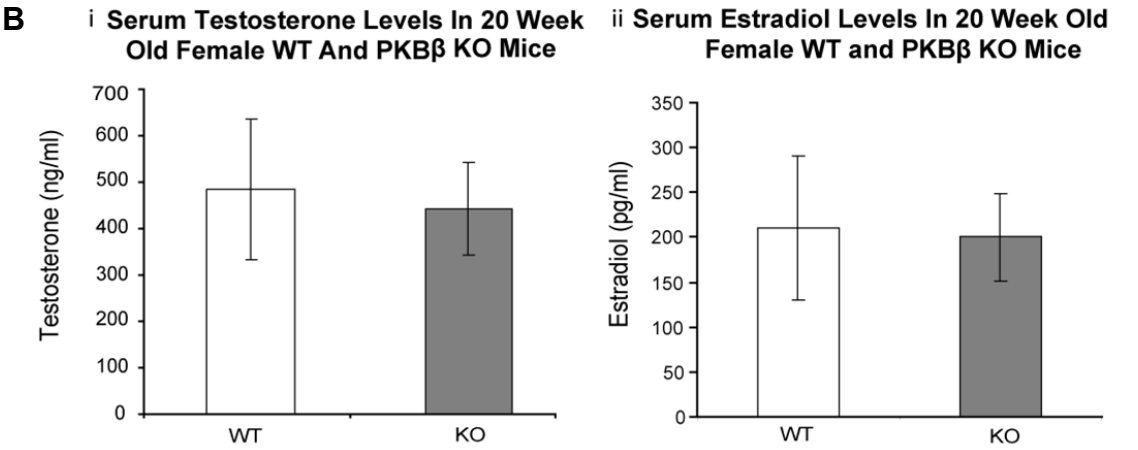

Figure 1.9. Analysis Of Young WT And PKBβ/Akt2 KO Mice Show Loss Of PKBβ/Akt2 Has No Significant Impact On Normal Ovarian Steroiodogenic Signaling Or Reproductive Function. (B) Circulating serum hormone levels of testosterone (i) and estradiol (ii) are similar in both WT and PKBβ/Akt2 KO animals, indicating steroidogenic production is unaffected by PKBβ/Akt2 loss under normal conditions.

Finally, reproductive function was assessed by analysis of litter sizes between WT and PKBβ/Akt2 KO animals and whilst a trend was seen toward a decrease in matings between PKBβ/Akt2 KO animals compared to WT, this was not statistically significant (Figure 1.9C).

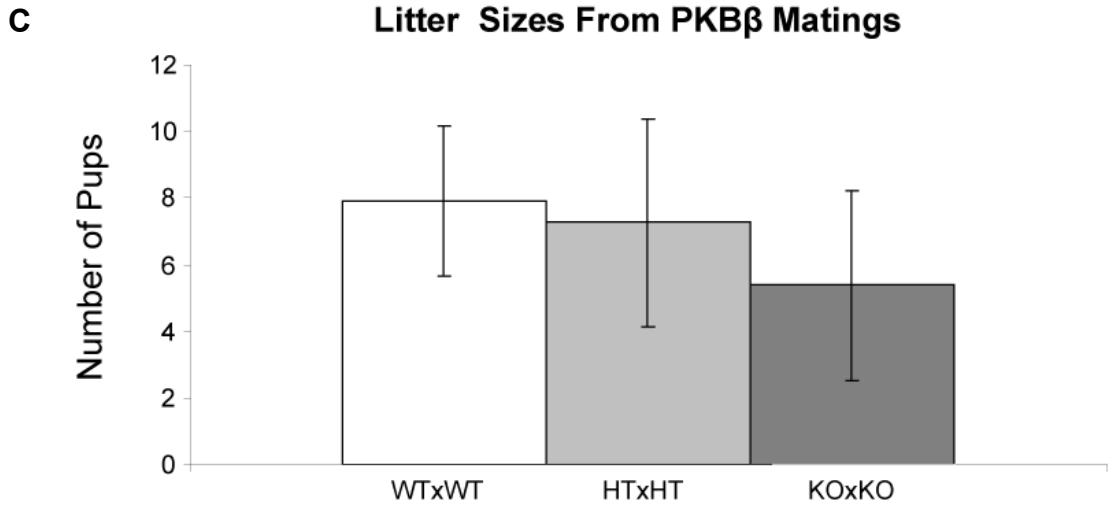

Figure 1.9. Analysis Of Young WT And PKBβ/Akt2 KO Mice Show Loss Of PKBβ/Akt2 Has No Significant Impact On Normal Ovarian Steroiodogenic Signaling Or Reproductive Function. (C) PKBβ/Akt2 KO mice are fertile and show similar litter sizes compared to matings from WT animals.

These findings indicate that compensatory mechanisms in the HPO axis in young PKBβ/Akt2 KO mice are sufficient to maintain normal ovarian function and suggest that co-operating dysfunction to imbalance these mechanisms may be required to unmask the effects of PKBβ/Akt2 loss in steroidogenesis.

2.5. Induction of PCOS Via Tonic LH Stimulation In PKBβ/Akt2 KO Mice Results In Increased Severity Of Polycystic Ovary Formation

As aged PKBβ/Akt2 KO ovaries showed cystic development and activation of androgenic steroidogenesis in thecal-interstitium, but ovaries from young mice failed to show any dysfunction, an experiment was designed to determine if a role for PKBβ/Akt2 in ovarian steroidogenesis could be revealed by inducing a state of hyperstimulated LH signaling. Since PCOS exhibits cystic development and increased testosterone synthesis, an *in vivo* model of PCOS induction by tonic stimulation with LH was chosen to determine if PKBβ/Akt2 loss could contribute to PCOS pathology. This model uses tonic LH stimulation which mimics the PCOS setting and exhibits features of PCOS pathology including increased steroidogenic signaling and testosterone production, resulting in cyst formation. Additionally, to counter possible effects of compensation by a potential increase in negative feedback to the pituitary, LH stimulation was also administered in the presence of a gonadotrophin releasing hormone antagonist (GnRHAnt). Consistent with previous reports, administration of LH with or without GnRHAnt led to development of hemorrhagic follicular cysts, whilst treatment with either vehicle or GnRHAnt alone did not result in cyst development (Figure 1.10A). On a background of PKBβ/Akt2 loss, whilst cyst development in ovaries was also unaffected by vehicle or GnRHAnt treatment alone, treatment with LH alone or with GnRHAnt led to an approximate three-fold increase in cystic area in the ovaries (Figure 1.10A).

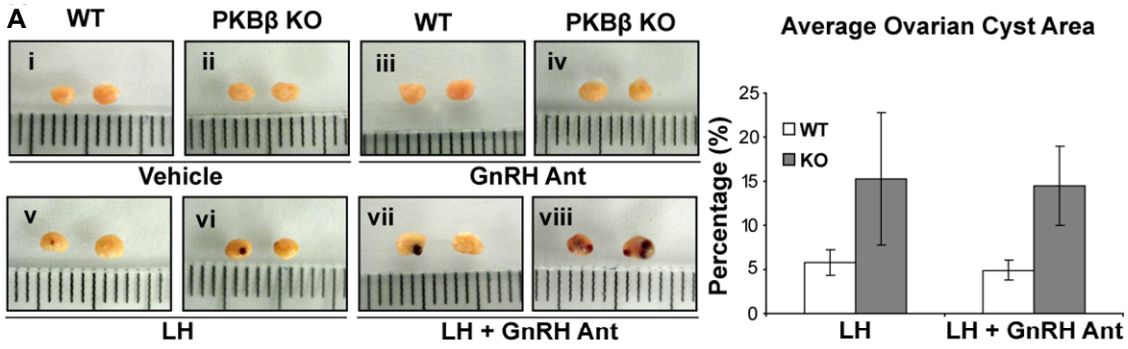

Figure 1.10. Induction Of PCOS Via Tonic LH Administration Results In An Increased Severity Of Ovarian Cysts In PKBβ/Akt2 KO Ovaries, With Formation Of Cysts Associated With ERK Activation And Lipid Accumulation In Steroidogenically Active Ovaries. (A) PKBβ/Akt2 KO ovaries showed an approximately three-fold increase in ovarian cyst area in LH treated ovaries (vi, viii) compared to WT (v, vii), independent of administration of a gonadotrophin releasing hormone antagonist. Treatment of WT and PKBβ/Akt2 KO mice with vehicle (i,ii) or gonadotrophin releasing hormone antagonist (iii,iv) alone had no effect on cyst formation.

Analysis of CREB phosphorylation showed that it was activated to support steroidogenesis in the ovaries of both WT and PKBβ/Akt2 KO mice (Figure 1.10B, i-iv), whilst activated ERK was strongly expressed in the thecal cells adjacent to the cystic lumen (Figure 1.10B, v-viii), reminiscent of that seen in the cysts of aged PKBβ/Akt2 KO mice. Strong ERK activation was more commonly seen surrounding cysts in PKBβ/Akt2 KO ovaries and interestingly, unlike in WT ovaries, strong ERK activation was also seen in thecal cells surrounding large follicles (Figure 1.10B, vi, viii, arrows), suggesting this may support increased steroidogenesis, follicular degeneration and cyst development leading to the increase in ovarian cyst area observed in PKBβ/Akt2 null ovaries.

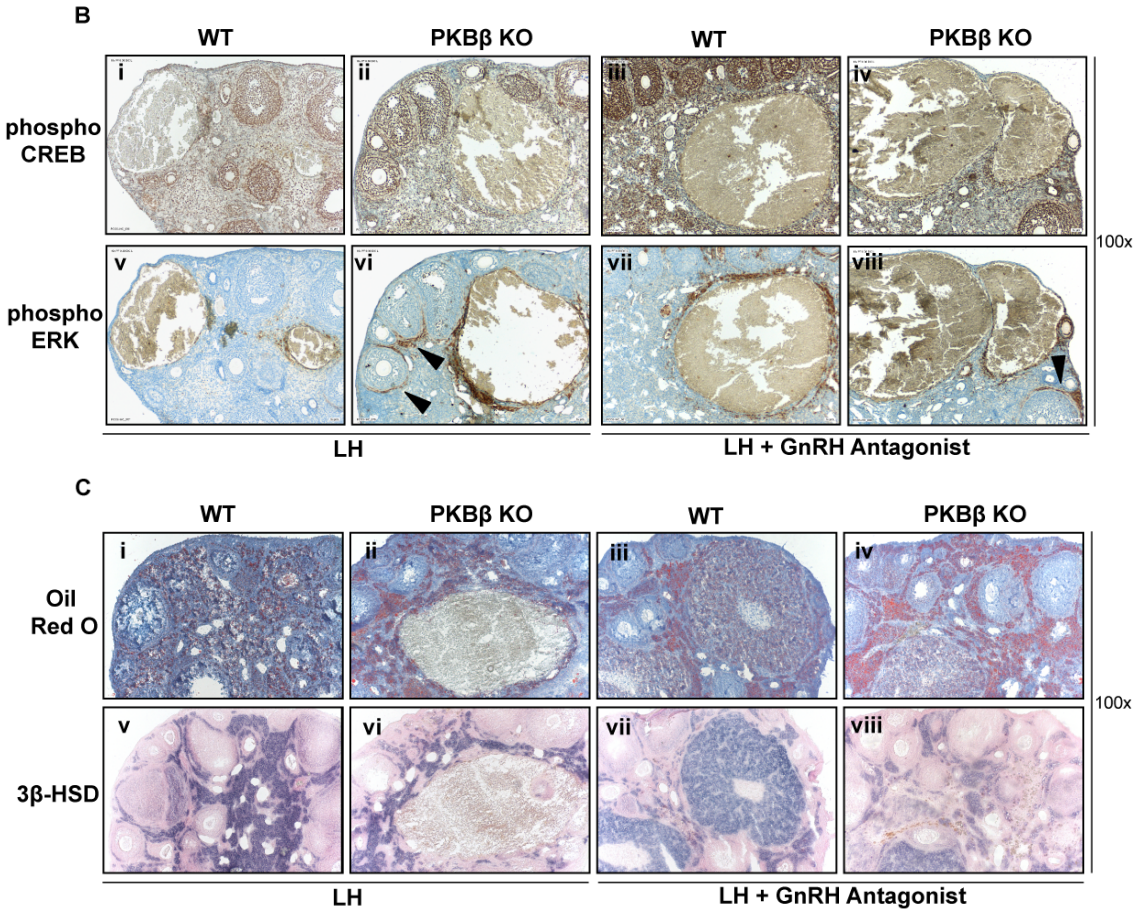

Figure 1.10. Induction Of PCOS Via Tonic LH Administration Results In An Increased Severity Of Ovarian Cysts In PKBβ/Akt2 KO Ovaries, With Formation Of Cysts Associated With ERK Activation And Lipid Accumulation In Steroidogenically Active Ovaries. (B) Steroidogenic signaling was active and seen in both ovaries from WT (i, iii, v, vii) and PKBβ/Akt2 KO (ii, iv, vi, viii) treated with LH. ERK however was also observed to be strongly active with increased theca thickness surrounding large follicles predominantly in PKBβ/Akt2 KO ovaries (arrows). (C) Increased lipid accumulation in ovaries treated with LH was also observed in PKBβ/Akt2 KO mice (ii, iv) in areas with active androgen steroidogenesis

[indicated by staining for 3β-HSD activity (v-viii)] compared to WT (i, iii). All magnifications for IHC are 100x.

Consistent with the observed CREB and ERK activation, cystic ovaries illustrated active steroidogenesis via 3β-HSD staining in the thecal-interstitial population of cystic ovaries (Figure 1.10C, v-viii) and increased lipid accumulation in this steroidogenic population in the PKBβ/Akt2 KO ovaries compared to WT animals (Figure 1.10C, i-iv), indicating PKBβ/Akt2 KO mice have increased cholesterol uptake that could also support androgen production in an environment of ERK activation.

Discussion

The PKB/Akt kinases have roles in diverse physiological functions and have been shown to play important roles in the actions of various hormones. However, whilst stimulation of PKB/Akt activity in the thecal-interstitium has been shown upon LH stimulation, the contribution of PKB/Akt isoforms to ovarian androgen production is undefined. This study provides *in vivo* evidence for an isoform specific role for the PKBβ/Akt2 isoform in thecal steroidogenesis and illustrates that loss of function of PKBβ/Akt2 in the presence of active thecal androgen steroidogenesis can support ovarian cyst formation and could contribute to PCOS pathology.

The findings of this study, show that specific loss of the PKBβ/Akt2 isoform in aged mice increases the incidence of cyst development and severity in both ovarian size and bilateral involvement. Ovarian cyst development has been linked in numerous studies to increased LHR signaling and subsequent testosterone biosynthesis in the thecal-interstitium compartment of the ovary. Postmenopausal women display follicular exhaustion that results in decreased conversion of testosterone to estrogen with increased LH and FSH as a consequence of loss of negative feedback upon the pituitary (Choi et al., 2007). Involvement of LH/FSH receptor deregulation in supporting cyst development has been illustrated in mouse models disrupting these sex hormones, with mice overexpressing LH displaying bilateral ovarian involvement, thecal hyperplasia, increased testosterone levels and cyst development, whilst LH or FSHR knockout mice also display cyst development (Danilovich and Ram Sairam, 2006; Huhtaniemi et al., 2006). This would appear to be reflected in the aged mice analysed in this study, with decreased or absent granulosa cells and follicular cyst development observed in WT, PKBα/Akt1 KO and PKBβ/Akt2 KO mice. However, only specific loss of PKBβ/Akt2 in this setting allows cysts to be permissive to severe cyst development. Ovaries from PKBβ/Akt2 KO mice show bilateral ovary involvement, thecal hyperplasia, increased testosterone levels and cyst development, all observed in LH overexpressing mice. This indicates that a consequence of loss of specific PKBβ/Akt2 functions in the aged ovary is exacerbated androgenic signaling. The findings of this study

suggest this is at least in part due to loss of functions that can control ERK activation and lipid accumulation, allowing increased testosterone production observed in the PKBβ/Akt2 mice, as proposed in the model below (Figure 1.11). This provides an important basis supporting further studies to determine the direct targets of PKBβ/Akt2 and how they function in ovarian steroidogenesis.

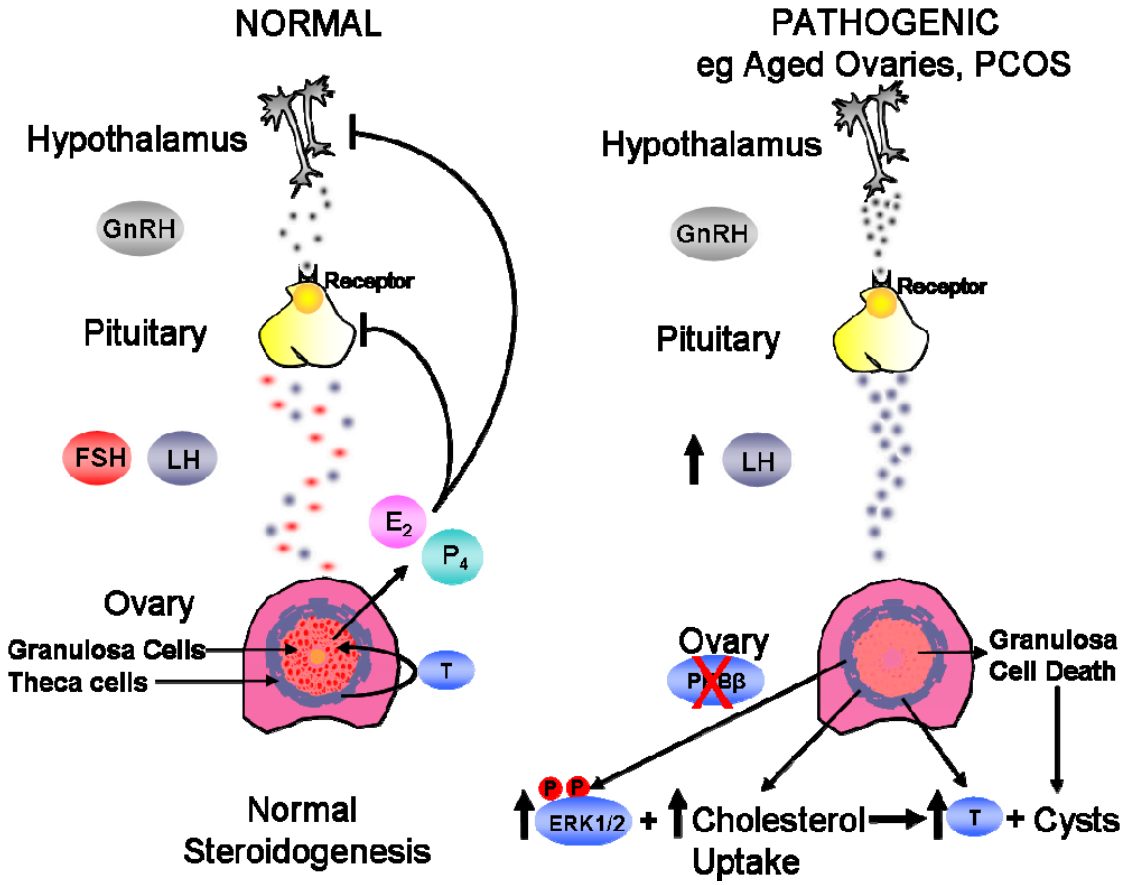

Figure 1.11. Proposed Model For Contribution Of PKBβ/Akt2 Loss To Development Of Ovarian Cysts And PCOS. Loss of PKB/Akt in the ovary in the presence of deregulated LH signaling results in thecosis. Increased testosterone production mediated by increased activation of ERK and cholesterol uptake promotes death of granulosa cells and the ovum leaving follicular cysts which can continue to increase in size in the presence of unchecked testosterone production.

Analysis of young PKBβ/Akt2 KO mice showed no significant effects on ovarian steroidogenesis or reproductive function. This indicates the role of PKBβ/Akt2 in these functions are not essential and are only unmasked upon the co-existence of another dysfunction. In these studies, increased LH-controlled androgenic signaling was the initiating factor for cyst development, whereupon loss of PKBβ/Akt2 functions could exacerbate the pathological consequences of this abnormality. Importantly, the effects of PKBβ/Akt2 loss are not confined

to aged mice, where other abnormalities or mutations that may be acquired in normal aging could co-exist with the loss of PKBβ/Akt2, but also in ovaries of young healthy mice stimulated with LH in the PCOS mouse model. Furthermore, the fact that no significant difference in ovarian cyst development in normal young mice exposed to the treatment of LH alone or LH with GnRHAnt, suggest that the dysfunction in these mice is most likely in positive stimulation of androgenic production rather than negative regulation of LH or deregulation at the level of the pituitary. This supports an intrinsic role for PKBβ/Akt2 within the ovary in functioning to control the amplitude of pathogenic androgenic responses, although *in vivo* deletion or specific inhibition of PKBβ/Akt2 in thecal cells needs to be performed to fully elucidate this.

Insulin resistance is established as the most common and detrimental co-morbidity in PCOS pathology. In PCOS patients, insulin resistance occurs in 50-70% of cases and 95% of obese sufferers. The central role for PKBβ/Akt2 in insulin signaling ensures that the findings of this study have a number of important implications for understanding how insulin resistance and signaling may be deregulated and contribute to PCOS development. PKBβ/Akt2 mice display peripheral insulin resistance due to impaired activation of PKBβ/Akt2 downstream signaling. Insulin resistance increases the severity of PCOS via multiple mechanisms. A number of mechanisms by which classical insulin-responsive tissues contribute have been reported. Insulin resistance in skeletal muscle and adipose tissue leads to decreased glucose uptake and storage, resulting in compensatory increases in insulin secretion from the pancreas, which can then amplify gonadotrophin actions in the ovary. Insulin resistance can be exacerbated by decreased glucagon synthesis and increased glucose production and release into the bloodstream by the liver. Additionally, increased free fatty acids released by the liver into the blood are not absorbed by insulin resistant adipose tissue that is defective in lypolysis and adipogenesis, allowing its utilization in the ovary for steroid synthesis. Insulin resistance also increases circulating bioactive testosterone by reducing hepatic steroid hormone binding protein that normally binds to testosterone to render it unable to stimulate androgen receptors. The increased testosterone levels disrupt follicogenesis in the ovary to promote cyst development.

In contrast to decreased sensitivity of classical insulin-responsive tissues, tissues involved in maintaining normal ovarian steroidogenesis, like the hypothalamus, pituitary and ovary are considered to maintain normal insulin sensitivity and thereby contribute to PCOS via increased insulin stimulation. The ovary is not involved in glucose/insulin homeostasis but is responsive to insulin, where it is considered a “co-gonadotrophin” due to the observations that it synergizes with gonadotrophins to amplify the cellular functions that they mediate. Accordingly, the mechanism by which insulin potentiates follicular cyst development is suggested to be an amplification of the classical LH stimulated steroidogenesis pathway. Indeed, in PCOS animal models, whilst chronic stimulation with LH stimulates polycystic ovaries (Bogovich, 1987; Bogovich, 2007), simultaneous administration of insulin results in an increase in both follicular cyst numbers and size (Poretsky et al., 1992). In androgen steroidogenesis, stimulation of both the InsR in the presence of LH both *in vivo* and in vitro has been shown to impact on various signaling pathways in the ovary, including the MAPK/ERK, PI3K/PKB and JAK/STAT signaling pathways (Lin et al., 1986; Duleba et al., 1999; Kwintkiewicz et al., 2006; Manna et al., 2006). In the pituitary it was recently shown in a diet-induced obesity mouse model that hyperinsulemia could increase LH release and testosterone production and that disruption of the InsR in the pituitary desensitized mice to LH secretion after gonadotrophin releasing hormone stimulation, suggesting the pituitary remains insulin-sensitive and hyperinsulemia promotes LH secretion to contribute to LHR hyperstimulation in PCOS (Brothers et al., 2010). Hyperinsulemia due to compensation by insulin-responsive tissues is seen in PKBβ/Akt2 mice and a role for this in enhancing insulin signaling in the ovary to exacerbate the PCOS phenotype is consistent with what is observed in this study. However, the loss of PKBβ/Akt2 in tissues of the hypothalamic-pituitary-ovarian axis, should impair insulin signaling in these tissues, therefore inhibiting the contribution of these tissues in promoting the PCOS phenotype, as suggested by decreased LH secretion upon gonadotrophin releasing hormone stimulation in hyperinsulemic pituitary-specific InsR knockout mice (Brothers et al., 2010) That our findings indicate loss of PKBβ/Akt2 increases androgen signaling in aged mice and the severity of PCOS with LH treatment, and independent of the pituitary/hypothalamic contributions after GnRHAnt

treatment suggests loss of PKBβ/Akt2 specifically within the ovary supports PCOS development. This would indicate that undefined PKBβ/Akt2-specifc substrates or interactors in the ovary control hyperandrogenic production and loss of this signaling promotes PCOS. Alternatively, compensation by other PKB/Akt isoforms, particularly PKBα/Akt1 that is well-expressed in the ovary, promotes pro-androgenic signaling. Opposing functions of these PKBα/Akt1 and PKBβ/Akt2 have been reported in various tissues, as has the existence of isoform specific substrates. It was observed in PKBβ/Akt2 KO ovaries that whilst increased activation of Akt was not apparent, no significant loss of expression was observed using a pan-Akt antibody (Appendix I), suggesting the other PKB/Akt isoforms can compensate to maintain PKB/Akt protein levels, although inappropriate functional compensation occurs. It should be noted that mice lacking both PKBβ/Akt2 and PKBγ/Akt3 developed more severe large hemorrhagic simple cysts (Appendix I). This could indicate that PKBγ/Akt3 is actively involved in compensating for PKBβ/Akt2 loss and that loss of its signaling or subsequent signaling solely through PKBα/Akt1 is a crucial component in promoting or supporting cystic pathology. To determine whether it is gain of PKBα/Akt1 and/or PKBγ/Akt3 specific signaling that may promote PCOS or if it is loss of PKBβ/Akt2 specific-signaling that may control PCOS and further, whether this could be also contributing to PCOS in human patients are crucial questions, particularly in terms of potentially targeting PKB/Akt signaling therapeutically in PCOS.

A number of findings from this study provide direction for new targets to therapeutically control cyst development both in severe cyst development like that seen in the aged mice and in PCOS. The combination of findings that PKBβ/Akt2 is not essential for normal reproductive and androgenic signaling, but does increase the severity of PCOS, highlights the potential of identifying targets of PKBβ/Akt2 that are affected upon loss of PKBβ/Akt2 in the milieu of increased LH/androgenic signaling, as restoring these functions in the pathogenic scenario by therapeutic means could specifically affect only cystic ovaries and not functions of unaffected ovaries. Furthermore, the identification *in vivo* of activated ERK, both in the fact that increased activation correlated with more severe cyst formation in aged mice and that it was specifically highly expressed in thecal cells adjacent to cysts that developed in the PCOS mouse

model, indicates inhibitors of ERK that are currently in clinical trials could have applications in treating ovarian cyst development in the aging population and PCOS. However, this should be approached with caution, as it has been reported that ERK signaling is lost in thecal cells derived from ovaries of PCOS patients (Nelson-Degrave et al., 2005). This could reflect a difference between the PCOS mouse model and the complexity of PCOS in human patients, or alternatively, a difference between thecal cell signaling in the ovarian environment compared to isolated PCOS thecal cells in the cell dish. This remains to be determined. The questions raised by the findings of this study, provide a basis for further investigation into both the role of ERK and PKB in PCOS, particularly as any therapeutics targeting these pathways that are currently in development and could become available in the near future, may have applications in PCOS to facilitate more effective and less invasive therapeutic treatments.

In conclusion, this study highlights for the first time *in vivo* a novel and specific role for loss of PKBβ/Akt2 in the development of ovarian cysts. Furthermore, this study identifies *in vivo* that thecal cell activation of ERK is strongly associated with cystic development. Through these findings this study identifies ERK and effectors downstream of PKBβ/Akt2 that display loss of function in the environment of increased LH androgenic in ovarian thecal cells, as potential targets for therapeutic intervention in the treatment and management of ovarian cysts and PCOS.

Materials And Methods

Reagents

Human LH (Lutophin) was obtained from Provet (Lyssach, BE). The gonadotrophin-releasing hormone antagonist (GnRHAnt) was generously provided by Dr Jean Rivier (The Salk Institute, San Diego, CA). Unless otherwise stated all other reagents were from Sigma (St.Louis, MO).

Mice

The PKBα/Akt1, PKBβ/Akt2 and PKBβγ/Akt2/3 mutant mice used in the study have been described previously (Yang et al., 2003; Dummler et al., 2006). Mice were housed in groups with 12-h dark-light cycles and with access to food and

water ad libitum, in accordance with the Swiss Animal Protection Laws. For PCOS induction experiments, age-matched WT and KO female mice were housed together between d21-d28 to promote synchronous estrous cycling. PCOS induction experiments were commenced at ~d28 (4wks). All procedures were conducted with the appropriate approval of the Swiss authorities.

Tissue Preparation For Histology

For histological analysis, anaesthetized mice were sacrificed, dissected and organs either immediately snap-frozen or fixed in 4% paraformaldehyde (PFA)-phosphate buffered saline (PBS). Snap frozen tissues were placed in a plastic cassette and covered with OCT compound, before being frozen by placing the cassette into a 2-methylbutane bath in dry ice. Frozen tissues were then stored at -80^{o}C until sectioned for use. Tissues placed in 4% PFA-PBS were allowed to fix overnight (~18hrs) at 4°C. Tissues were then subjected to a series of washes with PBS, 50% ethanol (EtOH)/PBS and 70%EtOH/PBS before being processed and embedded in paraffin using the Medite TPC15 Paraffin Processing Unit (Medite, Wintergarden, FL). Histological staining and immunohistochemistry (IHC) was performed on 12um frozen or 4um paraffin tissue sections, cut using a HM560H cryostat or M355S microtome (Thermo scientific, Fremont, CA).

Histological Staining

For hematoxylin and eosin (H&E) staining, sections were deparaffinized and stained according to the standard protocols using reagents purchased from Sigma (St.Louis, MO). Histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme activity was carried out according to a modified protocol of Klinefelter *et al.* (Klinefelter et al., 1987). Briefly, 12um ovarian sections were cut on poly-L-lysine coated glasses slides (Menzel-Gläser, Braunschweig, BRD) and covered with staining solution prepared by mixing equal volumes of solution A consisting of nitroblue tetrazolium (NBT) (#N6639, Sigma, St Louis, MO) and dehydroepiandosterone (DHEA) (#D1629, LKT Laboratories, St.Paul, MN) in PBS pH7.4 dimethylsulfoxide (DMSO) with solution B consisting of β-nicotinamide adenine dinucleotide (β-NAD) (#N7004, Sigma, St Louis, MO) in PBS pH7.4. Final concentrations were 0.25mM NBT, 1.5mM β-NAD, 0.2mM DHEA in PBS pH7.4. Tissue slides were allowed to stain for 90 minutes at 37^{o}C and fixed in 10% formalin in PBS with 5% sucrose,

pH 7·4 at 4°C for 5 minutes. Slides were then rinsed in distilled water and counterstained for 5 minutes with Nuclear Fast Red (#H-3403, Vector Laboratories, Burlingame, CA), rinsed again with distilled water, mounted and images taken under the microscope. Staining of lipids with Oil Red O was performed using the propolene glycol (PG) method. 12um fresh frozen sections were cut and air dried at RT before being fixed at 4°C in 10% formalin for 5 minutes. Sections were then rinsed in ddH2O three times and allowed to air dry at RT. Sections were then placed in 100% PG for 5 minutes before staining for 15 minutes with 0.5%(w/v) Oil Red O solution in PG pre-warmed to 60°C. Oil Red O solution was prepared by dissolving Oil Red O in PG at 90°C, filtering and allowing to stand at RT O/N. Staining was differentiated by placing slides in 85% PG for 5 minutes and then rinsing twice with double distilled water (ddH_2O). Slides were then counterstained with Gill's Haematoxylin (#GHS216, Sigma, St Louis, MO) for 15 seconds, rinsed three times in tap water, soaked in ddH_2O for 5 minutes and mounted.

Immunohistochemistry

4um sections were cut from paraformaldehyde-fixed, paraffin-embedded tissues and stained using the Ventana Discovery automated immunostainer (Ventana Medical Systems, Tucson, AZ). IHC was performed with or without cell conditioning using buffers CC1 or CC2, blocked with 5% normal donkey, goat or sheep serum for 1 hour. Primary antibodies diluted in Ventana antibody diluent were then applied and allowed to incubate for 1 hour to overnight at 25C. Primary antibodies and dilutions used were vimentin (#V2009) 1:100 (Biomedia, Foster City, CA), Muellerin inhibiting substance (MIS, sc-6886) 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA), Ki67 (#RM-9106-S0) 1:100 (thermo Scientific, Fremont, CA), pCREB S133 (#9198) 1:100, pERK1/2 T202Y204 (#4370) 1:125, GSK3b 1:100 (#9332), pGSK3α/β S21/9 1:50 (#9331), pPKB/AKT S473 1:25 (#4060), panPKB/AKT 1:125 (#4685), pS6 Ribosomal Protein S235/6 1:100 (#4858) pCREB S133 (#9198) 1:100, pERK1/2 T202Y204 (#4370) 1:125 (all Cell Signaling Technologies, Danvers, MA). After washing, sections were incubated with biotinylated donkey anti-mouse (#715-067-003) or anti-rabbit (711-067-003) secondary antibodies (Jackson Immuno Research Inc, West Grove, PA) for 32mins at 37C, before detection with HRP/DAB, OmniMap or UltraMap conjugates and counterstained

with haematoxylin (all Ventana Medical Systems, Tucson AZ). Photomicrographs were taken on a Nikon Eclipse E600 microscope (Nikon, Milville, NY).

Serum Hormone Measurement

Blood samples were collected by sublingual vein puncture into Microvette CB300 tubes (Sarstedt, Nümbrecht, DE) and serum separated by centrifugation at 5000xg for 10 minutes. To account for the daily variations in hormones levels, for all mice a morning sample was taken and a second sample taken 6 hours later. 50ul of each sample were then pooled and concentrated to 50ul by diethyl ether hormone extraction as described by (Wijayagunawardane et al., 2003). Briefly, 5 volumes of diethyl ether were added to the serum samples and the samples placed under agitation using a tabletop shaker at 250rpm for 30 minutes. Samples were then allowed to stand for 15 minutes and transferred to -80^{o}C for 1 hour. The upper steroid-containing diethylether layer was decanted, evaporated and the residue dissolved in 50ul of steroid-free serum (DRG Instruments GmbH, Marburg, Germany) at 250rpm for 5 minutes in an eppendorf table-top shaker. Concentrated hormone samples were then used in commercial enzyme-linked immunosorbent assay (EIA) kits (DRG Instruments GmbH, Marburg, Germany) to measure serum testosterone levels according to the manufacturer's instructions.

PCOS Induction And Cyst Measurement In Mice

Mice were subjected to the standard protocol for LH-induced PCOS by injection of 1.5U hLH twice daily, with or without GnRHAnt for 21 day as described by Bogovich *et al* (Bogovich and Richards, 1982; Bogovich, 1987). At day 21 mice were sacrificed and samples collected for further analysis. Formation of hemorrhagic cysts, observed in mice treated with LH +/- GnRHAnt, was quantified by sectioning through the ovaries and measuring every 100um the percentage area of the total ovary occupied by the hemorrhagic cysts using the ImageAccess Enterprise v10 software (Imagic Bildverarbeitung, Glattbrugg, Switzerland).

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II. RESULTS: Part II

Loss of Protein Kinase B beta (PKBβ/Akt2) Suppresses Pheochromocytoma Formation Induced By Pten Deficiency In Mice

1. Introduction

1.1. Cancer As A Disease Of Deregulated Cellular Signaling

Cancers arise from cells which acquire mutations that endow the cell with the ability for unchecked proliferation and protection from intrinsic apoptotic mechanisms designed to prevent cells from reaching such a state. These mutations result in various aberrations from overexpression, as is common with cell surface receptors, to loss of function, like that observed for tumour suppressor proteins like p53 and Pten. However, intrinsic to all tumour-promoting aberrations is deregulation of cellular signaling that is a necessity for successful tumour formation. Accordingly, kinases, in their capacity to regulate a wide variety of cellular functions, are commonly deregulated in tumour cells to support both positive pro-oncogenic signaling and inhibit negative pro-apoptotic signaling. Activated PKB/Akt, in its capacity to both promote proliferation and growth, and also inhibit apoptosis and cell cycle arrest, is thereby capable of mediating robust pro-oncogenic signaling. Accordingly, mutations that stimulate hyperactivation of PKB/Akt are notoriously common and inhibiting pro-oncogenic signaling by PKB/Akt holds great potential as a target for therapeutic intervention in tumours.

1.2. Deregulation Of The PI3K-Pten-PKB/Akt Pathway In Cancer

PKB/Akt is commonly activated in human tumours, primarily due to loss of upstream PI3K pathway regulation. As indicated in Figure 2.1, this includes overexpression and constitutively active signaling by membrane receptors like the ErbB receptor, activating mutations in PI3K, inactivating mutations in Pten and even mutation of PKB/Akt itself, all of which support the hyperactivation of PKB/Akt and result in inappropriate signaling to support growth and proliferation or increased survival (see appendix for further details on deregulation of specific upstream PI3K members in cancer).

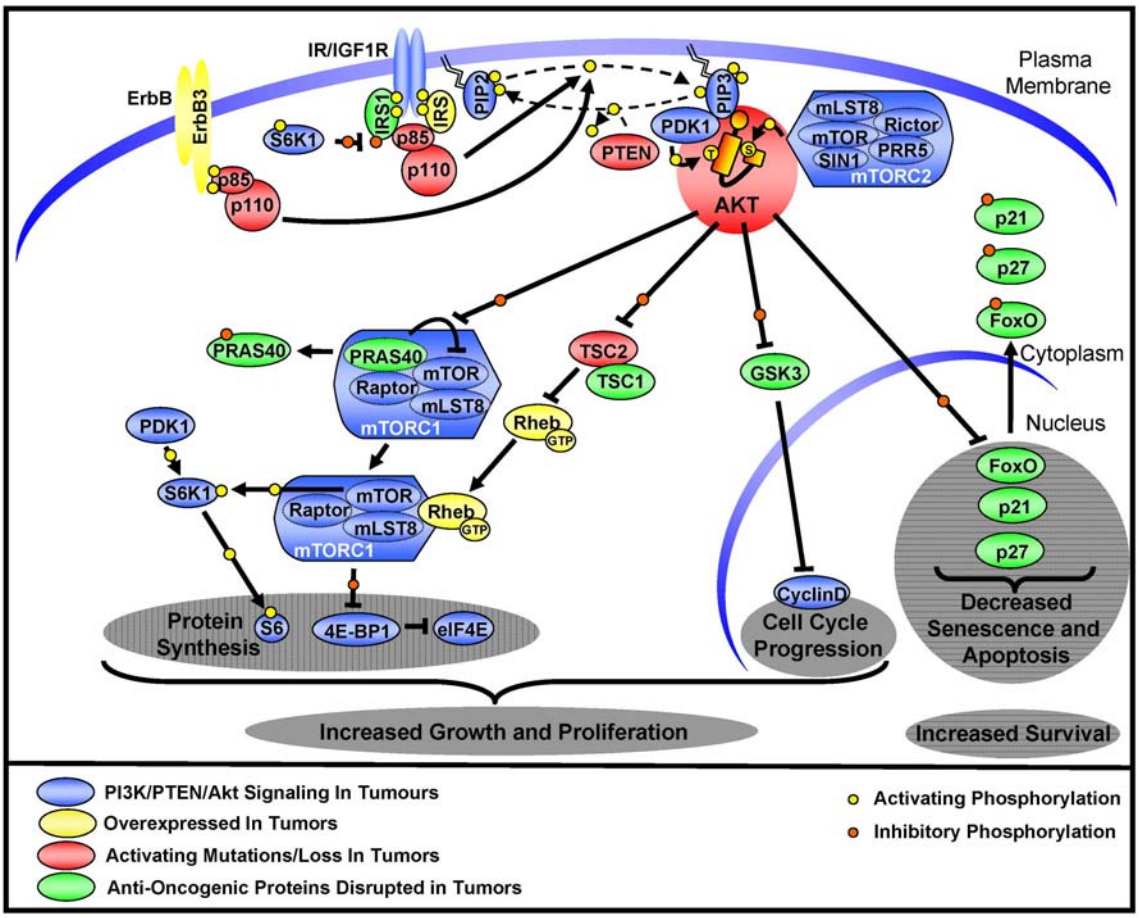

Figure 2.1. The PI3K-Pten-PKB/Akt Signaling Pathway And The Causes Of Its Hyperactivation In Tumourigenesis. Increased PKB/Akt activation can occur through overexpression of pathway components (yellow) or inhibitory mutation or complete loss of components (red). These events can lead to decreased activation of anti-oncogenic proteins (green) and increased growth, proliferation and survival signals to promote tumourigenesis. eIF4E, eukaryotic translation initiation factor 4E; LST8, target of rapamycin complex subunit LST8; PRR5, proline-rich protein 5; SIN1, SAPK-interacting protein 1; PIP2, PtdIns(3,4)*P*2; PIP3, PtdIns(3,4,5)*P*3 (Restuccia and Hemmings, 2010).

The capacity of activated PKB/Akt signaling to support tumour formation is best demonstrated by examination of the vast number of upstream activating aberrations and high incidence with which they can occur in a wide variety of tumour types (Table 2.1).

Gene	Mutation	Tissue	Incidence % (samples)	References
ErbB2	Point Insertions	Breast Lung Stomach Colorectal	4% (4/94) 4% (5/120) 5% (9/180) 3% (3/104)	(Stephens et al., 2004; Lee et al., 2006; COSMIC)
ErbB2	Amplification	Breast Ovary Stomach Oesophogeal	18-40% (19/103, 110/245, 34/86) 26% (31/120) 16% (27/166) 5-15% (7/145, 16/110)	(Slamon et al., 1987; Slamon et al., 1989; Reichelt et al., 2007; Marx et al., 2009)
IRS2	Amplification	Colon Brain	2% (3/146) 2% (2/103)	(Knobbe and Reifenberger, 2003; Parsons et al., 2005)
p85 (PI3K)	Deletions	Ovary Colon Brain	4% (3/80) 2% (1/60) 3-10% (1/30, 9/91)	(Philp et al., 2001; Mizoguchi et al., 2004; Parsons et al., 2008)
p110α (PI3K)	Various (especially Point mutants E542K/ E545K/ H1047R)	Colon Brain Stomach Breast Liver Lung Ovary Uterus	19-32% (6/32, 74/199) 7-27% (5/70, 10/105, 11/73, 4/15) 4-25% (4/94, 12/185, 3/12) 18-40% (13/53, 13/72, 19/92, 25/93, 28/70) 36% (26/73) 4% (1/24) 6-12% (11/167, 24/198) 36% (24/66)	(Bachman et al., 2004; Campbell et al., 2004; Samuels et al., 2004; Hartmann et al., 2005; Lee et al., 2005; Levine et al., 2005; Oda et al., 2005; Buttitta et al., 2006; Gallia et al., 2006; Velasco et al., 2006; Parsons et al., 2008)
p110α (PI3K)	Amplification	Lung Ovary Breast	33% (46/139) 25-58% (83/341, 7/12) 9% (9/92)	(Shayesteh et al., 1999; Campbell et al., 2004; Wu et al., 2005; Yamamoto et al., 2008)
K-Ras	Point (especially G12D)	Pancreas Colon Lung	75-95% (5/6, 12/16, 28/30, 21/22) 30-60% (10/29, 14/40, 37/61) 15-25% (22/129, 43/181)	(Almoguera et al., 1988; Smit et al., 1988; Suzuki et al., 1990; Burmer et al., 1991; Boughdady et al., 1992; Lemoine et al., 1992; Rodenhuis and Slebos, 1992)
PTEN	Promoter Methylation	Brain Breast Uterus	35-37% (22/60, 27/77) 34-48% (15/44, 43/90) 19% (26/138)	(Salvesen et al., 2001; Baeza et al., 2003; Garcia et al., 2004; Khan et al., 2004; Wiencke et al., 2007)
PTEN	Deletions Point LOH	Most Tissues (esp. Brain Prostate Uterus Colon)	16-31% (14/91, 13/42) 49% (25/51) 50% (16/32) 25% (14/57)	(Rasheed et al., 1997; Tashiro et al., 1997; Feilotter et al., 1998; Zhou et al., 1999; Kondo et al., 2001; COSMIC)
PDK1	D527E T354M	Colon	<1% (1/204) 1% (2/204)	(Parsons et al., 2005)
AKT1	E17K	Breast Colorectal Ovary Endometrium Skin Lung	4-8% (4/93, 5/61) 6% (3/51) 2% (1/50) 2% (2/89) <1% (1/137) 6% (2/36)	(Carpten et al., 2007; Davies et al., 2008; Kim et al., 2008; Malanga et al., 2008; Shoji et al., 2009)
AKT1	Amplification	Stomach Brain	20% (1/5) 1% (1/103)	(Staal, 1987; Knobbe and Reifenberger, 2003)
AKT2	S302G; R371H A377V	Colon Colon Lung	<1% (1/204) <1% (1/204) 1% (1/79)	(Parsons et al., 2005; Soung et al., 2006)
AKT2	Amplification	Colon Breast Ovary Head/Neck Pancreas	1% (2/146) 3% (3/106) 12-18% (16/132, 12/66) 30% (12/40) 20% (7/35)	(Cheng et al., 1992; Bellacosa et al., 1995; Ruggeri et al., 1998; Snijders et al., 2003; Parsons et al., 2005; Pedrero et al., 2005; Nakayama et al., 2006; Nakayama et al., 2007; Yu et al., 2009)
AKT3	E17K	Skin	2% (2/137)	(Davies et al., 2008)
AKT3	G171R	Brain	11% (1/9)	(Hunter et al., 2006)
AKT3	Amplification	Brain Liver	4-14% (4/230, 29/206) 30% (6/19)	(Hashimoto et al., 2004; CGARN, 2008; Ichimura et al., 2008)

Table 2.1: Common Upstream PKB/Akt Activating Mutations And Somatic PKB/Akt Mutations. Note PDK1, PKBβ/Akt2 and the PKBγ/Akt3 G171R somatic point mutants were detected in tumour samples and are hypothesized to promote activation due to the mutations occurring in kinase domains, however their activating potential has yet to be characterized Genes are listed in order of signal transduction along the PI3K-Pten-PKB/Akt pathway from receptor to PKB/Akt. Studies first reporting the indicated mutations and those with large sample sets were selected (Restuccia and Hemmings, 2010).

These observations have ensured that studies to understand PKB/Akt contributions to tumours have been extensive. Many of these studies have utilized mouse models providing a clear indication of the contribution of PKB/Akt to tumours resulting from various upstream mutations. These models

invariably result in neoplasia formation with similar signaling to that seen in human patients, validating the value of these models for studying the pathogenesis and progression of the human disease (Table 2.2).

Protein	Mutation	Effect	Phenotype	References
ErbB2	Tg-MG	O/E	Mammary tumors	(Muller et al., 1988)
+PTEN	cKO-MG	Loss	Acceleration of tumors	(Dourdin et al., 2008)
+Myr-AKT1	Tg-MG	O/E	Acceleration of tumors	(Young et al., 2008)
+AKT1	KO	Loss	Inhibition of tumors and metastasis	(Ju et al., 2007) (Maroulakou et al., 2007)
+AKT2	KO	Loss	Acceleration of tumors	(Maroulakou et al., 2007)
+AKT3	KO	Loss	No observable effect on tumorigenesis	(Maroulakou et al., 2007)
PolyMidT	Tg-MG	O/E	Mammary tumors	(Guy et al., 1992)
+IRS1	KO	Loss	Mammary tumors & metastasis	(Chen et al., 2006)
+IRS2	KO	Loss	Decreased mammary tumors	(Nagle et al., 2004)
+AKT1	KO	Loss	Inhibition of tumors	(Maroulakou et al., 2007)
+AKT2	KO	Loss	Acceleration of tumors	(Maroulakou et al., 2007)
+AKT3	KO	Loss	No observable effect on tumorigenesis	(Maroulakou et al., 2007)
IRS1	KO	Loss	Insulin resistance, reduced growth	(Araki, E. et al., 1994) (Tamemoto et al., 1994)
IRS1	Tg-MG	O/E	Mammary tumors and metastasis	(Dearth et al., 2006)
IRS2	KO	Loss	Diabetes	(Withers et al., 1998)
IRS2	Tg-MG	O/E	Mammary tumors and metastasis	(Dearth et al., 2006)
K-ras^{G12D}	KI-PtMt	G12D	Lung tumors	(Johnson et al., 2001)
+p85α$^{T208D/K227A}$	KI-PtMt	T208D/ K227A	Resistant to Ras binding and Ras-induced lung tumorigenesis	(Gupta et al., 2007)
Myr-p110α	Tg-Pr	O/E	Hyperplasia	(Renner et al., 2007)
p85α	cKO-Pr	Loss	No observable tumor phenotype	(Jia et al., 2008)
p85β	cKO-Pr	Loss	No observable tumor phenotype	(Jia et al., 2008)
PTEN	KO	Ht, hy/	MG, adrenal, uterine, prostate Neoplasia	(Di Cristofano et al., 1998) (Suzuki et al., 1998) (Podsypanina et al., 1999)
	Tg-Hy	Hy/+	Reduced neoplasia, increased latency	(Trotman et al., 2003)
+IRS2	KO	Loss	Decreased tumors in multiple tissues	(Szabolcs et al., 2009)
+p85α$^{+/-}$	KO	Ht	Increased GI polyps, PIN unaffected	(Luo et al., 2005)
+p85β$^{-/-}$	KO	Loss	Decreased PIN	(Luo et al., 2005)
+p85α$^{+/-}$β$^{-/-}$	KO	Ht/Loss	Increased GI polyps, PIN unaffected	(Luo et al., 2005)
+PDK1	KO	Hy/-	Inhibition of PTEN-driven tumors	(Bayascas et al., 2005)
+AKT1	KO	Loss	Inhibition of PTEN-driven tumors	(Chen et al., 2006)
PTEN	cKO-Sk	Loss	Susceptibility to carcinogens	(Inoue-Narita et al., 2008)
+B-raf^{V600E}	Tg-Sk	O/E	Metastatic melanoma	(Dankort et al., 2009)
PTEN	cKO-Pr	Loss	Metastatic prostate tumors	(Trotman et al., 2003;
+p85α	cKO-Pr	Loss	No effect on PTEN tumorigenesis	Wang et al., 2003)
+p85β	cKO-Pr	Loss	Loss of PTEN tumorigenesis	(Jia et al., 2008)
+mTOR	cKO-Pr	Loss	Inhibition of tumors	(Nardella et al., 2009)
+rictor	cKO-Pr	Loss	Inhibition of tumors	(Guertin et al., 2009)
AKT1	KO	Loss	Small, partial lethality	(Chen et al., 2001) (Cho et al., 2001a)
Myr-AKT1	Tg-Pr	O/E	High-grade PIN; 100% penetrance	(Majumder et al., 2003)
+p27	cKO	Loss	Progression to cancer	(Majumder et al., 2008)
Myr-AKT1	Tg-Lv	O/E	Insulinomas	(Alliouachene et al., 2008)
+S6K1	KO	Loss	Inhibition of insulinomas	(Alliouachene et al., 2008)
Myr-AKT1	Tg-Sk	O/E	Skin carcinomas, DMBA sensitive	(Segrelles et al., 2007)
Myr-AKT1	Tg-Tc	O/E	Thymic lymphoma with short latency	(Malstrom et al., 2001; Rathmell et al., 2003)
Myr-AKT1	Tg-MG	O/E	+DMBA: ER+ mammary tumors	(Blanco-Aparicio et al., 2007)
Myr-AKT$_{\Delta 11\text{-}60}$	Tg-Br	O/E	No tumor phenotype	(Holland et al., 2000)
+K-ras^{G12D}	PtMt-Br	O/E	Glioblastoma	(Holland et al., 2000)
+B-raf^{V600E}	PtMt-Br	O/E	Gliomas	(Robinson et al.)
AKT1^{E40K}	Tg-Tc	E40K	Peripheral lymphoma after long latency	(Malstrom et al., 2001)
AKT2	KO	Loss	Diabetes	(Cho et al., 2001b)
Myr-AKT2	Tg-Tc	O/E	Thymic lymphoma after long latency	(Mende et al., 2001)
AKT3	KO	Loss	Small brain	(Easton et al., 2005; Tschopp et al., 2005)
DNAPK$_{cs}$	KO	Loss	Thymic lymphomas	(Jhappan et al., 1997)
+AKT1	KO	Loss	Inhibition of DNAPK$_{cs}$–driven thymic lymphomas	(Surucu et al., 2008)

Table 2.2: Defining Mouse Models Of PKB/Akt Activation And Signaling In Tumourigenesis. *KO* whole body knockout, *Ht* heterozygous gene deletion, *hy* hypomorphic gene modification, *cKO* conditional tissue deletion, *Loss* protein loss, *Tg* transgenic, *O/E* protein over expression, *MG* mammary gland, *Pr* prostate, *Lv* liver, *Tc* T-*cell, Br Brain, KI knock-in gene mutation, PtMt genetic point mutant GI gastrointestinal.* Proteins modifications are listed in order of signal transduction along the PI3K-Pten-PKB/Akt pathway from receptor to PKB/Akt (Restuccia and Hemmings, 2010).

Whilst PKBα/Akt1 is most commonly associated with PKB/Akt hyperactivation in tumours, a number of studies have also illustrated a contribution of both PKBβ/Akt2 and PKBγ/Akt3 to pro-oncogenic signaling. One such study by Chen and colleagues examining the effect of ablation of PKBα/Akt1 on neoplasia formation from heterozygous Pten mice, clearly indicated that other PKB/Akt isoforms may play crucial roles in at least one examined tissue type, the adrenals. They observed an almost complete inhibition of tumour formation in all tissues except for the adrenal medulla where only a partial inhibition of pheochromocytoma formation was observed. These findings indicated that activation of the PKBα/Akt1 is the isoform responsible for neoplasia formation upon loss of Pten function in almost all tissues except the adrenal medulla, which is the tissue of origin for pheochromocytomas.

1.3. Pheochromocytomas And PKB/Akt

Pheochromocytomas are tumours of the adrenal medulla derived from chromaffin cells that synthesize, store, metabolize and secrete catecholamines like epinephrine/adrenaline. These functions of chromaffin cells are disrupted in pheochromocytomas resulting in hyperstimulation of hormone synthesis and release, particularly the catecholamines adrenaline/epinephrine and noradrenaline/norepinepherine. This elevated catecholamine biosynthesis and release is a clinical hallmark of pheochromocytomas and a major cause for the co-morbidities, particularly hypertension that is associated with the disease. Pheochromocytomas are considered extremely rare affecting between 3-8 people per million and are usually benign (Eisenhofer et al., 2004). However, ~10% are malignant and these are invariably fatal, as there is currently no effective treatments for malignant pheochromocytoma (Eisenhofer et al., 2004; Strong et al., 2008). Pheochromocytomas are highly heterogeneous with only ~10% being linked to familial inheritance due to mutations is diverse and functionally unrelated proteins, as indicated in Table 2.3.

Gene	Related Familial Syndrome	Incidence In PCC (Familial/Sporadic)	PCC	Location	Mutation/ Aberration	Pathway Activation
RET	*Multiple Endocrine Neoplasia Type 2*	*70%/<5%*	*Benign Malignant (3%)*	*AM AM+ExAM*	*Amp, ReArr Overexpression*	*Ras-Raf-MEK-ERK PI3K-PTEN-PKB/Akt*
VHL	*von Hippel-Lindau Syndrome Type1 & Type2*	*14%/2-11%*	*Benign Malignant (17%)*	*AM AM + ExAM*	*Deletion (Type1) Missense (Type2)*	*HIF stabilisation (mTORC1)-HIF1α-Cyclin D*
NF1	*Neurofibromatosis type1*	*~5%/~2%*	*Benign*	*AM*	*Loss (+/-) Loss (-/-)*	*Ras-Raf-MEK-ERK*
SDHD SDHB	*Hereditary Parasympathetic Paraganglioma Type 1 & 4*	*~1%/ ~3% <4%/4-10%*	*Benign*	*AM (+ExAM Familial)*	*Mut Very Rare Loss Of Activity*	*HIF Stabilisation (mTORC1)-HIF1α-Cyclin D*

Table 2.3 Genetic Mutations And Familial Syndromes Displaying PCC In Humans And Consequent Pathway Activation. AM; Adrenal Medulla, ExAM; Extra-Adrenal Medulla, RET; RET Oncogene; VHL; von Hippel-Lindau protein, NF1; Neurofibromin 1, SDH; Succinate Dehydrogenase Subunit, Amp; amplification, ReArr; rearrangement, Mut; Mutation Adapted from review by Dannenberg *et al* (2003)

As a consequence, the molecular mechanisms driving this tumour type remain largely unknown. Furthermore, other than Ki67 staining demonstrating increased proliferation, reliable markers distinguishing malignant pheochromocytomas from their benign counterparts remain undefined (Strong et al., 2008). However, PKB/Akt activation in human pheochromocytomas has been reported in a number of studies, although interestingly decreases, mutations or loss of Pten is rarely seen, suggesting PKB/Akt activation may contribute to pheochromocytoma formation independent of Pten status (Fassnacht et al., 2005; van Nederveen et al., 2006). Therefore, mouse models of Pten that display significant pheochromocytoma formation with clinical features seen in the human disease (Freeman, D. et al., 2006; Korpershoek et al., 2009) may not reflect a model for Pten status in the human disease, but rather PKB/Akt hyperactivation and provide a clinically relevant model for studying the contribution of activated PKB/Akt to human pheochromocytoma formation. The study by Chen and colleagues could show that ablation of PKBα/Akt1 was insufficient to prevent pheochromocytoma formation leading to the conclusion that PKBβ/Akt2, which is also strongly expressed in the adrenal medulla, was responsible for neoplasia formation in this setting. This indicated that the adrenal medulla may provide an appropriate setting to probe whether PKBβ/Akt2 contributes to tumour formation by utilizing the same signaling as PKBα/Akt1 does in other tissues, or if PKBβ/Akt2 acts via alternate and/or tissue specific signaling to promote and sustain tumour development.

2. Results

2.1. Ablation Of The PKBβ/Akt2 Isoform In Pten$^{+/-}$ Mice Impairs Whole Animal Growth And Leads To Reduced Adrenal Weight

Neoplasia development in Pten mice is well characterized, with significant pheochromocytoma formation observed in female mice between approximately 5-7 months (Chen et al., 2006). Mice were monitored and weights taken from the first until the seventh month. At seven months a trend towards a reduction in average weight of both female and male Pten$^{+/-}$ (SKO) mice and both wild-type (WT) and PKBβ/Akt2$^{-/-}$Pten$^{+/-}$ (DKO) mice was observed, although this was not significant in either sex and was more pronounced in female mice. (Figure 2.2, A, B, C).

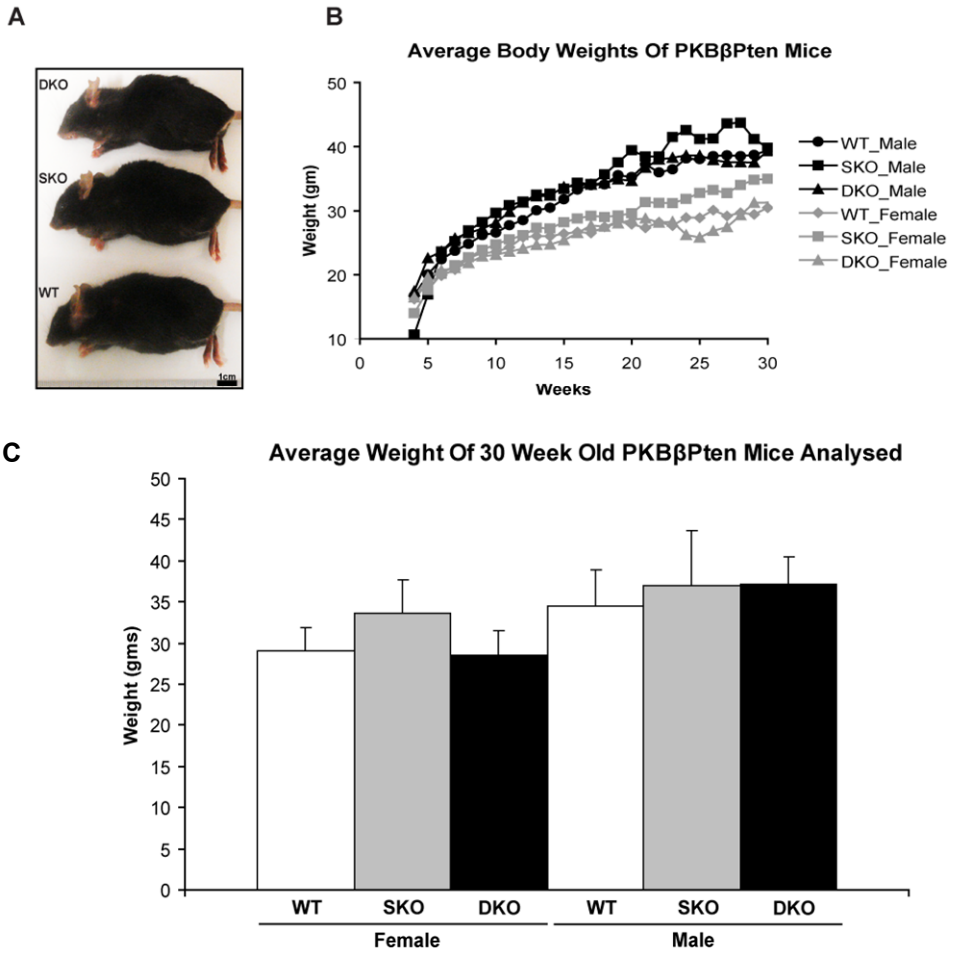

Figure 2.2. Ablation Of The PKBβ/Akt2 Isoform In Pten$^{+/-}$ Mice Impairs Whole Animal Growth (A) Appearance of WT, SKO, and DKO female mice at 30 weeks of age. Note the B-cell neck mass in the throat region of the SKO mouse. (B) SKO mice display increasing weight gain from 15-20 weeks until 30 weeks at which time (C) WT and DKO animals show a strong trend toward reduced animal weight compared to SKO animals. WT; wild-type, SKO; single knockout (Pten$^{+/-}$), DKO; double knockout (PKBβ/Akt2$^{-/-}$Pten$^{+/-}$)

Mice were then sacrificed and organs collected and weighed to determine changes resulting from loss of PKBβ/Akt2. Significant differences were observed in adrenals and B-cell tumours in SKO animals compared to both WT and DKO animals (Table 2.3).

		Adrenals	B-Cell (Neck Mass)	Brain	Liver	Mesenteric Lymph Node	Ovary	Pancreas	Uterine Horns	Anterior Prostate	White Adipose (Gonodal)
WT	Av. Wt.(gm)	0.006	0.002	0.519	1.306	0.017	0.009	0.312	0.079	0.044	0.115
	+/- SD	0.001	0.004	0.020	0.186	0.007	0.002	0.045	0.019	0.009	0.044
	(n)	(13)	(11)	(12)	(13)	(13)	(13)	(13)	(13)	(5)	(13)
SKO	Av. Wt.(gm)	0.018	0.504	0.630	1.759	0.046	0.012	0.357	0.317	0.107	0.081
	+/- SD	0.007	0.354	0.036	0.410	0.021	0.005	0.052	0.242	0.035	0.054
	(n)	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(9)	(11)
DKO	Av. Wt.(gm)	0.007	0.111	0.615	1.548	0.032	0.013	0.309	0.275	0.086	0.047
	+/- SD	0.001	0.070	0.024	0.256	0.017	0.003	0.056	0.141	0.025	0.018
	(n)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(12)	(6)
p-value	WT:SKO	<0.001	<0.001	<0.001	0.002	<0.001	0.037	0.032	0.002	0.002	0.110
	WT:DKO	0.326	0.001	<0.001	0.026	0.008	0.003	0.890	<0.001	0.008	0.001
	SKO:DKO	0.001	0.007	0.374	0.243	0.153	0.695	0.078	0.681	0.308	0.127

Table 2.3. Effect of Ablation Of The PKBβ/Akt2 Isoform In Pten$^{+/-}$ Female Mice At 30 weeks in various organs analyzed. Adrenals and B-cell tumour were significantly reduced in DKO mice compared to SKO mice. Other organs showed a trend to reducted weight or no noticeable effect. (see also Appendix II) WT; wild-type, SKO; single knockout (Pten$^{+/-}$), DKO; double knockout (PKBβ/Akt2$^{-/-}$Pten$^{+/-}$)

Whilst a significant difference between WT and DKO mice was observed in B-cell tumour formation, indicating that loss of PKBβ/Akt2 could reduce tumour burden, this was insufficient to prevent tumour formation (Table 2.3). Other organs like the uterus, prostate, mesenteric lymph node, pancreas and liver indicated a similar but milder trend towards a reduction in neoplasia burden upon loss of PKBβ/Akt2, which not significant (Table 2.3). Visually thyroids were also noticeably smaller in DKO mice compared to SKO mice (data not shown). Based upon these observations, the adrenals were selected for detailed examination and the analysis expanded to include PKBβ/Akt2$^{+/-}$Pten$^{+/-}$ (DHT) mice. As shown in Figure 2.3 A,B, a significant difference between SKO and all other genotypes was observed, indicating that even a reduction in PKBβ/Akt2 is sufficient to impair adrenal medulla hyperplasia.

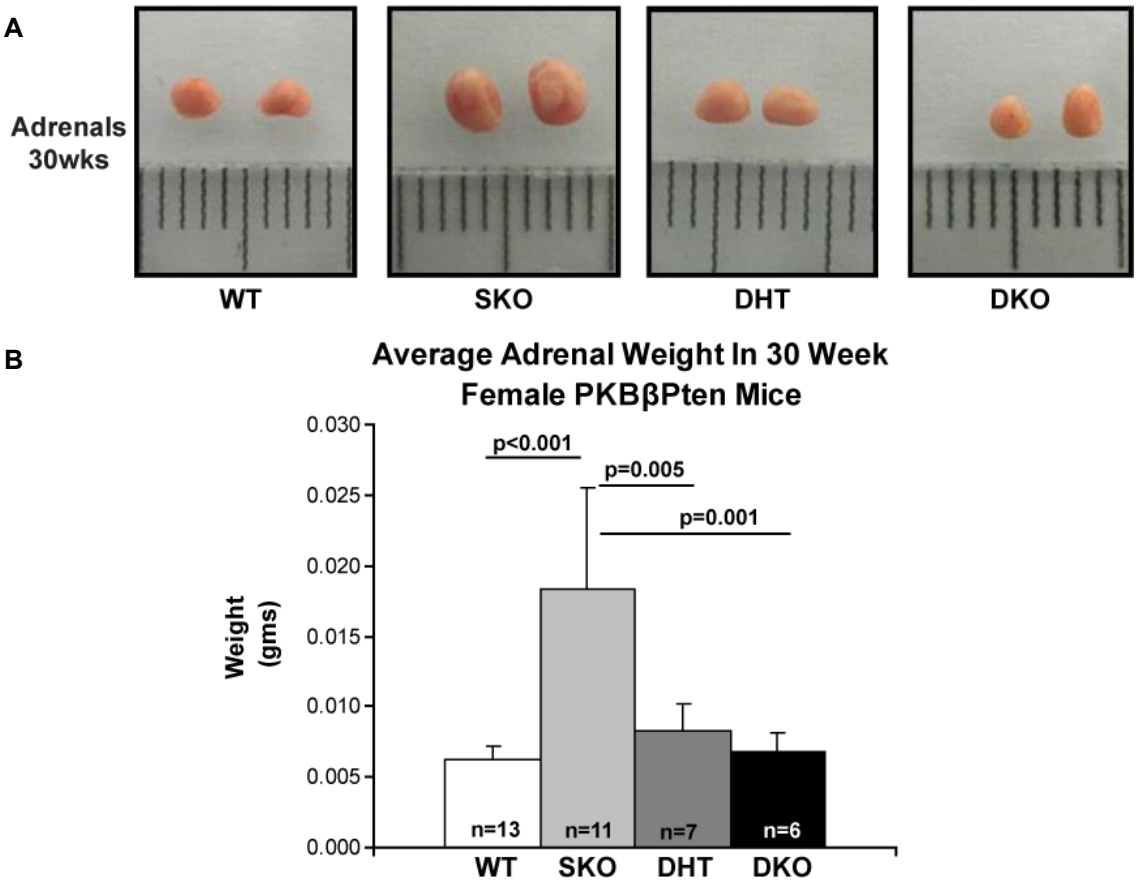

Figure 2.3. Ablation Of The PKBβ/Akt2 Isoform In Pten$^{+/-}$ Mice Significantly Reduces Adrenal Size and Weight. (A). WT, DHT and DKO adrenals are both visually smaller (B) and significantly reduced in weight compared to SKO animals at 30 weeks of age in female mice. WT; wild-type, SKO; single knockout (Pten$^{+/-}$), DHT; double heterozygous knockout (PKBβ$^{+/-}$ Pten$^{+/-}$), DKO; double knockout (PKBβ/Akt2$^{-/-}$Pten$^{+/-}$)

2.2. Decreased Adrenal Weight In PKBβ/Akt2$^{-/-}$Pten$^{+/-}$ Mice Reflects Suppression Of Pten$^{+/-}$ Induced Adrenal Medulla Neoplasia

To determine what effect loss of PKBβ/Akt2$^{-/-}$ was having on the adrenal gland that resulted in the observed significant reduction in weight, adrenals were subjected to histological analysis and the cortical and medullary regions measured for changes.

As indicated in Figure 2.4 A,B, reduction in PKBβ/Akt2$^{-/-}$ had little effect on the adrenal cortex, but significantly inhibited an increase in medullary size resulting from Pten reduction. A gene-dose dependant reduction in medullary area was observed with reduction of PKBβ/Akt2$^{-/-}$, although, interestingly, loss of PKBβ/Akt2$^{-/-}$ did not restore medullary area to that of WT, suggesting that loss of PKBβ/Akt2$^{-/-}$ cannot prevent changes involved in the initiation of neoplasia Figure 2.4 B,C.

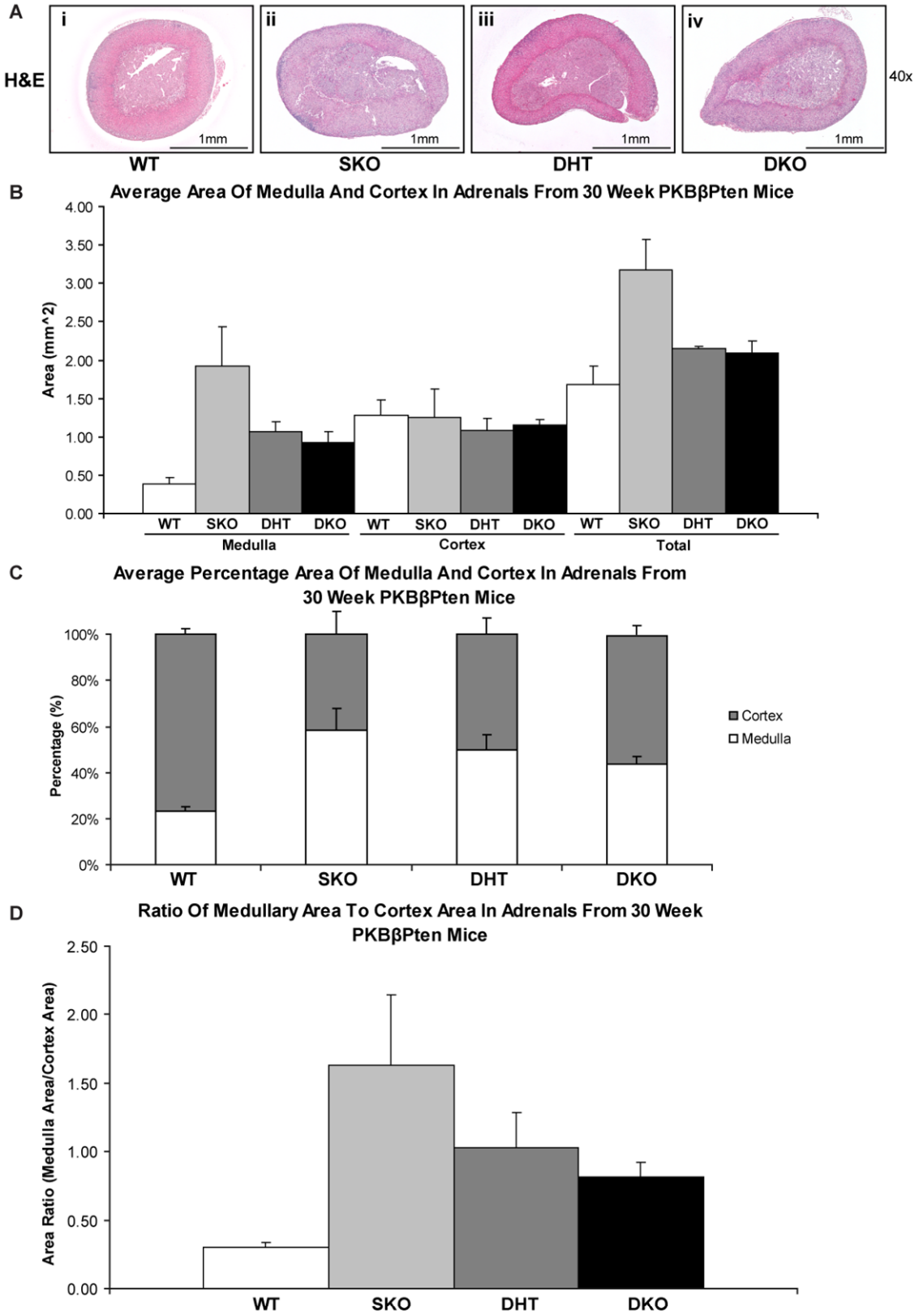

Figure 2.4. Decreased PKBβ/Akt2 In Pten$^{+/-}$ Mice Specifically Reduces Medullary Area In The Adrenals Indicating Suppression Of Pten-Driven Medullary Neoplasia. (A) Histological analysis of adrenals from WT, SKO, DHT and DKO mice by H&E staining illustrating the reduced medullary area in WT, DHT and DKO adrenals compared to SKO. (B) Quantitation of the area of medullary, cortical and total adrenal sizes and percentage composition of medulla and cortex (D) in analyzed mice. WT; wild-type, SKO; single knockout (Pten$^{+/-}$), DHT; double heterozygous knockout (PKBβ/Akt2$^{+/-}$Pten$^{+/-}$), DKO; double knockout (PKBβ/Akt2$^{-/-}$Pten$^{+/-}$)

Analysis of adrenals from 20week old mice showed a small but significant reduction in adrenal size in DKO compared to SKO and also between WT and DKO mice, suggesting that development but not initiation of neoplasia is impaired by PKBβ/Akt2$^{-/-}$ loss (Appendix II). To classify adrenal medullar enlargement as neoplastic and potentially as a pheochromocytoma, it has been previously reported that neoplasia can be distinguished by having a ratio between the medullary region and cortical region in excess of 1.0 (Szabolcs et al., 2009). Using this method of analysis, WT mice had a ratio of ~0.3, and DKO ~0.8, indicating that these adrenal were not displaying neoplastic growth. However, DHT mice were on the border of neoplastic growth with 1.0 and the SKO indicated strong neoplasia with a ratio of ~1.6.

2.3. PKBβ/Akt2$^{-/-}$Pten$^{+/-}$ Adrenals Show Impaired Proliferation

The proliferative effects resulting from Pten loss in mice has been extensively reported. Therefore adrenals were analyzed to determine if changes in proliferation could underlie the reduction in medullary neoplasia upon PKBβ/Akt2$^{-/-}$ loss. As DHT mice were on the border of neoplasia, further analysis was focused on WT, SKO and DKO to be able to clearly distinguish the functional and signaling determinants responsible for the observed differences between SKO and DKO adrenals. Immunohistochemical analysis of WT, SKO and DKO adrenals for BrdU incorporation as a marker for proliferation was performed (Figure 2.5 A) and quantified (Figure 2.5 B,C). Interestingly, loss of PKBβ/Akt2$^{-/-}$ had little effect on suppressing Pten induced proliferation in the cortex, however, in the medulla it was almost ten-fold lower, indicating the presence of PKBβ/Akt2 acts in medullary Pten-neoplasia by supporting proliferation (Figure 2.5 B,C).

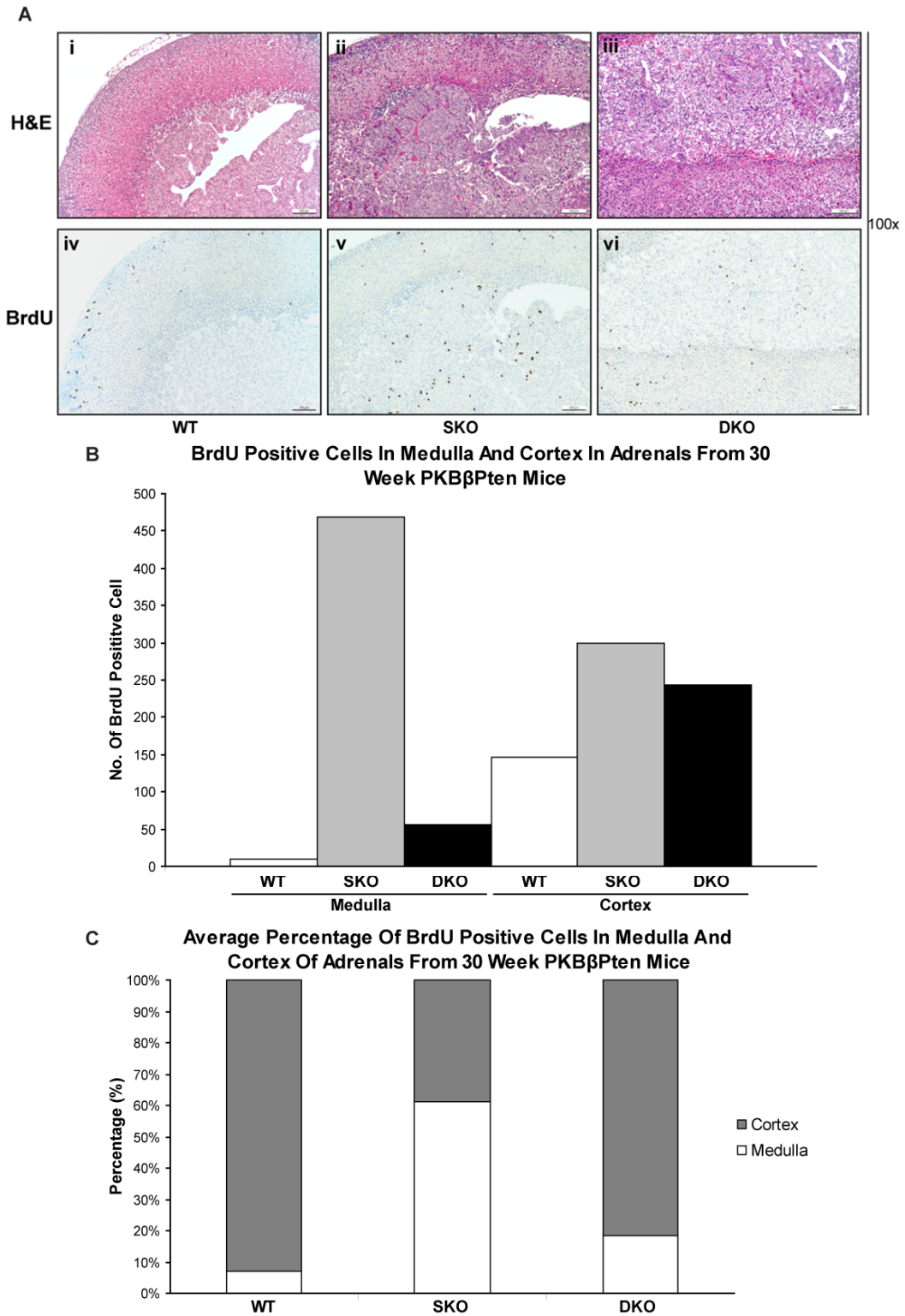

Figure 2.5. Loss of PKBβ/Akt2 In Pten$^{+/-}$ Mice Reduces Proliferation Specifically In The Adrenal Medulla Indicating Impaired Pten-Driven Proliferation. (A) Histological analysis of adrenals from (i) WT, (ii) SKO and (iii) DKO mice by H&E staining illustrating the medullary and cortical area, with increased BrdU staining observed primarily in the medulla of (v) SKO which is severely reduced in (vi) DKO and almost completely absent in (iv) WT medulla. Quantitation of total (B) BrdU positive cells in the medulla and cortex of analyzed mice, and expressed as a percentage of total BrdU positive cells (C). WT; wild-type, SKO; single knockout (Pten$^{+/-}$), DKO; double knockout (PKBβ/Akt2$^{-/-}$Pten$^{+/-}$)

2.4. PKBβ/Akt2$^{-/-}$Pten$^{+/-}$ Adrenals Display Impaired Activation Of PKB/Akt And Downstream Signaling Through mTORC1

Proliferative signaling driven by activated PKB/Akt has been studied in detail, including in the Pten mouse model, with numerous studies illustrating signaling via mTORC1 is central to this process. To determine whether differences in PKB/Akt activation and downstream signaling via mTORC1 could be contributing to the proliferative differences observed between SKO and DKO adrenals, immunohistochemistry examining this pathway was performed on SKO and DKO adrenals (Figure 2.6).

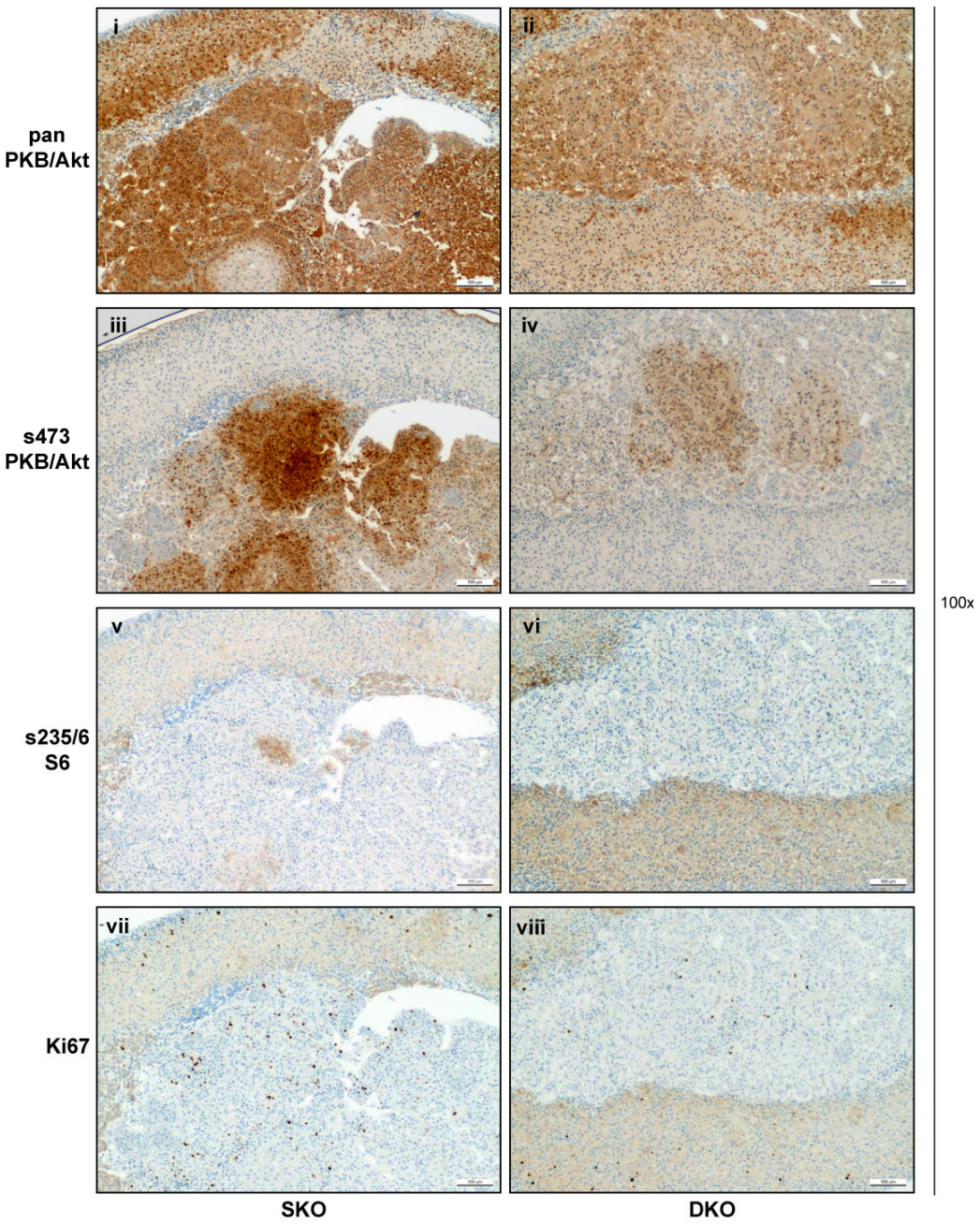

Figure 2.6. PKBβ/Akt2 Loss In Pten+/- Mice Results In Strong Reduction In Activated PKB/Akt And Signaling Via mTORC1 And Correlates With Decreased Proliferation In Adrenal Medulla. Immunohistochemical analysis of serial sections from SKO adrenals showing adrenal medulla expression of (i) pan PKB/Akt that displays robust activation of PKB/Akt (iii) and activation of ribosomal protein S6 (v) downstream of mTORC1 in neoplasic areas and correlating with high expression of the Ki67 proliferation marker (vii). Concomitant loss of PKBβ/Akt2 results in decreased panPKB/Akt expression (ii), activation (iv) and downstream proliferative signals (viii) through mTORC1 as illustrated by loss of S6 phosphorylation (vi) SKO; single knockout ($Pten^{+/-}$), DKO; double knockout ($PKB\beta/Akt2^{-/-}Pten^{+/-}$)

2.5. $PKB\beta/Akt2^{-/-}Pten^{+/-}$ Adrenals Exhibit Impaired Signaling Required For Catecholamine Synthesis And Decreased Adrenomedullin Expression

Human pheochromocytoma is characterized clinically by hypersecretion of catecholamines and currently analysis of catecholamines and their metabolites the metanephrines, remains the most reliable method for correct diagnosis of pheochromocytoma (Ram and Engelman, 1979; Eisenhofer et al., 2003). On a cellular level, the signaling pathways involved in activation of catecholamine secretion have been shown to involve both the ERK kinases and the transcription factor CREB (Hoeflich and Bielohuby, 2009; Mahata et al., 2011). Additionally, in both pheochromocytoma cells and in the clinic, pheochomocytomas have been shown to be strongly associated with increases in both adrenomedullin mRNA expression and circulating plasma concentrations (Kobayashi et al., 2004; Cotesta et al., 2005; Thouennon et al., 2010a), which is implicated in mediating growth-stimulatory and anti-apoptotic effects (Thouennon et al., 2010b). Furthermore, it has been shown that adrenomedullin and its growth-stimulatory effects are negatively regulated by Pten in the PC12 pheochromocytoma cell line (Betchen et al., 2006), suggesting it could be involved in pheochromocytoma formation in the Pten mouse model used in these studies. On the basis of this published data, activation of ERK, CREB and expression of adrenomedullin was examined in the adrenals from DKO compared to SKO by immunohistochemical analysis and qRT-PCR respectively. As shown in Figure 2.7 A, the adrenal medulla from SKO mice showed robust activation of ERK (i) and CREB (iii) indicating activation of signaling for production of catecholamines. Both ERK (ii) and CREB (iii) activation was greatly diminished in the adrenal medulla of DKO mice suggesting that PKBβ/Akt2 has suppressive effects not only on growth but on adrenal dysfunction observed in pheochromocytomas in human patients.

As a further indication that not only signaling pathways are activated, but that expression of secreted factors involved in pheochromocytoma are also affected, qRT-PCR was performed for dopamine β-hydroxylase (Dbh), the rate limiting enzyme for catecholamine synthesis and adrenomedullin that is frequenly observed to be upregulated in human pheochromocytoma (Figure 2.7B). In SKO adrenals, both Dbh and adrenomedullin were highly expressed and consistent with the other data showing DKO mice are not completely unaffected in medullary size, and neoplasia signaling, DKO mice showed a mild increase in Dbh and adrenomedullin expression compared to WT mice, but a dramatic reduction compared to SKO adrenals.

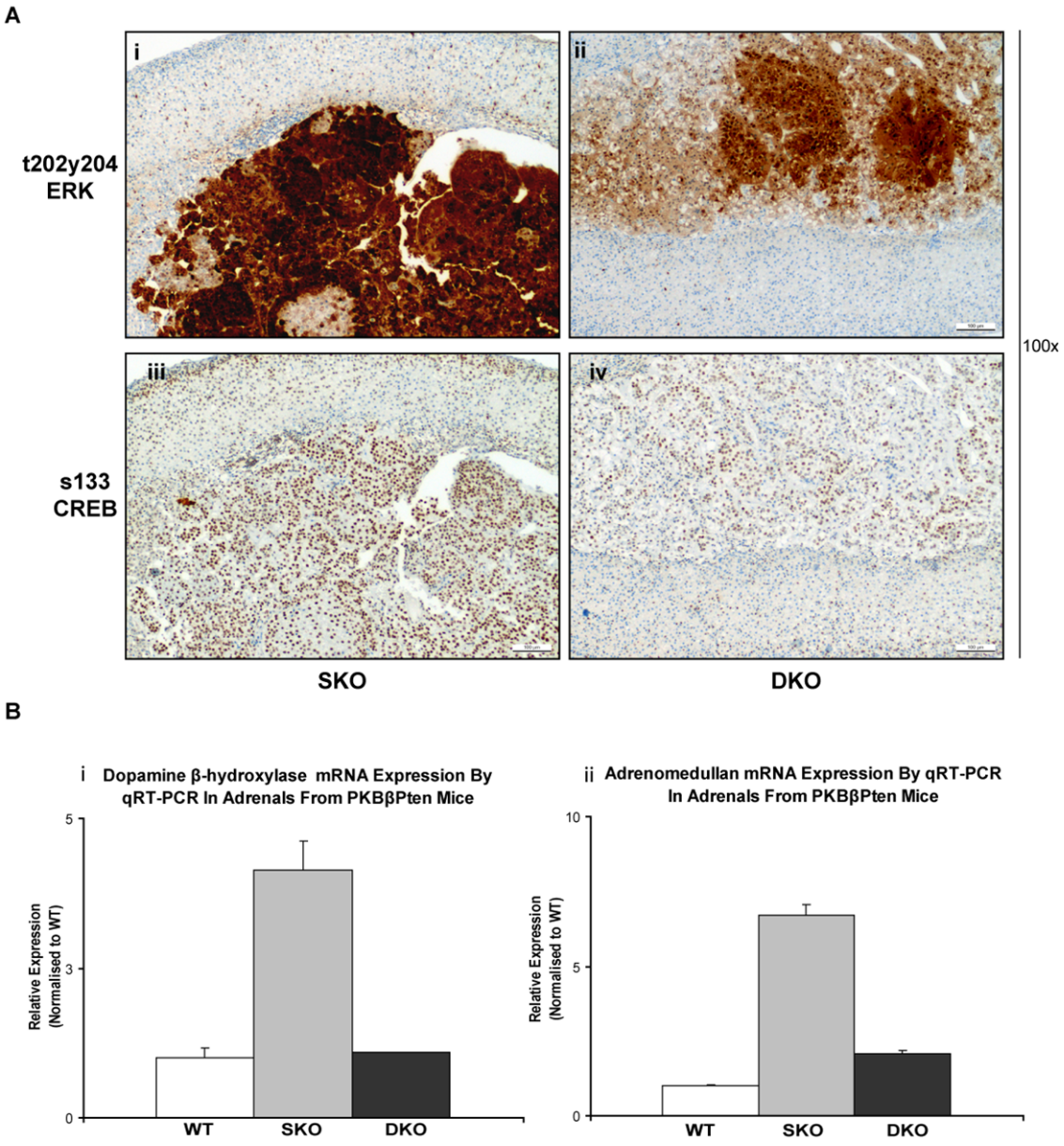

Figure 2.7. Increases In Catecholamine Signaling In Pten$^{+/-}$ Adrenals Is Dramatically Reduced Upon Concomitant Loss Of PKBβ/Akt2. Immunohistochemical analysis of serial sections from SKO adrenals showing robust adrenal medulla expression of activated (i) ERK and (iii) CREB involved in catecholamine synthesis, compared to DKO mice (ii and iv). (B) qRT-PCR analysis of (i) dopamine β-hydroxylase and (ii) adrenomedullin reveals robust upregulated in Pten$^{+/-}$ adrenals is restored to almost baseline WT levels by additional loss of PKBβ/Akt2. WT; wild-type, SKO; single knockout (Pten$^{+/-}$), DKO; double knockout (PKBβ/Akt2$^{-/-}$Pten$^{+/-}$)

3. Discussion

Pheochromocytomas are a poorly understood and understudied tumour, primarily due to it being both often benign and classified as a rare tumour type. However, evidence suggests that it is under-diagnosed and as malignant pheochromocytoma has a poor prognosis, invariably being fatal, these factors highlight a crucial need for both a greater understanding of the mechanisms driving this tumour type and development of effective therapeutics to treat malignant cases (Eisenhofer et al., 2004). A role for Pten and PKB/Akt activation in pheochromocytomas has been highlighted both *in vitro* and in the clinic. Pheochromocytoma cell lines, particularly the model PC12 cell line, have been used in numerous studies to illustrate a central role for PKB/Akt in both proliferation and cell survival (Andjelkovic et al., 1998; De Vita et al., 2000; Alvarez-Tejado et al., 2001; Powers et al., 2001; Fujita et al., 2006; Adler et al., 2009). Additionally, in human tumour samples activated PKB/Akt has been reported by various independent studies (Fassnacht et al., 2005; van Nederveen et al., 2006; Adler et al., 2009) and Pten loss also reported, albeit with low frequency (van Nederveen et al., 2006). However, Pten mouse models have provided further support for a crucial role of PKB/Akt activation in pheochromocytomas, with pheochromocytoma formation observed in multiple heterozygous Pten models, on various mouse backgrounds (Freeman, Dan et al., 2006). Recently it was also published that deletion S6K1 downstream of PKB/Akt and mTORC1 could rescue pheochromocytoma formation in Pten heterozygous mice, as ability attributed to the observation that S6K2 is not expressed in the adrenals and is therefore unable to compensate for S6K1 loss. The lack of expression of S6K2 was also observed in human adrenal samples, as was elevated S6K1 in human pheochromocytoma samples, illustrating the importance of this pathway downstream of PKB/Akt in pheochromocytoma and the use of the Pten mouse model to study mechanisms of pheochromocytoma formation (Nardella et al., 2011). Further, conditional deletion of Pten in the adrenals also results in malignant metastatic pheochromocytoma formation, suggesting a potential important role for hyperactive PKB/Akt in pheochromocytoma progression (Korpershoek et al.,

2009). Despite these connections between PKB/Akt and pheochromocytoma formation, analysis of the specific roles of the PKB/Akt isoforms has been limited (Andjelkovic et al., 1998). The observation that loss of PKBα/Akt1 was insufficient to prevent adrenal medulla neoplasia in the $Pten^{+/-}$ mouse model, despite it inhibiting neoplasia in all other analyzed tissues (Chen et al., 2006), indicated that this tumour type and model could provide valuable insights into the contribution of PKBβ/Akt2 to tumourigenesis.

To explore the contribution of PKBβ/Akt2 to $Pten^{+/-}$ adrenal neoplasia, we bred PKBβ/$Akt2^{-/-}$ with $Pten^{+/-}$mice to generate PKBβ/$Akt2^{-/-}Pten^{+/-}$ mice. Analysis of these mice at 30 weeks (Figure 2.2) indicated that PKBβ/Akt2 not only contributes to pheochromocytoma formation, but also to a neoplasia in a number of other tissues. In addition, to a significant reduction in neoplasia burden in B-cell tumours, a trend to reduced neoplasia was observed in prostate, uterus, liver and pancreas (Table 2.3). It was also noted at necropsy that even in male mice up to 11months of age, DKO failed to exhibit neoplasic infiltrates in the liver, whilst β-cell mass appeared to be increased on the surface of DKO pancreas' and DKO thyroids/parathyroid glands often appeared to be reduced in size compared to SKO littermates, suggesting PKBβ/Akt2 may well have subtle contributions to tumourigenesis and function in the context of Pten loss in these tissues. This was supported by preliminary analysis of prostate, uterus and thyroid tissues that indicated that PKBβ/Akt2 does contribute with various degrees to Pten-induced neoplasia in these tissues (see Appendix II and general discussion).

The most striking phenotype observed was the DKO adrenals, which both in appearance and size resembled that of WT animals (Figure 2.3 A,B). Histological examination of DKO adrenals revealed that they were not completely unaffected, although tumourigenesis was severely suppressed specifically in the adrenal medulla which showed little to no neoplasia (Figure 2.4 A-D). Interestingly, analysis of 20 week old mice indicated that mild changes in DKO tissue were already apparent, suggesting that PKBα/Akt1, which Chen and colleagues had shown was able to partially suppress adrenal tumourigenesis, can support initiation of adrenal tumourigenesis but PKBβ/Akt2 may be required for at least efficient neoplasia development, if not for progression itself (Appendix II). Furthermore, as loss of a single allele of

PKBβ/Akt2 was sufficient to severely retard neoplasia development (Fig 2.3 and 2.4), this suggest that if PKBβ/Akt2 specific substrates involved in pheochromocytomas could be identified and targeted, even mild reductions in their activities could provide valid and effective therapeutic benefits. However, it should be noted that decreased proliferation and signaling via mTORC1, which was identified to be decreased in DKO compared to SKO animals (Figures 2.5 and 2.6), can be utilized by all PKB/Akt isoforms. Consistent with a concept that PKBβ/Akt2 isoform-specific targets may provide the most effect treatment, it was recently shown that treatment of pheochromocytoma patients with the mTORC1 inhibitor RAD001 resulted in only a weak response (Druce et al., 2009). This supports the idea that mTORC1 is not the primary determinant driving malignant pheochromocytoma and hence not the most suitable target. However, the identification of strongly activated functional hormonal signaling via ERK and CREB in SKO that could be dramatically reduced in DKO adrenals (Figure 2.7A) is interesting for at least two reasons. Firstly, this provides another target that if used in combination therapy with an inhibitor like RAD001, could result in a more effective therapeutic response. Secondly, inhibition of ERK, in the context of inhibiting catecholamine synthesis, could result in a decrease in serious side-effects like hypertension, thereby providing a better quality of life for pheochromocytoma patients.

The ability for PKBβ/Akt2 ablation to almost completely restore the increase in Dbh and adrenomedullin expression in $Pten^{+/-}$ adrenals to that of wild-type animals suggests a possible specific role for PKBβ/Akt2 in regulating Dbh and/or adrenomedullin expression. Understanding the signaling by which PKBβ/Akt2 attains this could have particular promise therapeutically. Adrenomedullin is already strongly linked to pheochromocytoma proliferation and survival (Kobayashi et al., 2004; Cotesta et al., 2005; Zeng et al., 2006; Thouennon et al., 2010b). However, adrenomedullin is a potent vasodilator and can robustly induce angiogenesis and is linked to tumour angiogenesis (Nakamura et al., 2006; Kaafarani et al., 2009) as well as invasion (Keleg et al., 2007; Ramachandran et al., 2007) in a wide variety of tumour types (Zudaire et al., 2003; Nantermet et al., 2004; Betchen et al., 2006), resulting in it increasingly being examined as a possible drug target in cancer (Garcia et al.,

2006). Accordingly, inhibition of adrenomedullin expression supported by PKBβ/Akt2 could hold therapeutic benefits in multiple tumour settings.

This study provides strong evidence that PKBβ/Akt2 can mediate tumourigenesis in the setting of reduced Pten levels and that it is the major isoform involved in adrenal medulla pheochromocytoma formation. This involves increases in cell proliferation and functional activation of signaling known to promote catecholamine synthesis and release. Accordingly, this study indicates that therapeutic targeting of PKBβ/Akt2 signaling could have strong therapeutic potential in pheochromocytoma treatment.

Materials And Methods

Mice

The PKBβ/Akt2 and Pten mutant mice used in the study have been described previously (Di Cristofano et al., 1998; Trotman et al., 2003; Yang et al., 2003; Dummler et al., 2006). Mice were housed in groups with 12-h dark-light cycles and with access to food and water ad libitum, in accordance with the Swiss Animal Protection Laws. Mice were monitored by taking weekly weight measurements from 4 weeks of age until 30 weeks of age. All procedures were conducted with the appropriate approval of the Swiss authorities.

Tissue Preparation For Histology

For histological analysis, anaesthetized mice were sacrificed, dissected and organs either immediately snap-frozen or fixed in 4% paraformaldehyde (PFA)-phosphate buffered saline (PBS). Snap frozen tissues were placed in a plastic cassette and covered with OCT compound, before being frozen by placing the cassette into a 2-methylbutane bath in dry ice. Frozen tissues were then stored at -80^{o}C until sectioned for use. Tissues placed in 4% PFA-PBS were allowed to fix overnight (~18hrs) at 4°C. Tissues were then subjected to a series of washes with PBS, 50% ethanol (EtOH)/PBS and 70%EtOH/PBS before being processed and embedded in paraffin using the Medite TPC15 Paraffin Processing Unit (Medite, Wintergarden, FL). Histological staining and immunohistochemistry (IHC) was performed on 12um frozen or 4um paraffin tissue sections, cut using a HM560H cryostat or M355S microtome (Thermo scientific, Fremont, CA).

Hematoxylin and Eosin Staining And Adrenal Measurements

For hematoxylin and eosin (H&E) staining, sections were deparaffinized and stained according to the standard protocols using reagents purchased from Sigma (St.Louis, MO). Sections were quantified by sectioning through the adrenals to reveal the adrenal medulla. Three sections per animal were taken at least 100um apart and stained. Area of adrenal medulla and cortex were calculated by measuring the length and width of the total adrenal and medulla region using the ImageAccess Enterprise v10 software (Imagic Bildverarbeitung, Glattbrugg, Switzerland).

Immunohistochemistry

4um sections were cut from paraformaldehyde-fixed, paraffin-embedded tissues and stained using the Ventana Discovery automated immunostainer (Ventana Medical Systems, Tucson, AZ). IHC was performed with or without cell conditioning using buffers CC1 or CC2, blocked with 5% normal donkey, goat or sheep serum for 1 hour. Primary antibodies diluted in Ventana antibody diluent were then applied and allowed to incubate for 1 hour to overnight at 25C. Primary antibodies and dilutions used were anti-BrdU (#11 170 376 001) 1:1000 (Roche Applied Sciences, Rotkreuz, CH), Ki67 (#RM-9106-S0) 1:100 (Thermo Scientific, Fremont, CA), pPKB/AKT S473 1:25 (#4060), panPKB/AKT 1:125 (#4685), pS6 Ribosomal Protein S235/6 1:100 (#4858) pCREB S133 (#9198) 1:100, pERK1/2 T202Y204 (#4370) 1:125 (all Cell Signaling Technologies, Danvers, MA). After washing, sections were incubated with biotinylated donkey anti-mouse (#715-067-003) or anti-rabbit (711-067-003) secondary antibodies (Jackson Immuno Research Inc, West Grove, PA) for 32mins at 37C, before detection with HRP/DAB, OmniMap or UltraMap conjugates and counterstained with haematoxylin (all Ventana Medical Systems, Tucson AZ). Photomicrographs were taken on a Nikon Eclipse E600 microscope (Nikon, Milville, NY).

BrdU Analysis And Quantification

Animals were injected with 50mg/gm BrdU i.p. 24hrs prior to necropsy. Organs collection, processing and IHC was performed by standard procedures and using reagents stated in the preceding sections ‘Tissue Preparation For Histology’ and ‘Immunohistochemistry. Sections were quantified by sectioning through the adrenals to reveal the adrenal medulla. Three sections per animal

were taken at least 100um apart and stained and positive cells counted under the microscope. Area of adrenal medulla and cortex were calculated by measuring the length and width of the total adrenal and medulla region using the ImageAccess Enterprise v10 software (Imagic Bildverarbeitung, Glattbrugg, Switzerland).

RNA isolation, Amplification and qRT-PCR

Snap-frozen adrenals were placed in TRIZOL (Invitrogen, Basel, CH), homogenized and total RNA purified according to the manufacturer's instructions. Total RNA was then subjected to additional purification using the RNeasy mini columns (Qiagen, Germantown, MD) according to the manufacturer's instructions, eluted in 25ul of nuclease-free water and concentration and purify measured on a Nanodrop spectrometer (Thermo Scientific, Fremont, CA). cDNA amplification was performed on 1-2ug of RNA with 10ul of 2.5mM dNTPs (Roche Applied Sciences, Rotkreuz, CH), 1ug of oligo dT (Microsynth, Balgach, CH), 1ul of RNase inhibitor (#M0307), 1ul of Avian Myeloblastosis Virus reverse transcriptase (#M0277) (both from NEB, Ipswich, MA) in a final reaction volume of 20ul. PCR amplification was performed at 42°C for 60min followed by 95°C for 5min. Amplified cDNA was diluted to a concentration of 12.5ng/ul of original total RNA using nuclease-free water and stored at -20C for qRT-PCR. qRT-PCR was performed after primer validation and optimization of cDNA and primer concentrations. qRT-PCR was performed in a final reaction volume of 25ul using SYBR green PCR master mix from Applied Biosystems (Foster City, CA) and run on an ABI Prism 7000 qRT-PCR thermal cycler (Applied Biosystems, Foster City, CA). Primer sequences used for qRT-PCR were from PrimerBank [http://pga.mgh.harvard.edu/primerbank; (Spandidos et al., 2010)]. Sequences were: Adrenomedullin (f) GGAATAAGTGGGCGCTAAGTC and (r) CAAGAGTCTGGGTAGGAACTGT (PrimerBank ID: 6752988a2) and dopamine beta hydroxylase (f) GAGGCGGCTTCCATGTACG and (r) TCCAGGGGGATGTGGTAGG (PrimerBank ID: 20336728a1).

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III. GENERAL DISCUSSION

As the primary interest in studying the PI3K-PTEN-PKB/Akt signaling pathway is arguably the potential of therapeutically targeting its members and downstream signaling in cancer and other diseases, this general discussion will focus on the implications from the studies described in this thesis, in this broader context. Specific discussion of both the implications of targeting PKBβ/Akt2$^{-/-}$ and the PI3K-PTEN-PKB/Akt signaling pathway in ovarian dysfunction and pheochromocytoma can be found in the discussions in the respective sections of this thesis.

Therapeutic inhibition of the PI3K-PTEN-PKB/Akt pathway and downstream signaling is currently a major focus of drug development programs worldwide. This has been facilitated by extensive research to characterize the regulatory mechanisms of this signaling and define the transduction of signals from membrane to functional response. Whilst cell-based studies have been crucial in identifying mechanisms and signaling pathways, the relevance and potential of targeting these pathways is demonstrated through the use of mouse models that provide the physiological complexity of cross-talk between different cell and organs and reveals secondary effects on non-targeted tissues. Analysis of PI3K-PTEN-PKB/Akt pathway mouse tumour models has shown that they faithfully recapitulate many of the molecular changes seen in the human cancers they have been designed to represent and in doing so, they have highlighted a number of challenges in targeting this pathway and its signaling [for review see (Restuccia and Hemmings, 2010)]. Targeting upstream of Pten whilst effective in many cases and a current therapeutic option, as observed with ErbB2 overexpression, is undermined in the common scenario of loss or reduced Pten levels. Similarly, solely targeting the mTORC1 arm of downstream PKB/Akt signaling, is in most settings at best cytostatic or temporarily efficacious. Therefore, PKB/Akt as the point of convergence of the PI3K pathway and also divergence: as the effector kinase for the plethora of downstream signaling, is a prime candidate for effective therapeutic inhibition. However, as PKB/Akt has three isoforms and mediates, via its downstream signaling, a wide variety of crucial functions in the body, its inhibition poses

considerable challenges regarding isoform compensation and efficient inhibition to attain a feasible therapeutic window whereby therapeutic response outweighs off-targets effects. Indeed, this has been a particular challenge in the case of PKBβ/Akt2 due to the spectre of inducing insulin resistance or diabetes by its inhibition. This has resulted in efforts focusing on developing specific PKBα/Akt1 or pan PKB/Akt inhibitors. The potential of developing and using PKBα/Akt1 inhibitors is based upon its strong association with proliferation and anti-apoptosis coupled with its high occurrence of activation in a wide variety of tumour types (see general introduction Table 3 and Part 2 Table 2.3). Selective ablation of PKBα/Akt1 in mouse tumour models has provided proof-of-principle for the importance and effectiveness of PKBα/Akt1 inhibition (part 2 Table 2.2) and highlighted that even a reduction in PKBα/Akt1 can have considerable effects on tumour development (Chen et al., 2006). However, these inhibitors are potentially limited in their use against tumours displaying high PKBβ/Akt2 or PKBγ/Akt3 (see general introduction Table 3 and Part 2 Table 2.3). As selective inhibition of PKBβ/Akt2 could induce insulin resistance an alternative is the use of pan PKB/Akt inhibitors. Apart from potentially broad spectrum application, these inhibitors could prove to be effective in obtaining a therapeutic response, without insulin resistance, by administration of a concentration that sufficiently reduces activation of PKBβ/Akt2 in the tumour, whilst preventing compensation from other PKB/Akt isoforms and deleterious effects on PKBβ/Akt2 in insulin-sensitive tissues. Such compounds hold promise and support for this concept has been reported using mouse xenograph models (Rhodes et al., 2008). In relation to these therapeutic contexts, the studies performed in this thesis have a number of important implications and this is the focus of the following discussion.

Complete inhibition of PKBβ/Akt2 is deleterious due to its potential for development of insulin resistance and diabetics. The findings from work in this thesis suggest that this could also have an additional adverse metabolic reaction in promoting PCOS. Importantly, these studies suggest that reduction of PKBβ/Akt2 would not have such an effect, as heterozygous PKBβ/Akt2 mice do not appear to have an increased tendency to develop cysts (Appendix I) and show no apparent reproductive or steroidogenic dysfunction (Figure 1.9). Accordingly, in terms of potential off-target effects, this supports the concept for

partial inhibition of PKBβ/Akt2 as a viable therapeutic option. Conversely, in terms of the therapeutic potential of reducing activation of PKBβ/Akt2, this study demonstrates that adrenal medulla neoplasia is significantly reduced in heterozygous PKBβ/Akt2 mice (Figures 2.1, 2.2, 2.3) and therefore inhibitors that would elicit such an effect could be therapeutically beneficial as an anti-cancer treatment. Furthermore, the data from B-cell tumours suggests that this may be effective not only in pheochromocytomas, as heterozygous PKBβ/Akt2 mice also shows considerable reduction in tumour burden (P=0.031; Appendix II). However, in the formation of neoplasia in other tissues, reduction of PKBβ/Akt2 does not appear to have such a strong effect, suggesting application of such inhibitors would have to be specific to certain tissues.

Positive effects from partial inhibition of activated PKBβ/Akt2 in tissues outside of the adrenal and B-cell tumours may however still exist and contribute to the effectiveness of pan PKB/Akt inhibitors. Indeed, visually and based on weights of affected organs, a number of organs showed a trend toward a reduced tumour burden, particularly the uterus, prostate, mesenteric lymph nodes and thyroid, all of which are known to express at least moderate levels of PKBβ/Akt2. To explore this possibility, preliminary studies analyzing both the uterus and prostate were performed and showed noticeable differences on both a histopathological and molecular level (Appendix II). Uteri from PKBβ/Akt2$^{-/-}$ Pten$^{+/-}$ animals showed a striking difference with Pten$^{+/-}$ animals with proliferating cells in PKBβ/Akt2$^{-/-}$Pten$^{+/-}$ animals localized almost exclusively to the endometrial epithelium, whilst Pten$^{+/-}$ animals showed proliferation almost exclusively in the stromal myometrial compartment. It is well know that these two compartments communicate with each other, so interpreting whether this is cell autonomous or a response to effects from loss of PKBβ/Akt2 signaling in the adjacent tissue will require tissue specific ablation to confidently draw conclusions. A potential interpretation based on the current data, is that the loss of proliferation in the stromal compartment of PKBβ/Akt2$^{-/-}$Pten$^{+/-}$ uteri represents tumourigenic signaling driven by Pten reduction is exclusively mediated by PKBβ/Akt2 in this compartment and that in the endometrial compartment PKBβ/Akt2 has a inhibitory effect on Pten-induced neoplasia in the epithelial cells. A precedent for similar activity by the PKB/Akt isoforms has been reported and interestingly also in epithelial cells, when either PKBα/Akt1

or PKBβ/Akt2 were ablated in either the ErbB2 or Polyoma Middle-T breast tumour models (Maroulakou et al., 2007). Placing these findings in terms of pan PKB/Akt inhibitors, these findings suggest that such inhibition would be far more effective due to reduction of isoform specific functions of both PKBα/Akt1 and PKBβ/Akt2. This is further supported by the observation that the uteri from PKBβ/Akt2$^{+/-}$Pten$^{+/-}$ mice were smaller than uteri from either Pten$^{+/-}$ or PKBβ/Akt2$^{-/-}$Pten$^{+/-}$ mice, suggesting a partial inhibition of neoplasia in both compartments. Coupling inhibition of activated PKBβ/Akt2 with inhibition of activated PKBα/Akt1 that is presumably contributing to residual neoplasia signaling, based upon the fact that complete or partial ablation of PKBα/Akt1 in Pten$^{+/-}$ mice significantly suppressed endometrial neoplasia (Chen et al., 2006), suggests that pan PKB/Akt inhibitors could be more effective and have less adverse effects than single isoform inhibitors for tumours from either compartment.

Preliminary analysis of prostates from PKBβ/Akt2$^{-/-}$Pten$^{+/-}$ also indicated that PKBβ/Akt2 contributes to prostate neoplasia and inhibition of its functions could be beneficial in the therapeutic effects of pan PKB/Akt inhibitors. As was previously reported, complete or partial ablation of PKBα/Akt1 in the setting of Pten heterozygous prostates leads to considerable neoplasia suppression (Chen et al., 2006). Therefore, additional inhibition of potential PKBβ/Akt2 contributions would further support the anti-tumour activities of pan PKB/Akt inhibitors. The preliminary studies in the PKBβ/Akt2$^{-/-}$Pten$^{+/-}$ prostates showed an average ~15-20% reduction in weight at 30 weeks of age. However, no noticeable difference was noted in prostates of 20 week old mice. Analysis of the pathology of the adrenal glands and immunohistochemistry for downstream signaling showed little to no differences (Appendix I). However, analysis at 30 weeks of age showed noticeable differences pathologically in the number and pathology of affected glands, as well as in signaling involving mTORC1, p27 and ERK known to be activated in neoplastic signaling in these mice (Appendix II). This data suggests that PKBβ/Akt2 could contribute not to the initiation of prostatic neoplasia in the context of reduced Pten, but more importantly to the progression of the tumour. This suggests that inhibiting these contributions mediated by PKBβ/Akt2 by the use of pan PKB/Akt inhibitors would be far superior to isoform specific inhibitors and at least in this setting suggest that

they could also be effective in inhibiting progression and later stages of tumours, which is often the status of prostatic tumours affecting patients at the time of presentation. As this is often accompanied by additional reduction in Pten levels or even complete loss, it will be of considerable interest to determine what are the effects of reducing not only activation of PKBα/Akt1 but also PKBβ/Akt2 in this more aggressive and malignant tumour scenario.

IV. CONCLUSIONS

Collectively, the data contained in this thesis suggests that PKBβ/Akt2 does contribute to various aspects of tumourigensis in a tissue specific manner. Combining these finding with the published results on ablation of PKBα/Akt1 in $Pten^{+/-}$ mice advocates the use of pan PKB/Akt inhibitors and suggests that the PKB/Akt isoforms act not in the common concept of mutations and cellular aberrations in cancer being either drivers or passengers. Rather more appropriate may be a 'bad driver and nagging passenger' concept, whereby activation of one of the PKB/Akt isoforms, primarily PKBα/Akt1, may act like a bad driver of the tumour, but additional contributions by other activated PKB/Akt isoforms, primarily PKBβ/Akt2, act like a nagging passenger to exacerbate the negative effects or tumour progression mediated by the driving PKB/Akt isoform. Such a concept would explain the reported suppression of Pten-induced tumourigensis in prostate upon loss of the driving PKBα/Akt1 isoform in mice and also the observed unaffected initiation but decreased progression upon loss of PKBβ/Akt2 observed in this study. Similarly, this would be consistent with adrenals observations from this study where initial changes are observed on a molecular level in PKBβ/$Akt2^{-/-}$$Pten^{+/-}$ adrenals but these fail to progress to neoplasia, although restoration of a single isoform of PKBβ/Akt2 does allow mild, although still significantly suppressed neoplasia development. Importantly, it should be noted that the relationship of development to progression does not appear to be relative to the tissue expression levels of the PKB/Akt isoforms and hence total levels of PKB/Akt. Chen and colleagues in their analysis of the effects of loss of PKBα/Akt1 to Pten-induced tumourigensis analyzed expression levels between PKBα/Akt1 and PKBβ/Akt2. They could show that, for a poignant example, adrenal

expression of PKBβ/Akt2 was similar but still less than PKBα/Akt1 and the adrenal phenotype they observe is significantly less pronounced than that observed in this study. Conversely, when PKBβ/Akt2 was the major, potentially driving isoform, as seen in the thyroid where they reported PKBβ/Akt2 expression was two-fold greater than PKBα/Akt1 and significant PKBγ/Akt3 expression was noted, progression to tumours was still inhibited upon PKBα/Akt1 loss by 50% compared to $Pten^{+/-}$ mice. This is a simplified conceptual view compared and it remains to be seen whether the differences observed in these studies, using an essentially genetically homogenous and relatively mild tumour model, are relevant clinically in the context of tumours with multiple, highly heterogeneous aberrations and often more severe Pten loss. However, the comparative analysis of the data from the Chen study and this study raises sufficient questions to warrant further exploration of these questions and the relationships between PKBα/Akt1 and PKBβ/Akt2 in the contexts of tumour initiation and progression.

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VI. APPENDIX

Part I: Loss of Protein Kinase B beta (PKBβ/Akt2) Predisposes Mice To Ovarian Cyst Formation And Increases The Severity Of Polycystic Ovary Formation in vivo

Additional Data And Controls Related To Ovarian Phenotypes Observed In PKBβ/Akt2 Mice

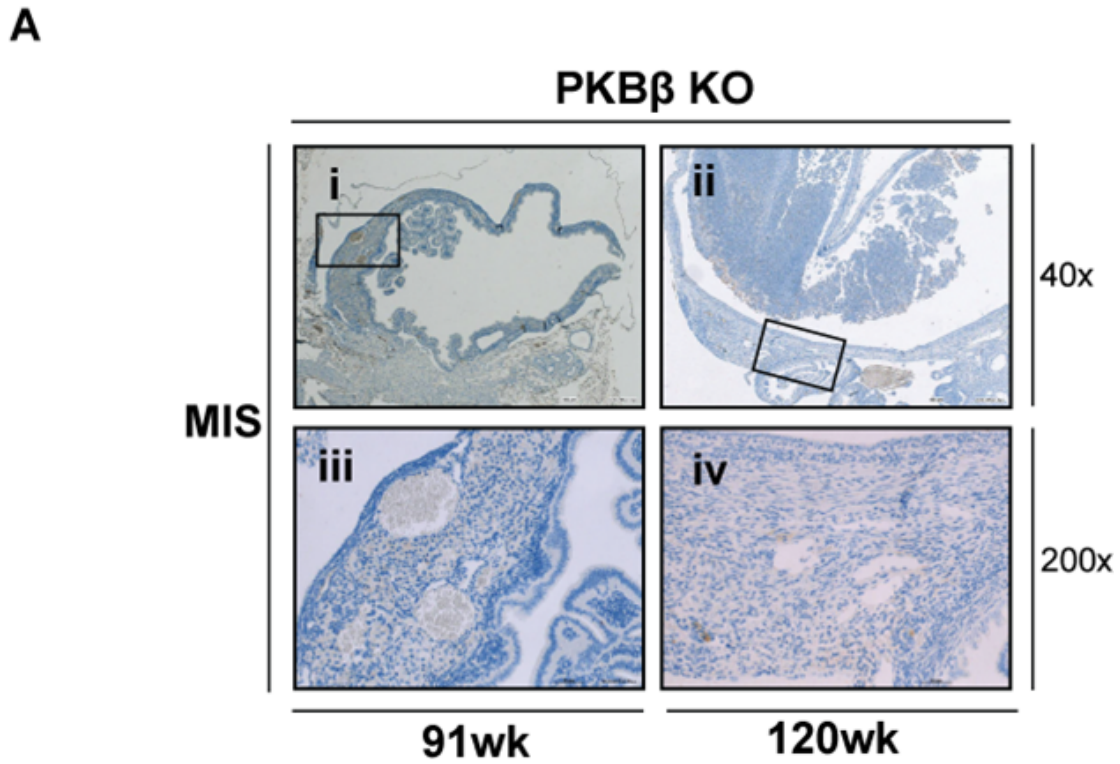


Figure A1.1 Aged PKBβ/Akt2 KO Ovaries Show An Absence Of Granulosa Cells Indicating Follicular Exhaustion. (A) Staining with anti-mullerian inhibiting substance to illustrate granulosa cells in aged PKBβ/Akt2 KO ovaries were completely negative, illustrating an absence of granulosa cells. magnification 40x (i, ii) and 200x (iii, iv).

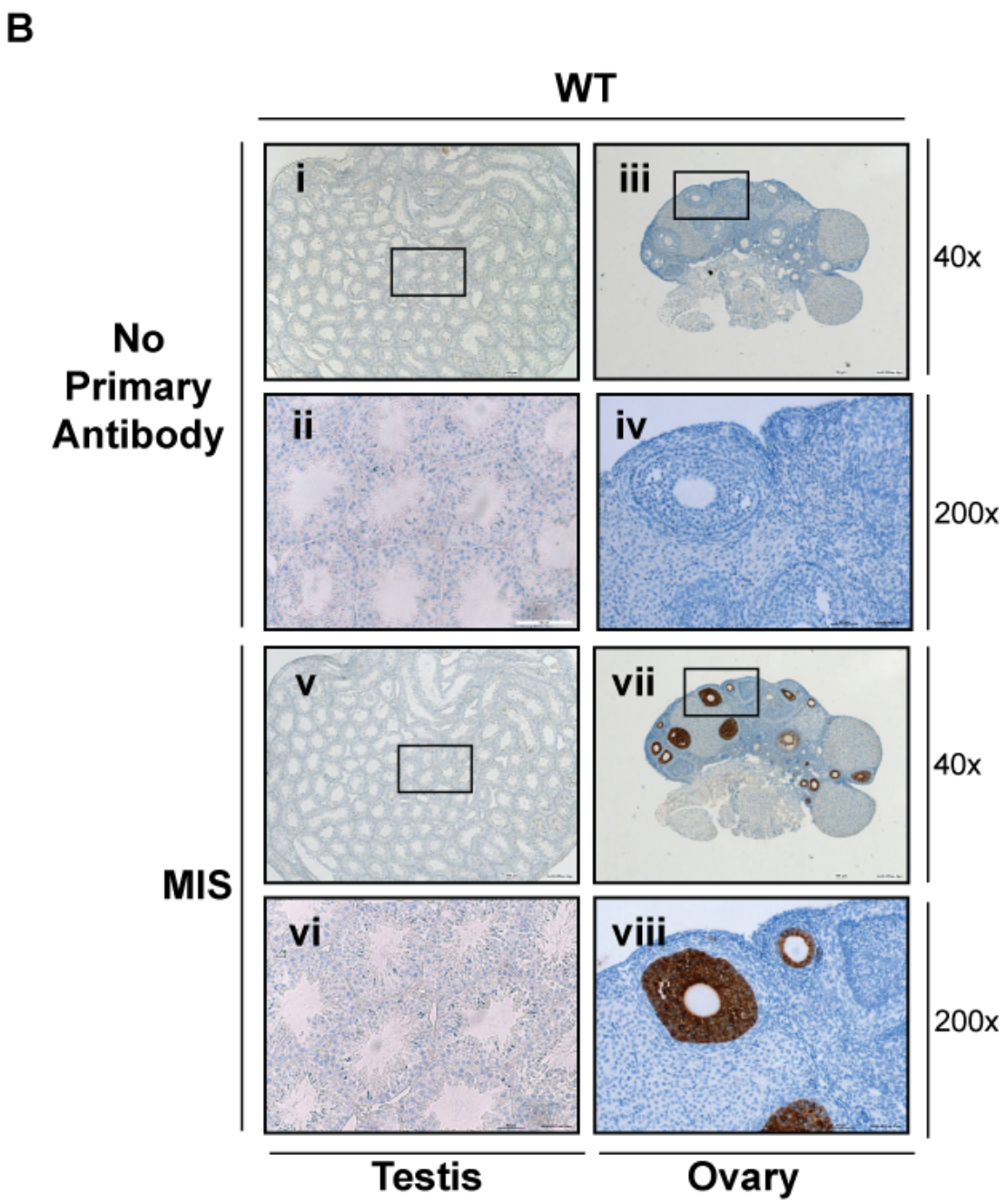


Figure A1.2 Aged PKBβ/Akt2 KO Ovaries Show An Absence Of Granulosa Cells Indicating Follicular Exhaustion. (B) Immunohistochemistry for anti-mullerian inhibiting substance showing specificity and positive reactivity of the antibody specifically to granulosa cells. No background was observed in the conditions used when the antibody neither was not applied (i-iv), nor was non-specific reactivity observed using testis negative control tissue. In contrast robust and specific staining was observed in the ovary solely in granulosa cells. Magnifications 40x (i, iii, v, vii) and 200x (ii, iv, vi, viii).

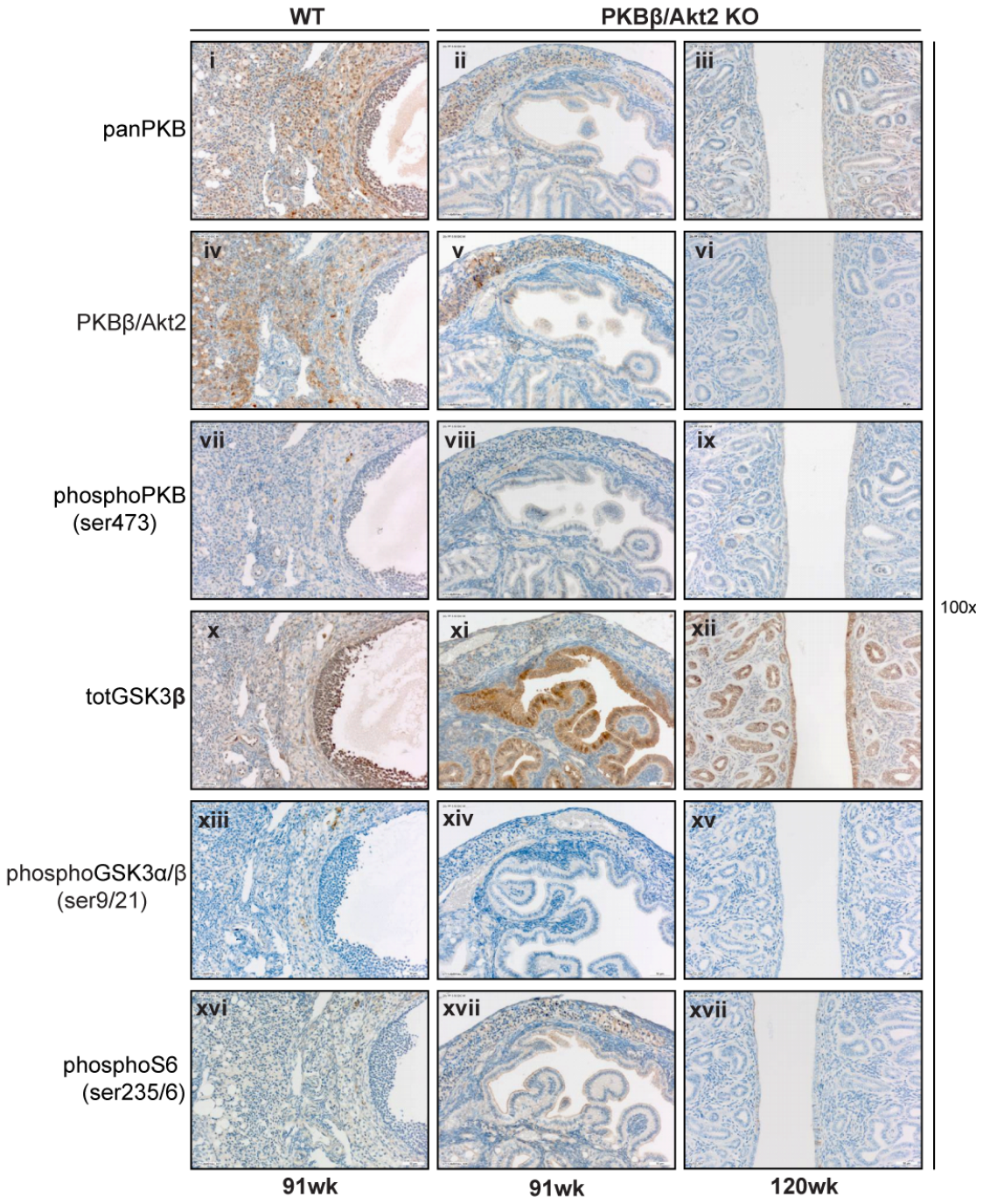


Figure A1.3 Aged PKBβ/Akt2 KO Ovaries Show No Significant Changes In PKB/Akt Activation And Downstream Signalling Compared To WT. Immunohistochemical analysis of PKBβ/Akt2 KO ovarian cysts illustrated no significant differences in downstream PKB/Akt signaling compared to aged WT ovaries. Magnification 100x.

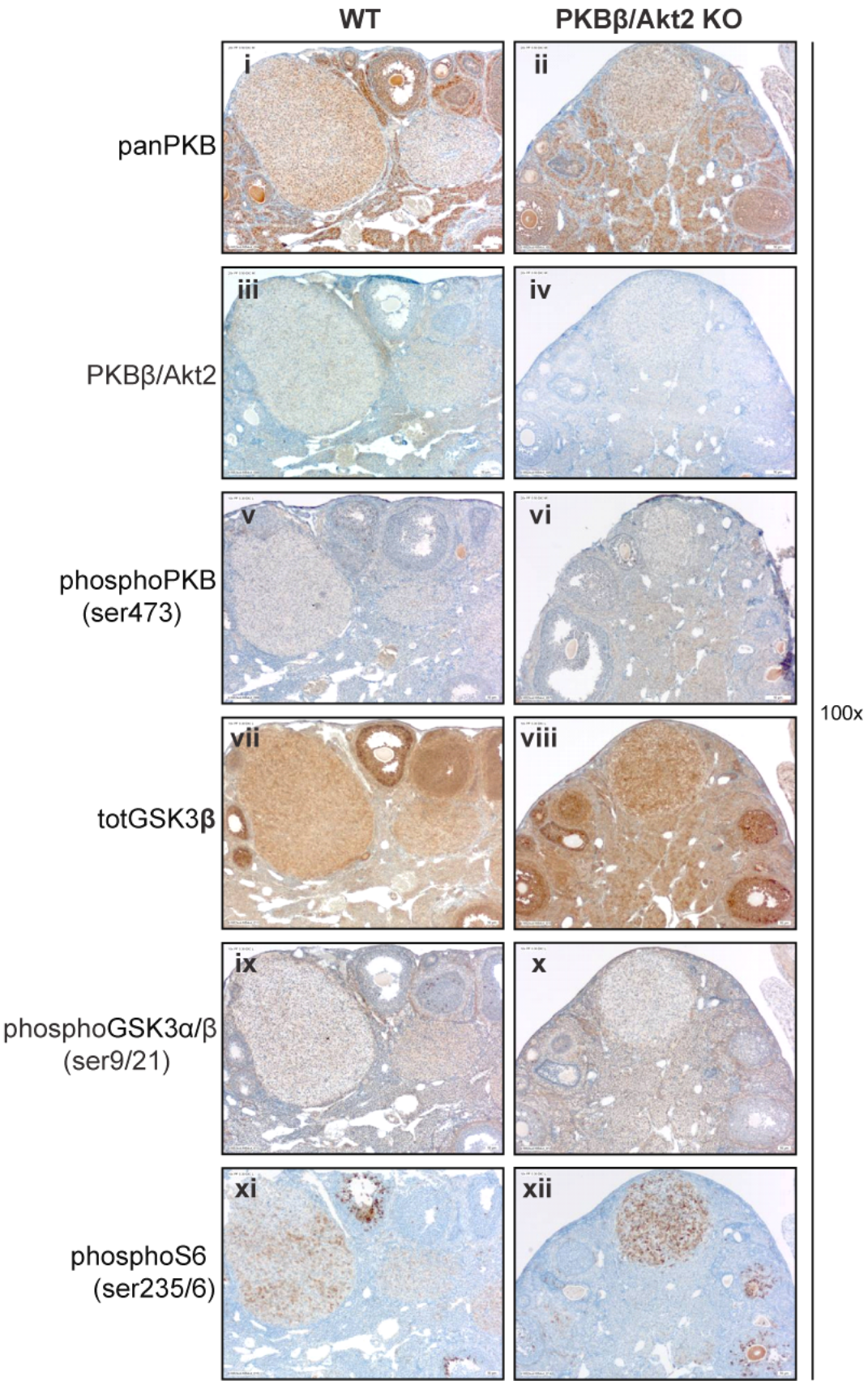


Figure A1.4 Young PKBβ/Akt2 KO Ovaries Show No Significant Changes In PKB/Akt Activation And Downstream Signaling Compared To WT. Immunohistochemical analysis of young WT or PKBβ/Akt2 KO ovaries illustrated no significant differences in downstream PKB/Akt signaling. Magnification 100x.

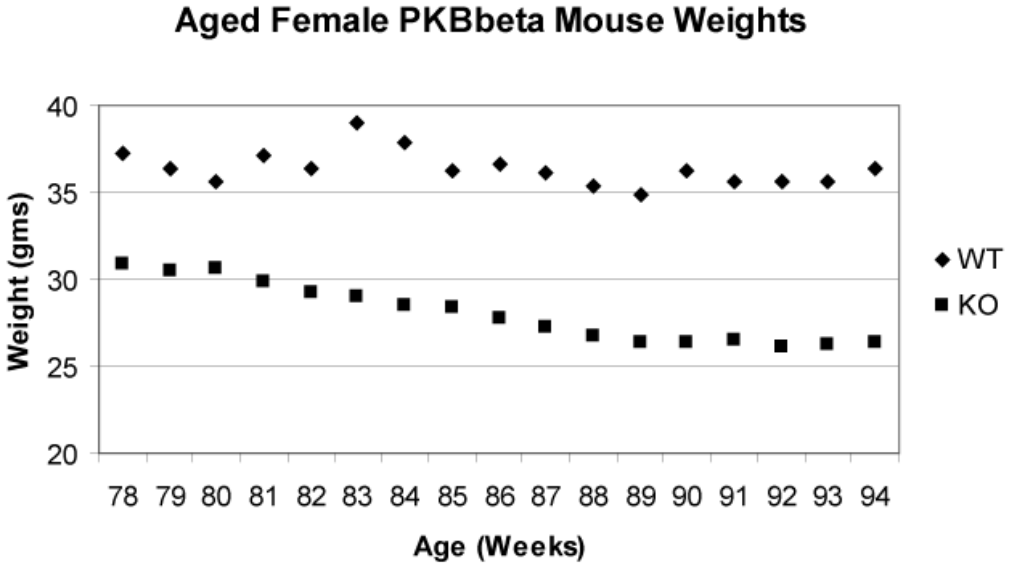


Figure A1.5 Aging PKBβ/Akt2 KO Mice Show Progessive Loss Of Weight Compared To WT Mice In Weeks Preceeding First Observed Cyst Development. PKBβ/Akt2 KO exhibited loss of weight reflected by very low levels of white adipose tissue at necropsy. As white adipose tissue is the most abundant extra-ovarian source of aromatase responsible for conversion of testosterone to estrogen, loss of white adipose and aromatase activity may reflect another mechanism contributing to increased testosterone levels in aged PKBβ/Akt2 KO mice.

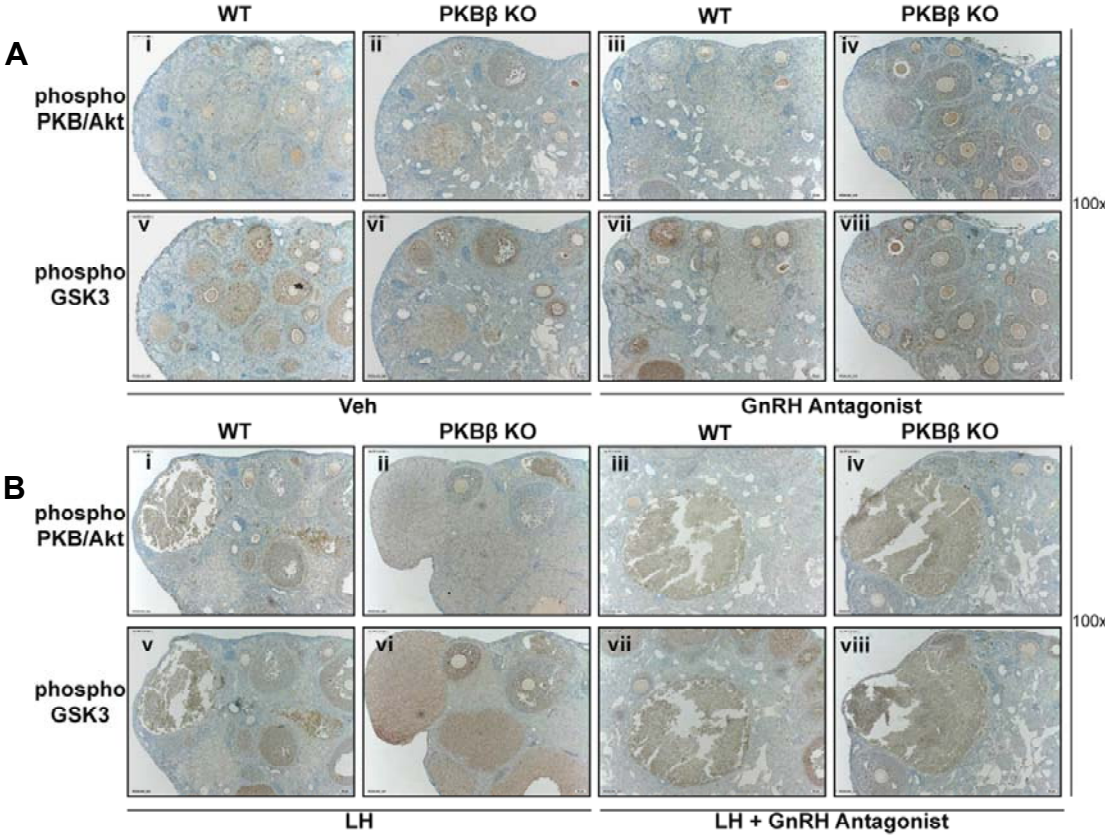


Figure A1.6 Treatment Of Mice In PCOS Induction Experiments Show No Significant Differences In PKB/Akt Activation And Downstream Signaling. Activation of PKB/Akt and downstream signaling was active in control (A) vehicle or GnRH antagonist treated and experimentally treated (B) LH or LH + GnRH antagonist ovaries from WT (i, iii, v, vii) and PKBβ/Akt2 KO (ii, iv, vi, viii) mice, however, no significant differences were observed between animals. All magnifications for IHC are 100x.

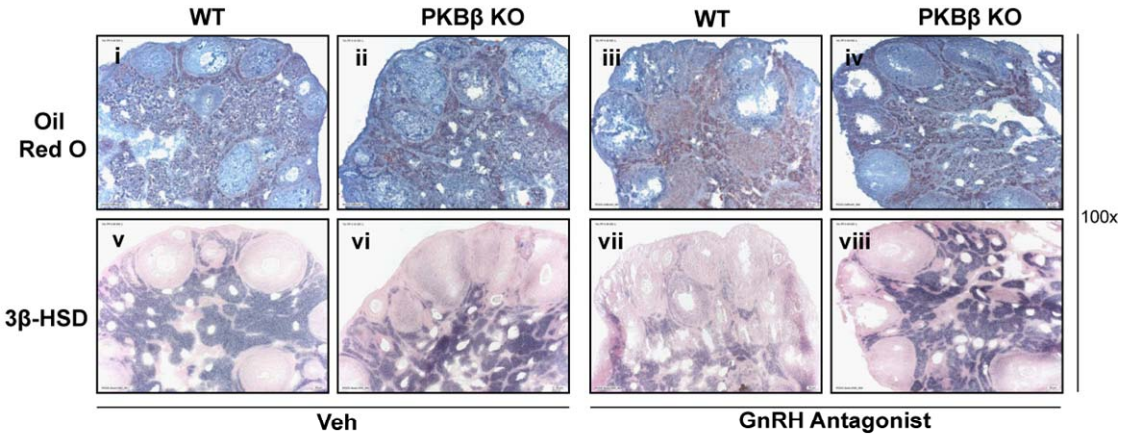


Figure A1.7 Controls Showing Oil Red O Staining And 3β-HSD Activity In Vehicle And GnRH Antagonist Treated Mice. No significant differnces in lipid accumulation in control ovaries treated with vehicle or GnRH antagonist were observed in PKBβ/Akt2 KO mice (ii, iv) in areas with active androgen steroidogenesis [indicated by staining for 3β-HSD activity (v-viii)] compared to WT (i, iii). All magnifications for IHC are 100x.

Protein	PKB β KO	FSHR +/-	FSHR KO	LH O/E	LH KO	LHR KO
Ovary						
Size	–	–	↓↓↓	–	↓↓↓	↓↓↓
Cell Abnormalities	interstitial	–	↑Interstitial	↑ thecal	–	–
Cysts	+++	–	++	+	+	–
-Unilaterial	√	–	√ (tumours)	–	–	–
-Bilateral	X(√)	–	–	√	√	–
-Serous	√	–	√	–	√	–
-Heamorrhagic	X(√)	–	–	√	–	–
Tumours	X	–	sertoli-leydig	granulosa leutomas	–	–
Uterus						
Size	↑↑↑+cysts	↑↑+ cysts	↓↓↓	–	↓↓↓	↓↓↓
Epithelium	hyperplasia	↑ hyperplasia	↓	–	–	↓
Stroma	–	–	↓	–	–	↓
Myometrium	↓	–	↓	–	–	↓
White Adipose	↓↓↓	–	↓↓↓	–	–	–
Follicle Stimulating Hormone	?	↑	↑↑	?	–	↑
Leutinizing Hormone	?	↑↑	↑	↑↑↑	?	↑
Testosterone	↑	↑	↑↑	↑↑	?	?
Estrogen	?	↓↓	↓↓↓	↑	↓	↓
Progesterone	?	↓	↓↓↓	↑↑↑	↓	↓
Reference		Danilovich et al	Balla et al; Danilovich et al; Kumar et al; Abel et al	Matzuk et al; Milliken et al	Pakarainen et al	Zhang et al; Lin et al; Lei et al

Figure A1.8 Ovarian Phenotypes Of Mouse Models Disrupting Gonadotrophin Signaling. Ovarian and steroidogenic phenotypes of mice with genetic modification of the gonadotrophin receptors, compared to that observed in aged PKBβ/Akt2 KO mice. -; no change/ not applicable, +; positive, √; observed, x; not observed, ↑; increased, ↓; decreased, ?; not reported/determined.

Additional Data And Controls Related To Ovarian Phenotypes Observed In PKBβ/γ-Akt2/3 Mice

Mouse Line	PKB α/Akt1		PKB β/Akt2			PKB βγ/ Akt2/3
	WT	KO	WT	HT	KO	DKO
No. Of Animals	6	14	15	6	18	4
No. Of Animals With Ovarian Cysts	2 (33%)	7 (50%)	7 (47%)	3 (50%)	14 (78%)	4 (100%)
-Unilateral/Bilateral	2/0	5/2	5/2	2/1	7/7	2/2
-Maximum Diameter (mm)	5	8	8	8	40	20
-Average Diameter (mm)	3.5	4.5	4.3	4.3	9.6	14.3
-Earliest Detection (wks)	98	91	91	89	91	80
Mean Age Of Animals (wks)	~112	~108	~108	~100	~106	~112
Age Range of Animals	98-121	91-128	91-128	89-108	91-134	80-127

Figure A1.9 Observed Cyst Formation In PKBβ/γ-Akt2/3 DKO Mice Compared To WT, PKBα/Akt1 and PKBβ/Akt2 Mice. Additional ablation of PKBγ/Akt3 on a PKBβAkt2 background leads to 100% penetrance, increased size and earlier presentation of cysts. WT; wild-type, HT; heterozygous, KO; knockout, DKO; double knockout.

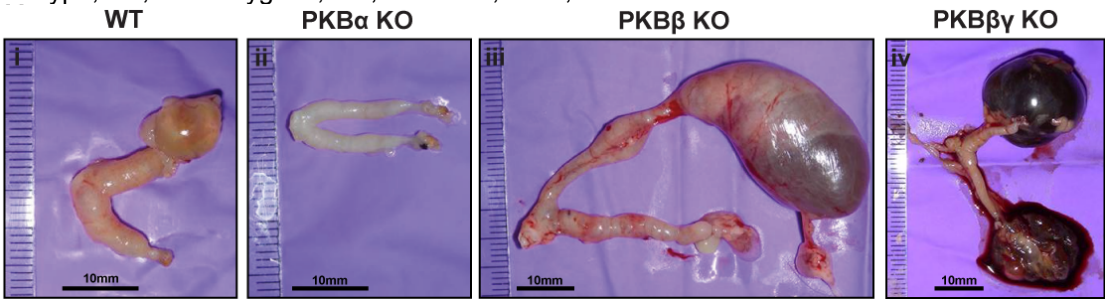


Figure A1.10 Presentation Of Cyst From PKBβ/γ-Akt2/3 DKO Mice Compared To WT, PKBα/Akt1 and PKBβ/Akt2 Mice. Cystic ovaries isolated from WT (i) and PKBα/Akt1 KO (ii) mice fail to show atresia or small ovarian cyst formation, whilst PKBβ/Akt2 KO (iii) and PKBβ/γ-Akt2/3 DKO (iv) mice show severe ovarian cyst formation, with Akt2/3 DKO showing increased hemorrhagic and bilateral cyst involvement.

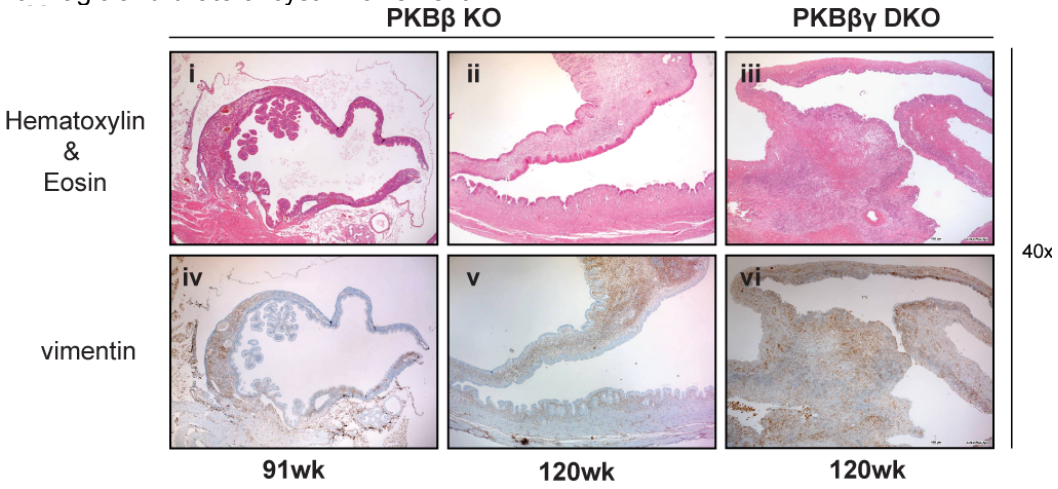


Figure A1.11 Histology Of Cyst From PKBβ/γ-Akt2/3 DKO Mice Compared To PKBβ/Akt2 Mice Illustrating Thecosis. Cysts from PKBβ/γ-Akt2/3 DKO (iii, vi) mice show increased poorly differentiated stromal cell content (iii) by heamatoxylin and eosin staining and vimentin (vi) staining reflecting of thecal-interstitial hyperplasia compared to early 91wk old (i,iv) and late 120wk old (ii,v) aged PKBβ/Akt2 KO mice.

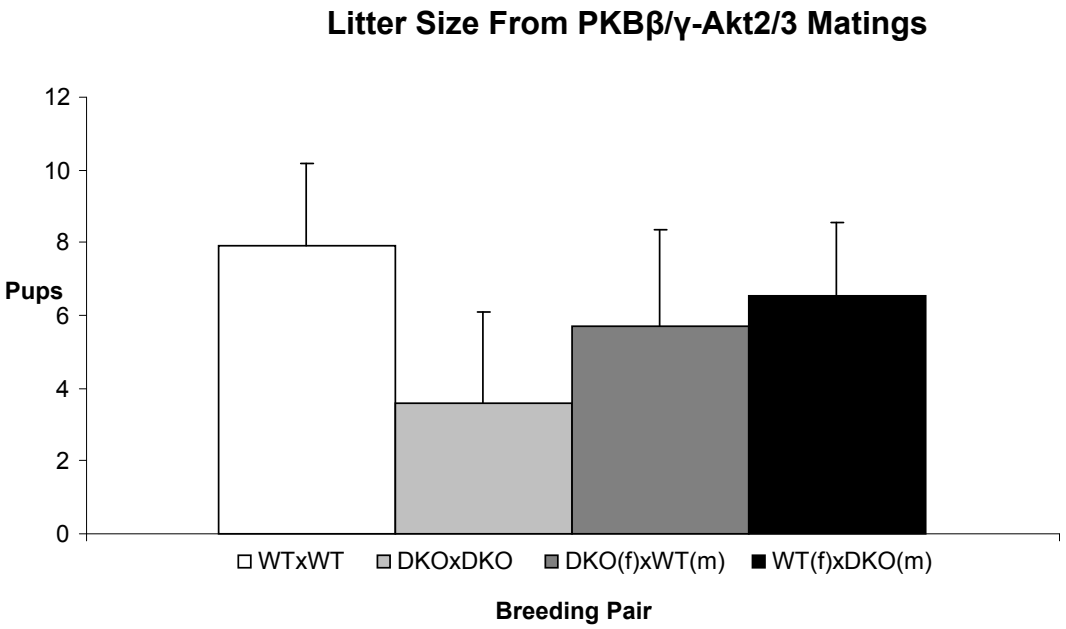
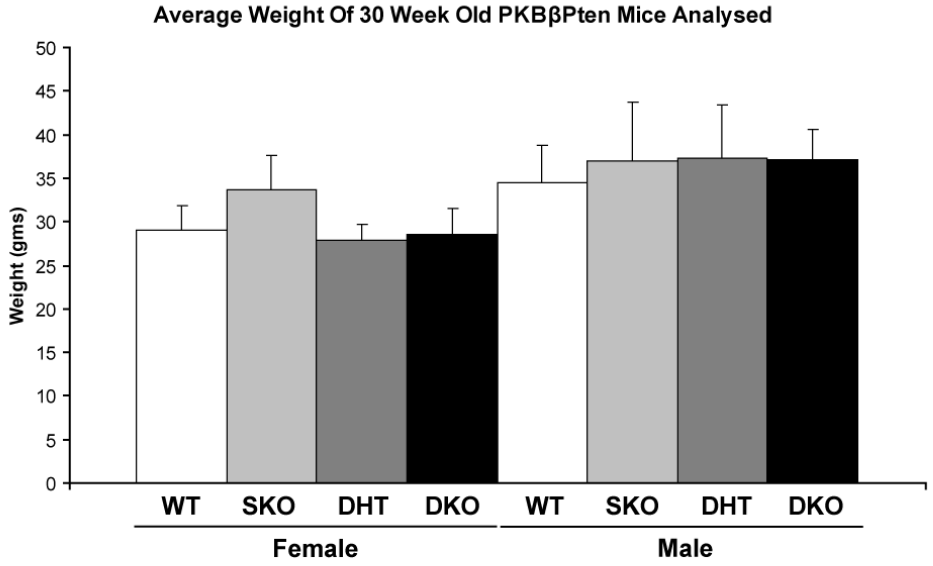


Figure A1.12 Litter Sizes From PKBβ/γ-Akt2/3 DKO Matings Compared To WT. Matings from PKBβ/γ-Akt2/3 DKO mice show a strong trend towards lower litter sizes suggesting reproductive fertility is compromised in female PKBβ/γ-Akt2/3 DKO mice.

Part II: Loss of Protein Kinase B beta (PKBβ/Akt2) Suppresses Pheochromocytoma Formation Induced By Pten Deficiency In Mice

Additional Data On Adrenal Phenotypes Observed In PKBβ/Akt2Pten Mice

A



B

		Adrenals	B-Cell (Neck Mass)	Brain	Liver	Mesenteric Lymph Node	Ovary	Pancreas	Uterine Horns	Anterior Prostate	White Adipose (Gonodal)
WT	Av. Wt.(gm)	0.006	0.002	0.519	1.306	0.017	0.009	0.312	0.079	0.044	0.115
	+/- SD (n)	0.001 (13)	0.004 (11)	0.020 (12)	0.186 (13)	0.007 (13)	0.002 (13)	0.045 (13)	0.019 (13)	0.009 (5)	0.044 (13)
SKO	Av. Wt.(gm)	0.018	0.504	0.630	1.759	0.046	0.012	0.357	0.317	0.107	0.081
	+/- SD (n)	0.007 (11)	0.354 (11)	0.036 (11)	0.410 (11)	0.021 (11)	0.005 (11)	0.052 (11)	0.242 (11)	0.035 (9)	0.054 (11)
DHT	Av. Wt.(gm)	0.008	0.150	0.619	1.333	0.031	0.010	0.325	0.170	0.087	0.061
	+/- SD (n)	0.002 (7)	0.090 (5)	0.029 (7)	0.265 (7)	0.013 (7)	0.002 (7)	0.030 (7)	0.098 (7)	0.019 (4)	0.023 (7)
DKO	Av. Wt.(gm)	0.007	0.111	0.615	1.548	0.032	0.013	0.309	0.275	0.086	0.047
	+/- SD (n)	0.001 (6)	0.070 (6)	0.024 (6)	0.256 (6)	0.017 (6)	0.003 (6)	0.056 (6)	0.141 (6)	0.025 (12)	0.018 (6)
p-value	WT:SKO	<0.001	<0.001	<0.001	0.002	<0.001	0.037	0.032	0.002	0.002	0.110
	WT:DHT	0.005	<0.001	<0.001	0.802	0.006	0.254	0.517	0.004	<0.001	0.014
	WT:DKO	0.326	0.001	<0.001	0.026	0.008	0.003	0.890	<0.001	0.008	0.001
	SKO:DKO	0.001	0.007	0.374	0.243	0.153	0.695	0.078	0.681	0.308	0.127
	SKO:DHT	0.004	0.031	0.562	0.038	0.112	0.338	0.192	0.179	0.099	0.412
	DKO:DHT	0.109	0.137	0.792	0.165	0.843	0.083	0.525	0.157	0.973	0.218

Figure A2.1 Comparison Of The Effects In DHT Mice On Whole Body (A) And Organs Weights (B) With WT, SKO and DKO Mice. Reduction in PKBβ/Akt2 levels on the background of Pten heterozygosity in adrenals and B-cell neoplasia strongly reverses the Pten neoplasia phenotype, although exhibits generally only weaker effects on weight gain in other organs. WT; wild-type, SKO; single knockout ($Pten^{+/-}$), DHT; double heterozygous ($PKB\beta/Akt2^{+/-}Pten^{+/-}$), DKO; double knockout ($PKB\beta/Akt2^{-/-}Pten^{+/-}$)

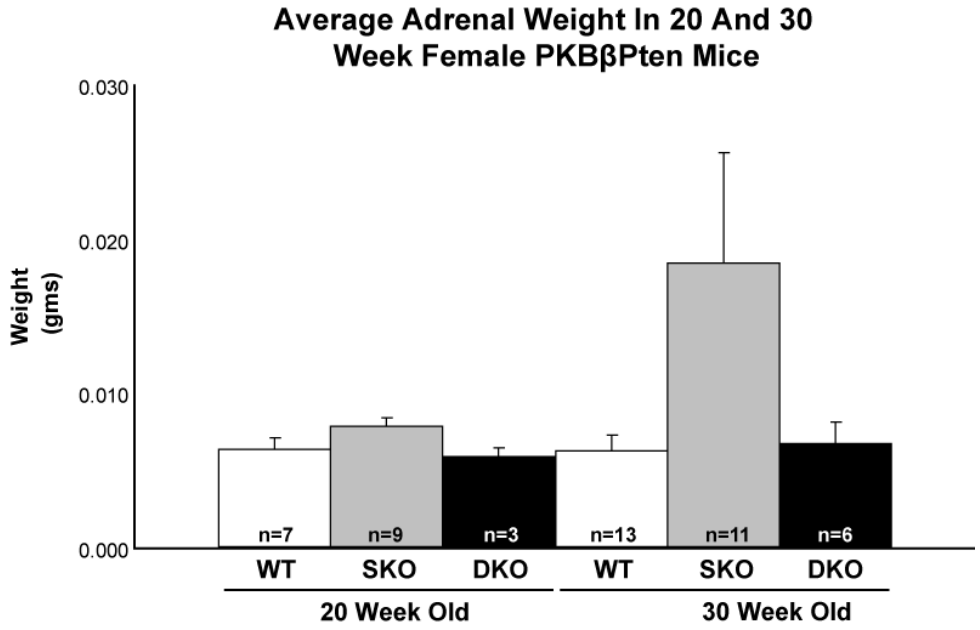


Figure A2.2 Comparison Of The Effects On Adrenal Weights Between WT, SKO and DKO Mice at 20 and 30 weeks. Loss of PKBβ/Akt2 on the background of Pten heterozygosity in adrenals is efficient in preventing increases in tumour burden in early (20 week old mice) and effectively halts tumour progression with similar tumour weights seen in 20 week and 30 week old mice. WT; wild-type, SKO; single knockout ($Pten^{+/-}$), DKO; double knockout ($PKB\beta/Akt2^{-/-}$ $Pten^{+/-}$)

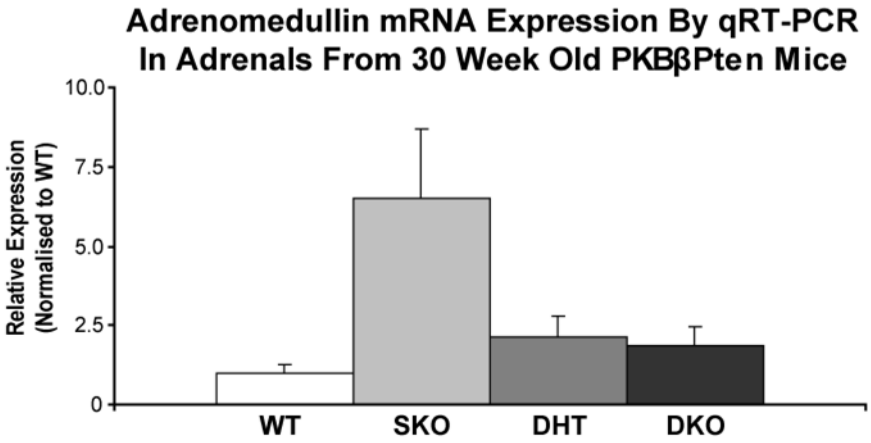
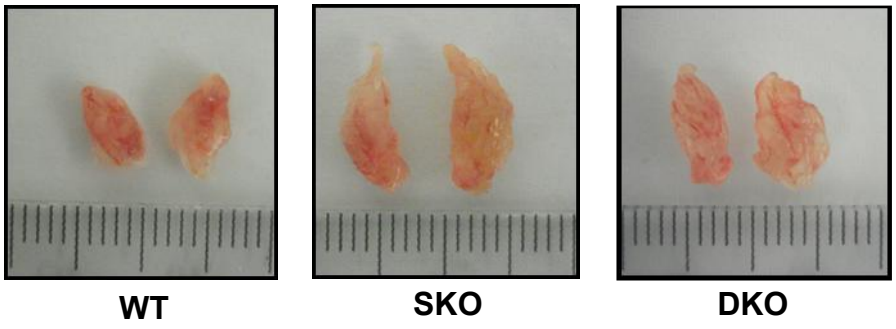


Figure A2.3 Effect Of Loss Of A Single Isoform Of PKBβ On Adrenomedullin mRNA Expresion Compared To WT, SKO and DKO Mice In 30 Week Old Mice. Reduction or loss of PKBβ/Akt2 on the background of Pten heterozygosity in adrenals is strongly suppresses increases in adrenomedullin expression known to have growth promoting effects and associated with pheochromocytomas. WT; wild-type, SKO; single knockout ($Pten^{+/-}$), DHT; double heterozygous ($PKB\beta/Akt2^{+/-}Pten^{+/-}$), DKO; double knockout ($PKB\beta/Akt2^{-/-}Pten^{+/-}$)

Additional Data On Prostate Phenotypes Observed In PKBβ/Akt2Pten Mice

A



B

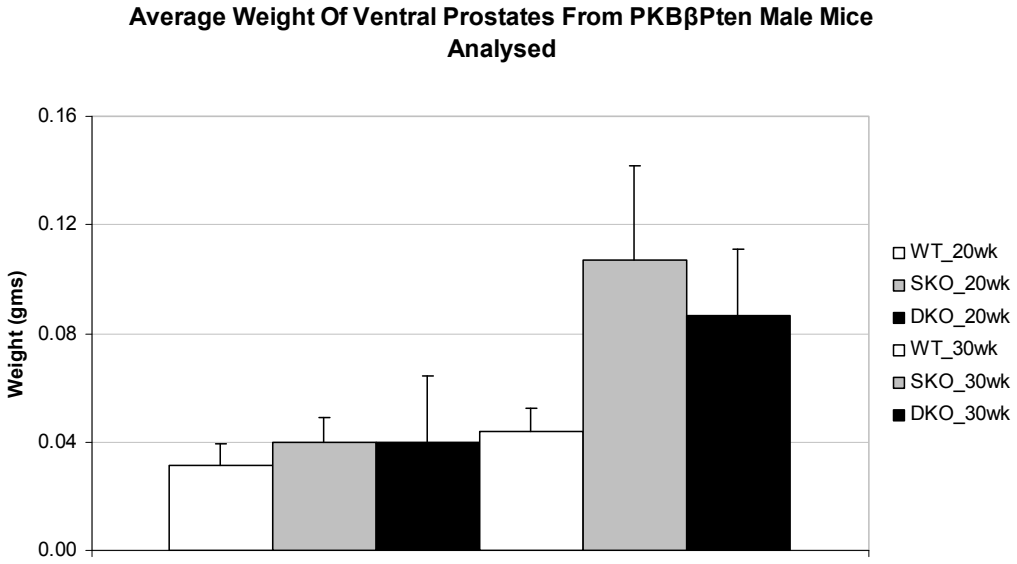


Figure A2.4 Effects Of Ablation Of The PKBβ/Akt2 Isoform In Pten$^{+/-}$ Mice On Prostate Presentation And Weight In 20 and 30 Week Old Mice. (A). Presentation of WT, SKO and DKO prostates from 30 week old mice shows a mice decrease in prostate size in DKO animals (B) Prostate weights at 20wks and 30wks from WT, SKO and DKO mice. WT; wild-type, SKO; single knockout (Pten$^{+/-}$), DKO; double knockout (PKBβ/Akt2$^{-/-}$Pten$^{+/-}$).

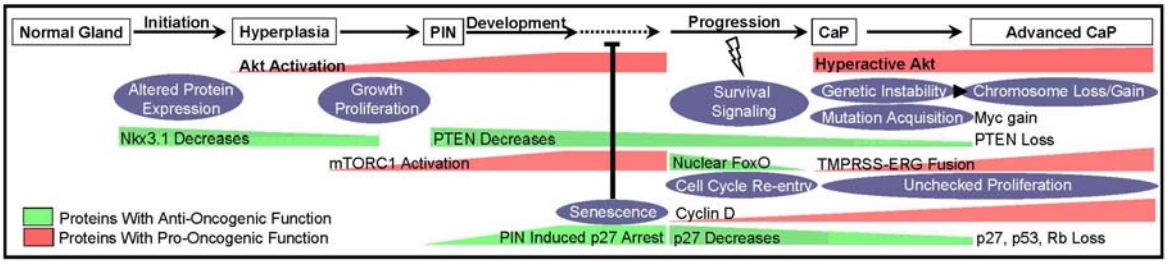


Figure A2.5 PKB/Akt Activation And Associated Events During Tumour Development In The Prostate. Initiation of tumourigenesis and hyperplasia occur through altered protein expression, which promotes AKT activation, mTORC1 activation and PIN development. p27-induced senescence prevents progression to CaP, which is overcome by AKT signaling combined with changes in the expression and/or activity of other proteins and genes. CaP displays high AKT activation, supporting proliferation, survival and acquisition of mutations with increasing genetic instability, leading to the gross chromosomal losses and gains that are characteristic of advanced malignant CaP (Restuccia and Hemmings, 2010).

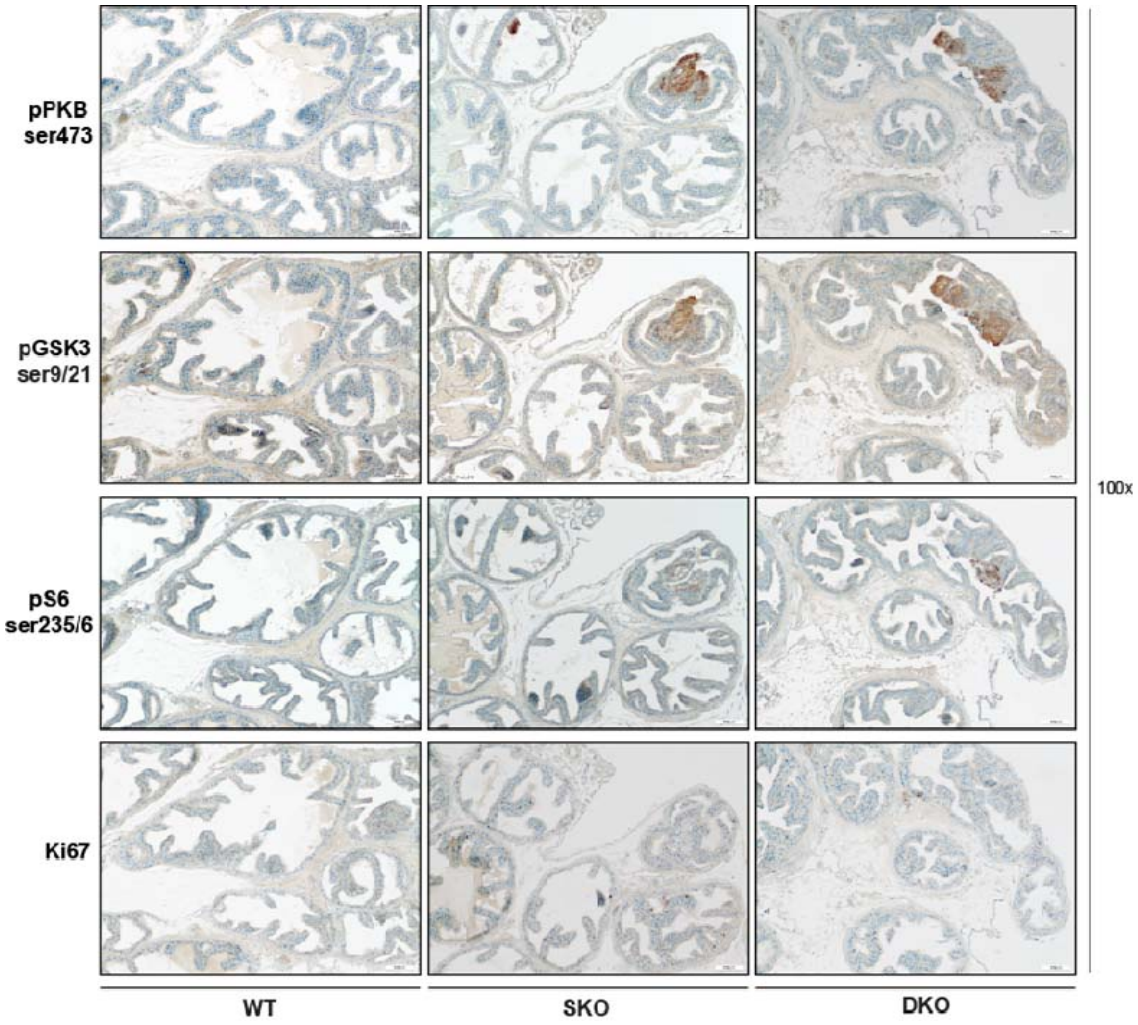


Figure A2.6. Effects On Downstream Signaling In Prostates From 20 Week Old PKBβ/Akt2Pten Mouse By Immunohistochemistry. Downstream signaling in prostates from SKO and DKO mice show no difference in neoplasia and downstream signaling, indicating PKBβ/Akt2 is dispensible for early prostate neoplasia resulting from Pten deficiency. WT; wild-type, SKO; single knockout ($Pten^{+/-}$), DKO; double knockout ($PKB\beta/Akt2^{-/-}Pten^{+/-}$).

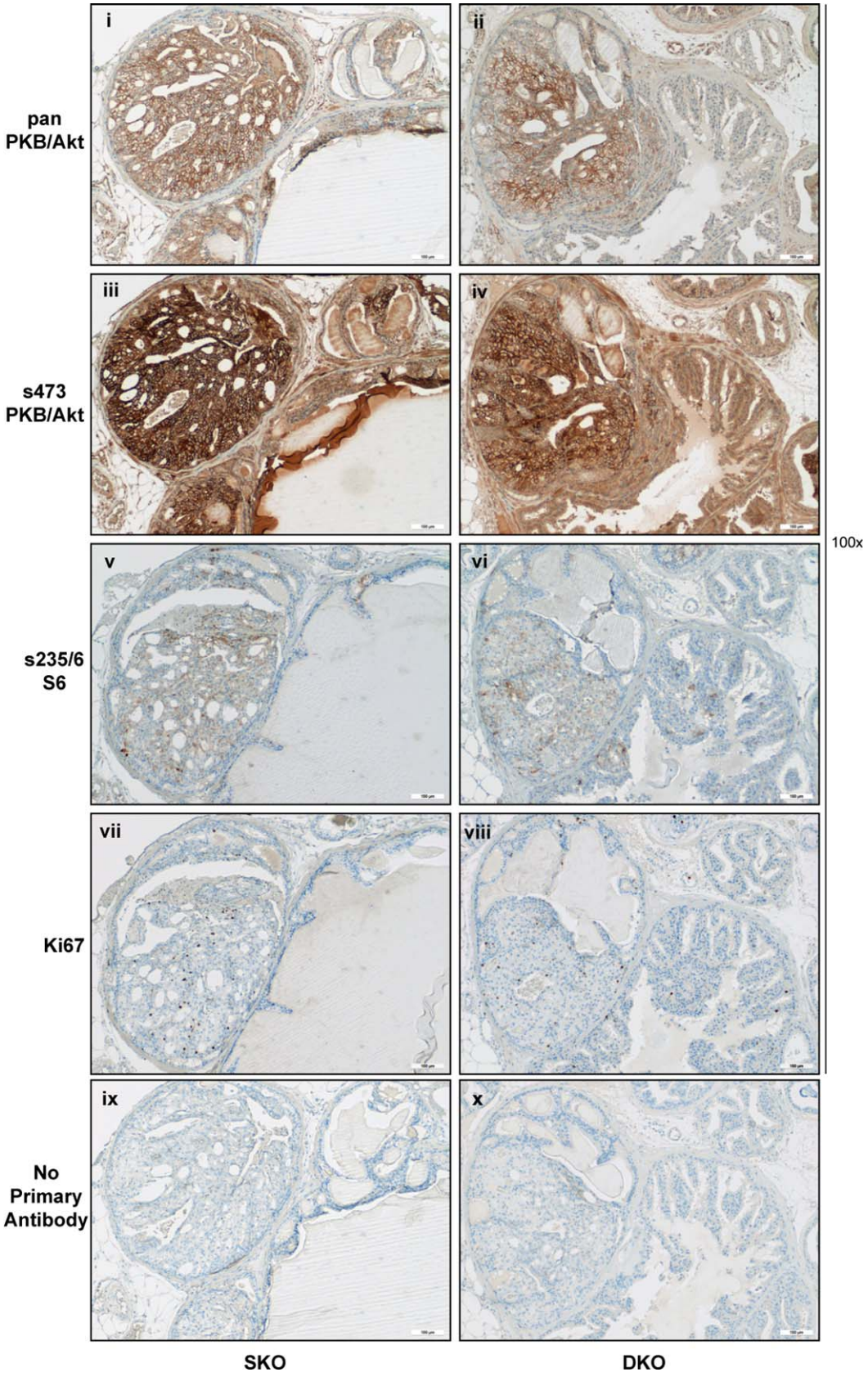


Figure A2.7. Effects On Downstream Signaling In Prostates From PKBβ/Akt2Pten In 30 Week Old Mouse By Immunohistochemistry. DKO prostates show mild decreases in PKB/Akt activation, signaling through mTORC1 and proliferation compared to SKO prostates, indicating PKBβ/Akt2 may contribute progression of prostate neoplasia resulting from Pten deficiency. SKO; single knockout ($Pten^{+/-}$), DKO; double knockout ($PKB\beta/Akt2^{-/-}Pten^{+/-}$).

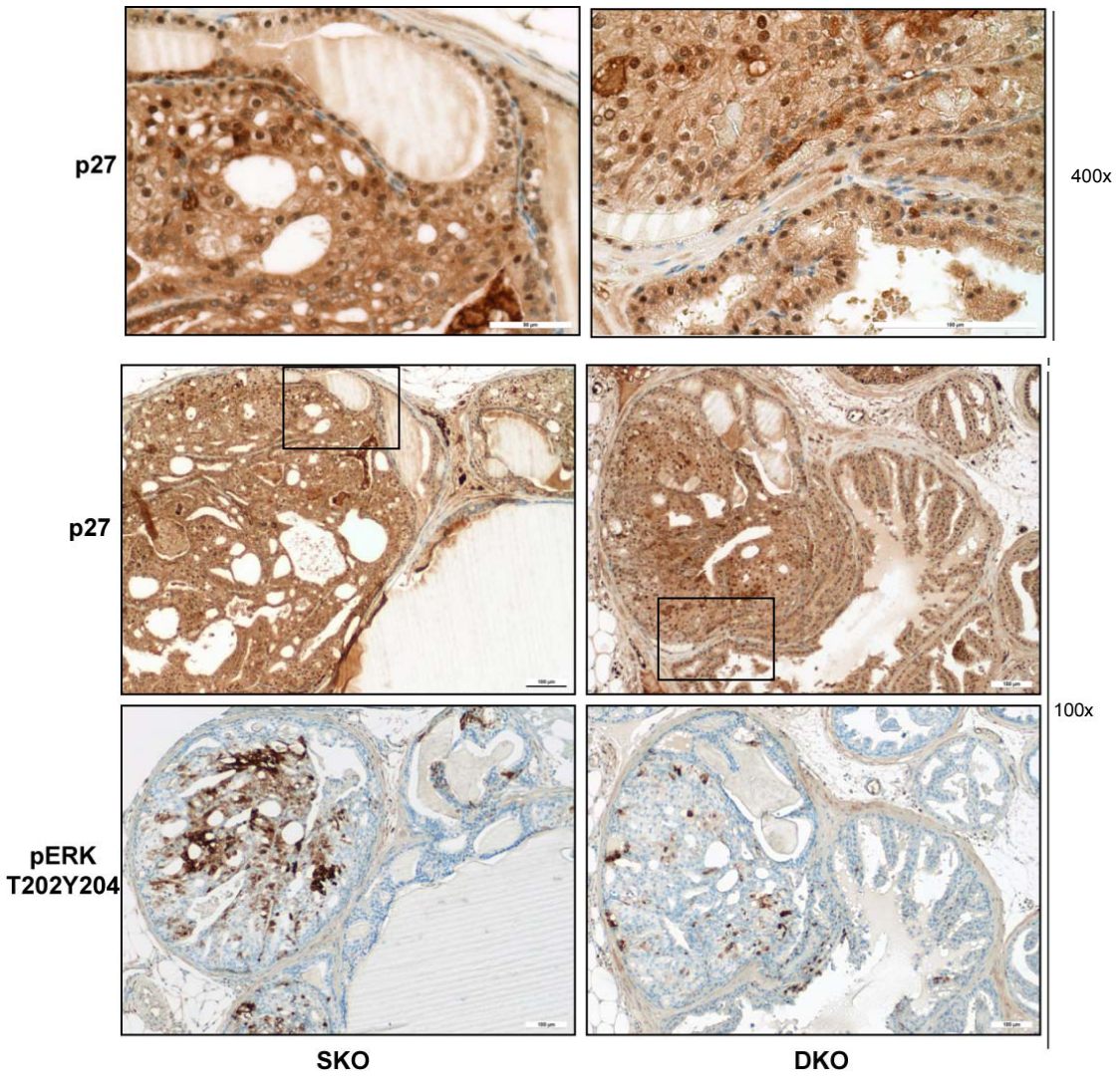


Figure A2.8 Effects On Signaling Related To Prostate Neoplasia Progression From PKBβ/Akt2Pten In 30 Week Old Mouse By Immunohistochemistry. Consistent with a role for PKBβ/Akt2 in contributing to progression of prostate neoplasia resulting from Pten deficiency, DKO prostates show stronger nuclear p27, representing the known p27-mediated senescence induced by Pten deficiency and significantly decreased ERK activation, than SKO prostates. SKO; single knockout ($Pten^{+/-}$), DKO; double knockout ($PKB\beta/Akt2^{-/-}Pten^{+/-}$).

Additional Data On Uterine Phenotypes Observed In PKBβ/Akt2Pten Mice

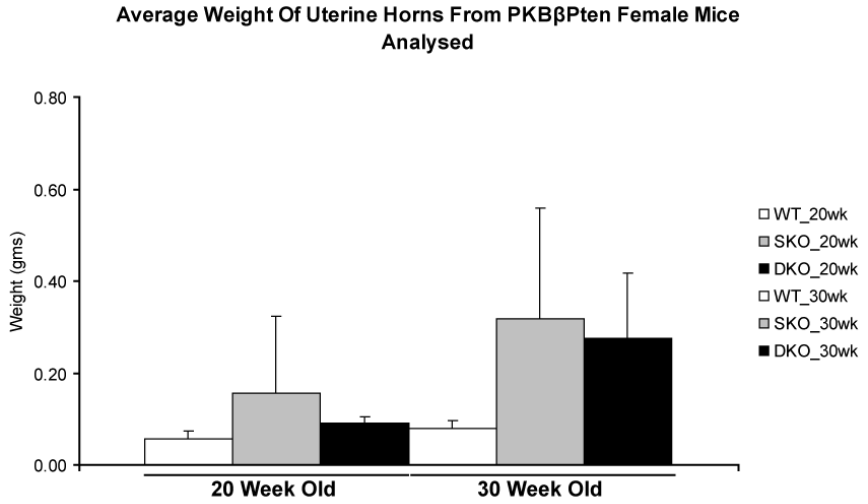


Figure A2.9 Effects Of Ablation Of The PKBβ/Akt2 Isoform In Pten$^{+/-}$ Mice On Uteri Weight at 20wks and 30wks. Loss of PKBβ/Akt2 on the background of Pten heterozygosity in uteri is shows a trend but no significant difference compared to SKO mice at 20 weeks of age, which is even less pronounced 30 weeks of age. WT; wild-type, SKO; single knockout (Pten$^{+/-}$), DKO; double knockout (PKBβ/Akt2$^{-/-}$Pten$^{+/-}$)

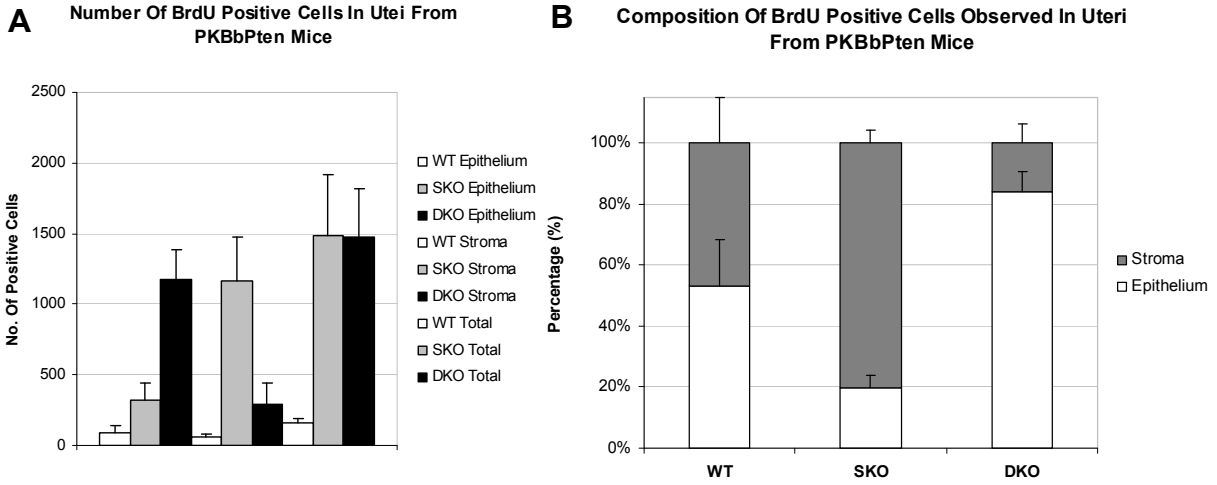


Figure A2.10 Quantification Of BrdU Positive Cells In Myometrium And Endometrial Compartments Of 30 week Old PKBβ/Akt2Pten Mice. Loss of PKBβ/Akt2 on the background of Pten heterozygosity in uteri is shows a striking difference between proliferation in SKO myometrial and epithelia compartments, with almost ~80% of proliferating cells observed in the epithelial compartment of DKO mice whilest 80% of proliferating cells are localized to the myometrial compartment of SKO mice. WT; wild-type, SKO; single knockout (Pten$^{+/-}$), DKO; double knockout (PKBβ/Akt2$^{-/-}$Pten$^{+/-}$)

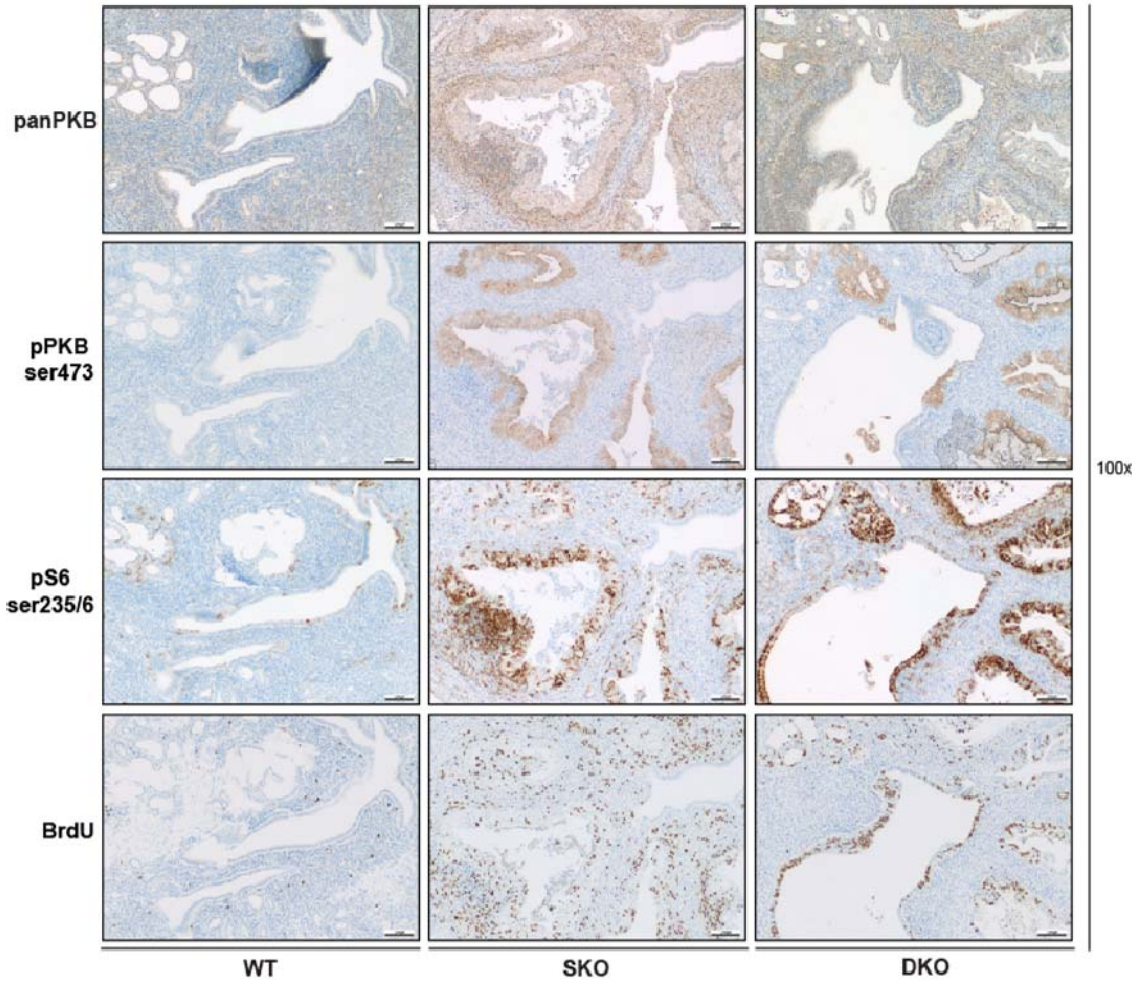


Figure A3. Effects On Downstream Signaling In Uteri From PKBβ/Akt2Pten In 30 Week Old Mouse By Immunohistochemistry. Immunohistochemistry on uteri shows that BrdU staining in DKO mice is almost exclusively endometrial and correlates with strong mTORC1 activation, indicated by phosphorylated S6. Interestingly, in comparison to DKO uteri, uteri from SKO mice show BrdU staining primarily in the stromal but not epithelial compartment, despite strong epithelial activation of PKB/Akt and mTORC1. WT; wild-type, SKO; single knockout ($Pten^{+/-}$), DKO; double knockout ($PKB\beta/Akt2^{-/-}Pten^{+/-}$)

The Epidermal Growth Factor Receptors (ErbB)

The ErbB tyrosine kinase family consists of four members; ErbB1/EGF, ErbB2/Neu/Her2, ErbB3 and ErbB4. These receptors dimerise with each other to mediate extracellular signals upon ligand stimulation. ErbB2 signaling is found in many cancer types and activates multiple signaling pathways, particularly the MAPK pathway, although dimerisation with ErbB3 leads to binding of the p85 subunit of the PI3 Kinase and activation of this pathway. ErbB2 is of particular importance in breast cancer where amplification or overexpression of ErbB2 is seen in ~20% of cases and results in dimerisation with the ErbB3 receptor and constitutive PI3K pathway activation and PKB hyperphosphorylation. Blocking this interaction with Gefitinib, a humanized ErbB2 antibody, results in decreased PKB phosphorylation and tumour remission in ErbB2 overexpressing patients. However, more recently resistance to Gefitinib has been shown as a result of various downstream mutations including in Ras, Src and importantly, loss of PTEN. These observations correlate well with recent studies in mice. In these studies promotion of breast cancer via estradiol required ErbB2 and signaled via the PI3K/Akt1 pathway (Lehnes et al., 2007). Further, Gefitinib prevented cancer progression in mice overexpressing activated ErbB2 (Piechocki et al., 2008), with an even greater effect seen with combination therapy blocking Raf-1 (Hausherr et al., 2006). Conversely, activation of downstream PKB signaling by loss of PTEN accelerated tumour progression (Dourdin et al., 2008). Activated PKBα/Akt1 in the mammary glands of mice overexpressing mammary ErbB2, showed a similar acceleration without activation of ErbB3 (Young et al., 2008). Consistent with the importance of PKB hyperactivation, Rapamycin that acts on the mTOR complex 1 downstream of PKB has also been show to inhibit tumour progression (Mosley et al., 2007). This is further emphasized by the observation that ablation of Akt1 in mice inhibits breast tumour formation (Maroulakou et al., 2007), indicating that multiple downstream substrates of PKB are involved in the tumour progression and development. As mentioned,

whilst resistance can involve other pathways, the activation of the PI3 kinase signaling arm seems crucial to achieving resistance and illustrates the usefulness of therapeutics targeting this pathway.

Insulin Receptor Substrates (IRS)

IRS molecules bind to the insulin and IGF-1 receptors and mediate downstream signaling through MAPK and PKB. The two major forms of the IRS molecules are IRS1 and IRS2 and both have been linked to poor prognosis in patients. Both isoforms have been shown to positively regulate extracellular signals from the IR/IGF1R via PKB and loss of either molecule in mice results in diabetic and growth retardation phenotypes (Tamemoto et al., 1994; Withers et al., 1998) Overexpression of IRS1 or 2 in mice in the mammary gland leads to tumourigenesis and metastasis (Dearth et al., 2006) and loss of IRS2 leads to decreases in tumour development (Nagle et al., 2004), an effect also seen in tumour development in Pten +/- prostates (Szabolcs et al., 2009). Unexpectedly, IRS-1 loss leads to mammary tumour metastasis (Chen et al., 2006) and increased anti-apoptosis (Nagle et al., 2004). This apparent contradiction is due to a negative feedback loop downstream of PKB which results in IRS1 phosphorylation at ser307 and prevents signaling to PI3K (Aguirre et al., 2002; Carlson et al., 2004). Thus, when IRS2 alone can transduce signaling, an increased activation of the PI3K pathway occurs and concomitant increases in PKB activation and signaling. These findings indicate the IRS molecules are important in tumourigenesis and mediate this, at least in part, through modulating PI3K activation.

Phosphoinositol-3-Kinase (PI3K)
[For Review See Wu, Yan And Backer, 2007]

The phosphoinositol-3-kinase family exists as 3 classes; Class I, Class II and Class III. PKB activation requires conversion of PIP2 to PIP3 which is mediated by Class IA PI3K. Class 1A PI3K is composed of one of five regulatory subunits (p50α, p55α, p55γ, p85α and p85β) that bind to RTKs and one of three catalytic subunits (p110α, p110β and p110δ) that bind to the regulatory subunit. When

complexed, they execute the attachment of a phosphate group onto the PIP2 molecule to generate PIP3. All components are ubiquitously expressed except for the p110δ (only expressed in leukocytes). Mutation of the p110α subunit has been found in ~9-30% of tumours of the breast, prostate, cervix and endometrium (Catalog of Somatic Mutations in Cancer, www.sanger.ac.uk/genetics/CGP/cosmic; (Miyake et al., 2008)). These mutants include E542K, E545K which result in a ras-dependant gain of function and H1047R that is proposed to lead to ras-independent gain of function. In all cases hyperphosphorylation of PKB occurs. Truncation mutants of H1047R (Kwon et al., 2008) that fail to stimulate PKB are non-oncogenic and overexpression of PTEN that reverses the conversion of PIP2 to PIP3 mediated by PI3K prevents tumour formation, together suggesting that the oncogenic potential of PI3K activation is mediated exclusively via PKB. Recent studies in mice have shown that PI3Kα is the primary isoform mediating insulin and growth signaling to PKB/Akt (Jia et al., 2008). It can also contribute to more diverse cancers including thyroid (Furuya et al., 2007), pituitary (Lu et al., 2008), ovary (Wu et al., 2007) and lung (Yang et al., 2008) and importantly is required for ras driven tumourigenesis (Gupta et al., 2007). More recently a contribution by PI3Kβ to tumour promotion has also been reported and mouse models have illustrated a crucial role for this subunit in prostate cancer, where ablation of the PI3Kβ subunit prevented tumour formation in a PTEN null prostate cancer model, with concomitant loss of ser473 staining of PKB observed (Jia et al., 2008). Further support for a crucial role in prostate cancer was shown in a recent paper that showed PI3Kβ mediated androgen receptor transactivation and overexpression resulted in androgen independent transactivation that required its lipid kinase activity (Zhu et al., 2008). Consistent with the finding that PI3Kβ is required for prostate cancer in a PTEN null setting, loss of PI3Kβ in prostate cancer cell lines by RNA silencing prevented tumour formation upon injection into nude mice (Wee et al., 2008; Zhu et al., 2008). These findings indicate whilst PI3Kα is the most commonly involved subunit in tumourigenesis, the other isoforms may play a more important roles in a tissue specific manner.

Phosphatase And Tensin Homolog (PTEN)

The phosphatase and tensin homolog is a phosphatase that is distinguished by its preference for phosphoinosityl substrates. In this capacity PTEN acts as the phosphatase of the PI3K pathway, reversing the kinase actions of PI3K by dephosphorylating PIP3 to PIP2. In doing so, PTEN acts as the major negative regulator of the PI3K pathway and PKB activation. Hereditary genetic mutations of PTEN give rise to Cowden's syndrome, which predisposes affected individuals to tumour development. Even more striking are the somatic mutations, gene deletion, inactivation and silencing of PTEN leading to both protein reduction or loss, which are found in a diverse range of cancers, and make PTEN one of the most commonly mutated proteins in cancer. These PTEN mutations drive tumour development by hyperphosphorylated PKB, which inhibits apoptosis and promotes survival, proliferation and growth. PTEN deletion in mouse models is embryonically lethal (Stambolic et al., 2000), however, loss of a single allele leads to hyperplastic and dysplastic tissues in multiple organs and further reduction of PTEN (Trotman et al., 2003) leads to complete, often aggressive and invasive, tumour formation.

In response to PTEN mutations and loss being found in a broad spectrum of tumour types, more recent studies have utilized conditional PTEN loss to explore its ability to cause tumours in specific organs, including skin (Inoue-Narita et al., 2008), uteri (Hernando et al., 2007; Daikoku et al., 2008), lung (Yanagi et al., 2007; Dave et al., 2008), thyroid (Yeager et al., 2007), intestines (He et al., 2007), ovary (Fan et al., 2008; Reddy et al., 2008), bone (Ford-Hutchinson et al., 2007), prostate (Di Cristofano et al., 2001; Trotman et al., 2003; Wang et al., 2003; Ratnacaram et al., 2008) and liver (Stiles et al., 2004; Horie et al., 2006; Sato et al., 2006). Mouse models of PTEN loss or reduction have been combined with overexpression or loss of components of the PI3K pathway to determine the importance of PTEN loss and subsequent PKB activation, as well as to probe for PKB independent roles of the PI3K pathway in tumour development. These models have included ErbB2 upstream of the PI3K pathway (Dourdin et al., 2008), p110δ (Janas et al., 2008), p110β (Wee et al., 2008; Zhu et al., 2008) or p85 (Luo et al., 2005) subunits of PI3K, PKBα/Akt1 (Chen et al., 2006) itself and mTORC2 via rictor ablation (Guertin et

al., 2009) or via mTOR kinase ablation (Nardella et al., 2009). In addition, the role of other pathways and their potential contribution to PI3K pathway driven tumourigenesis by co-operation with PTEN have been probed by mutations of proteins with both a putative and established roles in tumour development. In these cases PTEN loss has been combined with activation of the FBJ murine osteosarcoma viral oncogene homolog (Fos) (Yao et al., 2008), overexpression of the Ras homology enriched in brain (Rheb) (Nardella et al., 2008) or loss of the tuberous sclerosis protein 2 (TSC2) (Ma et al., 2005), the adenomatosis polyposis coli protein (APC) (Shao et al., 2007), the Von Hippel-Lindau protein (VHL) (Frew et al., 2008), the Rac activator T cell invasion and metastasis gene 1 protein (TIAM1) (Strumane et al., 2008) and the LKB1 tumour suppressor (Huang et al., 2008), the cell cycle inhibitors p18 (Bai et al., 2006), p27 (Di Cristofano et al., 2001) and the tumour suppressor p53 (Chen et al., 2005). In addition, recent interest in the regulation of PTEN localization, particularly in the nucleus, and PIP3 phosphatase independent activities of PTEN have gained much interest and been addressed in various experimental designs. The former area has been addressed with studies into import and export of PTEN including interesting studies into the role of Nedd4 (Trotman et al., 2007; Wang et al., 2007; Fouladkou et al., 2008), oxidative stress (Chang et al., 2008) and the TSC2/mTOR/S6K pathway (Liu et al., 2007). Studies addressing PTEN effects independent of its phosphatase activity on PIP3 have examined the roles of PTEN in the FBXW7 and PTEN regulation of mTOR (Mao et al., 2008), PTEN regulation of p300-dependent hypoxia inducible factor 1 (Emerling et al., 2008), PTEN regulation of Src family kinases (Dey et al., 2008) and JNK signaling (Vivanco et al., 2007).

The prevalence and variety of PTEN deregulation in tumour development, underscored by both clinical data and experimental models, highlights the selective advantage that activation of PKB signaling can confer upon a tumour in both development and progression.

3-Phosphoinositide-Dependent Protein Kinase 1 (PDK1)

The 3-Phosphoinositide-Dependent Protein Kinase 1 (PDK1) is part of the AGC protein kinase family and is crucial in the PI3K pathway through its ability to phosphorylate PKB at the Thr308 site in the T-loop of the catalytic domain performing the initial step in PKB activation. In addition however, PDK1 can phosphorylate at least 22 other protein kinases, including the p90 ribosomal S6 kinases (RSK), protein kinase C isoforms (PKC), the serum and glucocorticoid-induced protein kinase (SGK) and the p70 ribosomal protein kinase (S6K1) downstream of mTORC1. Knockout mice of PDK1 are embryonically lethal and in order to determine the contribution of PDK1 signaling via PKB and its other kinase substrates *in vivo* both hypomorphic and knock-in mouse models were developed by the Alessi laboratory. The hypomorphic model can facilitate generation of mice with 80-90% loss of PDK1 (Bayascas et al., 2005), whilst more selective analysis of the downstream signaling can be ascertained via the two knock-in models. The first knock-in model; a PDK1 Leu155Glu mutant, prevents PDK1 from interacting with and phosphorylating its substrates (McManus et al., 2004), whilst the second; the PDK1 K465E mutant is unable to bind phosphoinositides via the PDK1 PH domain, resulting in the selective loss of signaling through PKB (Bayascas et al., 2008). The PDK1 hypomorphic mice are small but otherwise normal, a phenotype observed in the K465E mutant in addition to insulin resistance. These phenotypes are similar to those seen with knockout of PKBα/Akt1 or PKBβ/Akt2 respectively. PDK1 loss also provides a similar protective effect to that seen with PKBα/Akt1 loss in $PTEN^{+/-}$ mice. PDK1 hypomorphic protein loss on a $PTEN^{+/-}$ background resulted in longer tumour latency, decreased number of tumours and longer survival times. Furthermore, the tumours that did develop showed Ki67, phospho-S6 and cytoplasmic FoxO1 similar to that seen in the $PTEN^{+/-}$ only mice. This suggests the driving mechanism of the tumours that develop from $PDK1^{fx/-}PTEN^{+/-}$ mice is similar to $PTEN^{+/-}$ driven tumours and signals via PKB. This provides even more evidence that PKB is the crucial signaling node in PI3K mediated tumour development. It will be interesting to see if selective loss of PKB activation via the K465E mutant on the background of PTEN reduction or loss can abrogate tumour development.

Part IV: Publications

Akt/PKB-mediated phosphorylation of Twist1 is crucial for breast cancer metastasis
Xue G, Restuccia DF, Hynx D, Dirnhofer S, Hess D and Hemmings BA. *Cancer Cell (in revision)*

Loss of Protein Kinase B beta (PKBβ/Akt2) predisposes mice to ovarian cyst formation and increases the severity of polycystic ovary formation in vivo
Restuccia, DF Hynx, D and Hemmings BA. *Dis Model Mech. (in revision)*

From man to mouse and back again: advances in defining tumour AKTivities in vivo.
Restuccia DF, Hemmings BA. *Dis Model Mech. 2010 Nov-Dec;3(11-12):705-20.*

PKB/AKT phosphorylation of the transcription factor Twist-1 at Ser42 inhibits p53 activity in response to DNA damage.
Vichalkovski A, Gresko E, Hess D, Restuccia DF, Hemmings BA. *Oncogene 2010 Jun7;29(24):3554-65*

Cell signaling. Blocking Akt-ivity.
Restuccia DF, Hemmings BA. *Science. 2009 Aug 28;325(5944):1083-4.*

Targeting the Kinome II
Hemmings BA, Restuccia D, Tonks N. *Curr Opin Cell Biol. 2009 Apr;21(2):135-9.*

Marked structural and functional heterogeneity in CXCR4: separation of HIV-1 and SDF-1alpha responses.
Sloane AJ, Raso V, Dimitrov DS, Xiao X, Deo S, Muljadi N, Restuccia D, Turville S, Kearney C, Broder CC, Zoellner H, Cunningham AL, Bendall L, Lynch GW. *Immunol Cell Biol. 2005 Apr;83(2):129-43.*

Complete publications can be found at the end of this thesis

CURRICULUM VITAE

PERSONAL DETAILS

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EDUCATION

Postgraduate: July 2005 - June 2011	Doktorat der Philosophie Philosophisch-Naturwissenschaftliche (Biochemie) Universität Basel / Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland.
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RESEARCH EXPERIENCE

Ph.D. Candidate July. 2005 – June 2011	***Friedrich Miescher Institute for Biomedical Research, Growth Control Group, Switzerland*** Dr. Brian A Hemmings FRS *Project: The Contribution of Protein Kinase B beta (PKBβ/Akt2) to Endocrine Metabolism and Tumourigenesis in vivo*
Temporary Laboratory Mouse Colony Manager Dec. 2009 – July 2010	***Friedrich Miescher Institute for Biomedical Research, Growth Control Group, Switzerland*** Dr. Brian A Hemmings FRS
Research Assistant March 2003 – June 2005	***The Kolling Institute for Medical Research, Department of Molecular Medicine, University of Sydney, Australia*** Dr. Carolyn Scott *Project: The Role of the M6P/IGF-II Receptor in Cancer Cell Migration and Invasion*
Undergraduate Project Jan. 2001- Nov. 2002	***Cellular and Molecular Biology Research Unit (CMPRU), Department of Oral Pathology and Oral Medicine, University of Sydney, Australia*** Associate Professor Hans Zoellner *Project: Chemokine (C-X-C motif) Receptor 4 (CXCR4) Expression on Endothelial Cells and Relevance to Angiogenesis*.

CURRICULUM VITAE

SCIENTIFIC PUBLICATIONS AND PRESENTATIONS:

Publications:

Akt/PKB-mediated phosphorylation of Twist1 is crucial for breast cancer metastasis
Xue G, Restuccia DF, Hynx D, Dirnhofer S, Hess D and Hemmings BA. *Cancer Cell (under review)*

Loss of Protein Kinase B beta (PKBβ/Akt2) predisposes mice to ovarian cyst formation and increases the severity of polycystic ovary formation in vivo
Restuccia, DF Hynx, D and Hemmings BA. *Dis Model Mech. (in revision)*

From man to mouse and back again: advances in defining tumour AKTivities in vivo.
Restuccia DF, Hemmings BA. *Dis Model Mech. 2010 Nov-Dec;3(11-12):705-20.*

PKB/AKT phosphorylation of the transcription factor Twist-1 at Ser42 inhibits p53 activity in response to DNA damage.
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Cell signaling. Blocking Akt-ivity.
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Targeting the Kinome II
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Marked structural and functional heterogeneity in CXCR4: separation of HIV-1 and SDF-1alpha responses.
Sloane AJ, Raso V, Dimitrov DS, Xiao X, Deo S, Muljadi N, Restuccia D, Turville S, Kearney C, Broder CC, Zoellner H, Cunningham AL, Bendall L, Lynch GW. *Immunol Cell Biol. 2005 Apr;83(2):129-43.*

Public Presentations and Conferences Attended:

FMI 40th Anniversary Symposium 2010 (Poster)
PKBβ Contributions To Ovarian Abnormalities
Restuccia, DF Hynx, D and Hemmings BA. *Friedrich Miescher Institute, Basel, Switzerland*

FMI/UCL Joint PhD Metting 2009 (Poster)
PKBβ Contributions To Ovarian Abnormalities
Restuccia, DF Hynx, D and Hemmings BA. *Friedrich Miescher Institute, Basel, Switzerland*

2nd International Conference on Angiogenesis and Tumour Microenvironment 2008

CSHL Meeting on PTEN/PI3 Kinase Pathways in Health and Disease 2008

Targeting the Kinome 2006

Australian Society for Medical Research Conference 2004 (Oral)
A Step In The Right Direction? Career Opportunities For Graduates and How to Pursue Them.
Restuccia D. *Kolling Institute of Medical Research, Uni. of Sydney.*

CURRICULUM VITAE

SCIENTIFIC PUBLICATIONS AND PRESENTATIONS:

Public Presentations and Conferences Attended:

Australian Society for Medical Research Conference 2004 (Poster)
The role of the M6P/IGF-II receptor in cancer cell migration and invasion
Scott CD., Restuccia D., Lee JS., Weiss J. *Kolling Institute of Medical Research, Uni. of Sydney.*

2nd International GH-IGF Symposium 2004 (Poster)
The role of the M6P/IGF-II receptor in cancer cell migration and invasion
Scott CD., Restuccia D., Lee JS.,Weiss J. *Kolling Institute of Medical Research, Uni. of Sydney.*

The 2nd Biannual University of Sydney Colleges of Health Sciences Conference 2002 (Oral)
Variable expression of different CXCR4 structural isoforms between different cell types
Restuccia, D, Lynch, G, and H Zoellner, H. *Cellular and Molecular Biology Research Unit, Uni. of Sydney.*

16th Lorne Cancer Conference 2003 (Poster)
The G-Coupled receptor CXCR4 differs both structurally and functionally between cells.
Lynch, G[1,5,7], Deo, S[5], Restuccia, D[7], Sloane, A[1,2], Xiao, X[4], Raso, V[3], Muljadi, N[1,2], Dimitrov, D[4], Cunningham, A[1,2], Zoellner, H[7], Bendall, L[5]. *[1]Centre for Virus Research & [5]Westmead Inst. for Cancer Res, Westmead Millennium Institute, & [7]Cell and Mol Path Res Unit, Westmead Hospital Dental School & Westmead Hospital, Uni. of Sydney, Westmead, NSW & [2]Nat Centre for HIV Virol Res, NSW. [3]Boston Biomed Res Inst & Harvard Med School, MA. [4]Lab Exp & Comput Biol, NCI, NIH, MD. [6]Dep Micro & Immunol, Uniform Serv Uni of Health Sci ,MD, USA*

Westmead Hospital Research Week 2002 (Poster)
CXCR4 heterogeneity and functional variability between functionally different cell types.
Restuccia, D[*#], Lynch, G[*], Zoellner, H[*], Thompson, C[#]. *[*]Dept. Oral Pathology and [#]Dept. Infectious Diseases, Uni. of Sydney.*

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Targeting the Kinome II

Editorial overview

Brian A Hemmings, David Restuccia and Nick Tonks

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Brian Hemmings' group is based at the Friedrich Miescher Institute in Basel. For the past 27 years he has been struggling with the Human Kinome, a family of approximately 500 proteins. His group has made many important breakthroughs, such as the discovery of PKB/Akt and NDR kinase's, the role of DNA-PK in regulating PKB following DNA damage, and identification of CTMP as a mitochondrial protein. He contributed to the elucidation of the PI3-kinase/PTEN/PKB signaling pathway and the identification of GSK3 as the first substrate. Recently the role of the NDR signaling pathway in centrosome duplication was established.

David Restuccia

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David Restuccia is a PhD student in the Hemmings laboratory.

Nick Tonks

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Nick Tonks' group works at Cold Spring Harbor Laboratory in New York. While a postdoc with Eddy Fischer at the University of Washington in Seattle, he was the first to purify a protein tyrosine phosphatase. The enzyme, PTP1B, was the prototype for a large family of PTPs (~100 genes in humans), which have been the focus of his research throughout his independent career. He has made several important contributions to our understanding of the structure, regulation, and function of the PTP family.

The topic of this edition of Current Opinion in Cell Biology originated some 22 years ago in Basel, when Novartis gave approval for an ambitious project to isolate protein kinase inhibitors. The first fruit of this research was the development of Glivec, an inhibitor of the Abl tyrosine kinase. While originally registered for treatment of Philadelphia chromosome-positive chronic myeloid leukemia (CML), the identification of several other tyrosine kinases that it effectively inhibits has seen its application extended to other malignancies, including the often inoperable and metastatic GIST type of gastrointestinal cancer. In 2006, the 20th anniversary of that Novartis decision, another ambitious project brought the leading experts in protein kinase inhibitor research to Basel for a meeting to celebrate this odyssey. Over 600 scientists from 30 different countries heard 36 presentations on all aspects of kinase biology (see details on the meeting report by Bozulic *et al.* [1]). Against this background, this issue revisits many of the areas discussed at the 2006 meeting. Overall, 23 articles written by former speakers on the 2006 program are presented, highlighting significant advances in the development of additional Kinome-targeted therapies.

Eddy Fischer and Ed Krebs won their 1992 Nobel Prize for work in the mid 1950s describing reversible protein phosphorylation, but it took a further 25 years for kinase deregulation to be linked to disease. In 1978 it was shown that transformation by the Rous Sarcoma Virus is mediated by a protein kinase, v-Src. One year later, Tony Hunter and co-workers, working on v-Src and Polyomavirus middle T kinase activities, identified a new type of protein modification, tyrosine phosphorylation. The response was a keen interest that led rapidly to the identification of novel tyrosine kinases (TK), including Abl and EGFR in 1980 and v-Erb and BCR-Abl five years later. In 2001, the first small-molecule TK inhibitor directed against the Bcr-Abl protein, later known as Glivec, was approved for CML. In the first paper, Tony Hunter presents a comprehensive account of the past 30 years of TK research, reviewing the developments that have led to current understanding of TK function, signaling mechanisms, deregulation in disease, and the development of targeted TK therapies. Further, he discusses the current challenges of resistance, the development of second-generation inhibitors, combinational therapy regimes, and the potential of tailored tumor therapy.

Specific kinases signal by phosphorylation of a defined substrate motif. These motifs are often common, yet kinases phosphorylate only a discrete set of substrates, suggesting additional levels of kinase regulation. The review by Pawson and Kofler addresses this question by examining the role of intramolecular, allosteric, and protein–protein interactions. Using data on various TKs, they illustrate how linkage of additional SH2, SH3, or other domains commonly found in TKs ensures substrate specificity, despite the conserved SH2 and catalytic domains. They examine how additional

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domains can localize the kinase in proximity to their substrates, while unselective substrate association is prevented by inhibitory intramolecular bonds. This can be extended to non-TK, as shown by the activation of PKA, which is kept inactive by binding of regulatory subunits to the catalytic subunit. Attachment of both substrates and the PKA regulatory domains to the scaffold protein AKAP ensures, when activation occurs by increases in local cAMP, that kinase activities are restricted to those substrates attached to the AKAP scaffold. Such domains involved in localization and inhibition thus provide important targets for inhibition of selected kinases.

VEGFs and their TK receptors are involved in both angiogenesis and lymphangiogenesis, where signaling through VEGF-A/VEGFR-2 and VEGF-C/VEGF-D/VEGFR-3 are the major pathways, respectively. Further members of the VEGF and VEGFR families are involved primarily in modifying the angiogenic process after initiation. While abnormalities in knockout mouse models emphasize the crucial nature of angiogenesis and lymphangiogenesis in development, their importance in pathogenic settings, especially cancer, has instigated numerous attempts to exploit the therapeutic potential of their inhibition. Lohela *et al.* review this work and the understanding it has fostered of this system in pathological settings. Evidence indicates that anti-VEGF mAbs can inhibit tumor growth by preventing neoangiogenesis and also increase vessel stability, which supports perfusion and access of chemotherapeutic agents to the tumor. Preclinical models targeting VEGF-C/VEGF-D/VEGFR-3 block lymphangiogenesis that allows tumor access to the lymphatic network and thus acts to suppress metastatic spread. Interesting results also indicate that the targeting of individual components involved in angiogenesis and lymphangiogenesis or combined therapy against novel modulators like neurophilin nerve growth factors may also bring additional therapeutic benefit to pathologies such as rheumatoid arthritis, human lymphodema and cancer.

TGF-β family cytokines signal by binding to and activating hetero-dimeric serine–threonine protein kinase receptors. Phosphorylation of receptor activated Smads (R-Smads) results in complex formation with SMAD4, translocation to the nucleus, and in conjunction with other transcription factors, the regulation of genes involved in growth arrest, apoptosis and epithelial-to-mesenchymal transition (EMT). Specificity of signaling is attained via diverse mechanisms, including receptor–ligand binding affinities, both receptor and Smad post-translational modifications, negative feedback by inhibitory Smads, and the nuclear export of Smads. In tumor pathogenesis, TGF-β members initially inhibit growth and promote apoptosis. However, the tumor overcomes TGF-β growth arrest at later stages, while utilizing the TGF-β ability to promote EMT, thereby increasing invasion and metastasis. Recent data on the mechanisms underlying tumor inhibitory and EMT promoting effects are addressed by Heldin *et al.* Escape from the growth inhibitory effects of TGF-β has been linked to loss of plasminogen activator inhibitor-1 by as yet unknown mechanisms resulting in sustained activation of the growth-promoting kinase Akt. Akt itself and the downstream regulated target mTOR have been shown to interfere with Smad signaling. Decrease in TGF-β-induced apoptosis has been shown also to involve increases in pro-survival signaling mediated by Akt as well as survivin. Loss of pro-apoptotic effectors Bim and p53, the latter being co-regulated by Smad and p38 proteins, has also been observed. Interestingly, it has been found that p38 can be activated by specific TGF-β receptor complexes through a novel TRAF6-mediated apoptosis pathway. An interesting mechanism for EMT induction in tumors has also been reported. Tumors increase TGF-β release, which acts on the surrounding stroma to induce stromal cytokine production. These cytokines act on the tumor to enhance EMT, invasiveness, and metastasis through complex pathways that involve Notch, JNK1, and FAK signaling. Transcriptional induction of Snail and Twist also induces EMT, and this can be initiated by TGF-β-promoted SMAD3 signaling but inhibited by SMAD2 signaling. These findings promote novel signaling components indirectly associated with Smad signaling, as well as components within Smad signaling, as therapeutic targets to prevent the shift from tumor inhibition to EMT promotion.

ErbB receptor tyrosine kinases (RTKs) form heterodimers upon ligand binding and transducer signals via multiple intracellular cascades. Mutation and overexpression lead to activation, growth promotion, and tumorigenesis. The often observed PI3K mutations or low PTEN also imply that signaling via the PI3K/PTEN/AKT pathway is crucial for tumor progression. Indeed, therapeutic monoclonal antibodies (mAbs) and TK inhibitors of ErbB RTKs, ErbB2 and EGFR, are used clinically and their therapeutic benefit is correlated with phospho-AKT levels.

Resistance to these therapies is seen, however, and the interplay between ErbB, PI3K signaling, and resistance common to ErbB2+ breast and non-small cell lung cancer is the focus of the paper by Hynes and MacDonald. A commonly observed mechanism promoting resistance is the acquisition of mutations downstream of ErbB2 in p110α and PTEN that restore AKT signaling. Compensatory mechanisms increasing insulin-like growth factor 1 receptor signaling, MET receptor levels, and protease cleavage of the mAb inhibited ErbB2 (that allows it to bind the ErbB3), all reactivate AKT signaling (illustrated on the cover of this issue of Current Opinion in Cell Biology). These findings suggest that combined targeting of both the receptor and downstream AKT activation is

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needed. This is the focus of the review by Klein and Levitzki, who deliver a thorough overview of current therapies in clinical trials targeting ErbB receptor, PI3K, AKT, and mTOR. They discuss the different classes of classical drugs and the potential advantages of these newer drugs. Insights into strategies to improve therapeutic efficacy by combinational treatment and by novel more potent ErbB receptor therapies are also presented.

The potential of targeting PI3K/AKT/mTOR goes beyond ErbB2-driven tumors. This pathway is the most commonly mutated and hyperactivated pathway in human cancers and offers targets for therapeutics across a diverse range of cancer types. Brachmann *et al*. review clinical data from approved and new-generation inhibitors in trials in this context. They also highlight the benefits of both selective and combined inhibition of components of this pathway. Recent data show that the PI3K isoforms have different functions and contributions depending on the cancer setting. Accordingly, the review by Jia *et al*. focuses on the potential benefits of selective PI3K isoform inhibition. Recent advances in the biological regulation and actions of the downstream mTOR protein are reviewed by Polak and Hall. They illustrate how newly identified components in mTOR signaling could provide novel therapeutic targets that are more selective in cancer and metabolic therapies. This is made especially relevant by recent data showing that mTOR has crucial roles in both tissue and whole organism metabolism, given the beneficial and adverse metabolic effects seen in mouse models. Patients have shown good tolerance of the selective inhibition of mTORC1, although responses are often varied. Recent data pertaining to possible mechanisms underlying these observations, particularly feedback and compensatory effects, are reviewed in the paper by Lane and Breuleux. In this context, they discuss the importance of optimizing mTORC1 inhibition by combinational therapies and patient profiling. The application of mTORC1 inhibition in an expanded therapeutic spectrum beyond malignant tumors is the topic reviewed by Plas and Thomas. This includes data on immunosuppression and cardiovascular disease, as well as encouraging findings on the treatment of benign tumor syndromes.

Genome integrity is maintained through checkpoints that recognize DNA-damage and coordinate repair. The mammalian PI3 kinase-related kinases, ATR, ATM and DNA-PK, are central to the checkpoint signal and maintenance of their function is essential to prevent mutation acquisition during S-phase. Friedel *et al*. review recent findings important for the maintenance of genome integrity by checkpoint initiation. They discuss mechanisms of cell-cycle arrest, activation of repair, and the restart of S-phase after the DNA-damage response that are initiated by the phosphorylation of canonical ATM/ATR downstream kinases, Chk1 and Chk2. Reinhardt and Yaffe focus on these effector kinases and the identification of an interesting new effector kinase complex of p38 Mitogen- Activated Protein Kinase (MAPK) and Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 (MK2). They document the contributions of these effector kinase complexes to the induction of growth arrest through the activation of diverse kinase signaling cascades. Bozulic and Hemmings review the novel role of AKT in the DNA-damage response, with recent data showing that DNA-PK phosphorylates and activates AKT1 on the important serine-473 residue after DNA damage induced by γ-irradiation. Interestingly, DNA-PK and AKT are both in the cytoplasm and the nucleus, and their coordinated activation affects AKT substrates GSK3, FOXO4 and MDM2. These proteins are involved in p53 regulation, and in fact, p21 induction by p53 is impaired in AKT1 knockout cells. AKT1 activation is enhanced after DNA-damaging cancer therapies and radiotoxicity is increased with simultaneous targeting of AKT1. Collectively, these reviews illustrate novel signaling in the DNA-damage response that may be targeted to minimize off-target genotoxic stress and improve cancer radiotherapy.

Protein Kinase C (PKC) is a family of nine isoforms in three classes that are particularly important in T cell activation and adhesion. This promotes PKC as a therapeutic target in T-cell-dependent immune responses. Baier and Wagner review progress in PKC isoform signaling in this setting, with a brief discussion of the implications of preclinical studies. The review by Roffey *et al*. documents the current status of PKC isoform knockout mouse models illustrating the role of PKC beyond T cells and its potential as a target in neurological disorders, metabolism, and cancer. They address the major challenges in the design of isoform-specific inhibitors and some of the approaches taken and present an overview of PKC inhibitors in clinical trials.

The crucial structural element of a kinase is the ATP-binding pocket, which is exploited by small-molecule inhibitors like Glivec and erlotinib to provide effective therapies. However, the selectivity of inhibition by kinases relies on other flexible elements, such as the highly conserved DRG element, which can enhance binding or sterically interfere with small-molecule inhibitors. Different conformational changes upon activation alter exposed and hidden residues that may stabilize inhibitors. Structural biology has generated insights into these interaction sites between different but often highly conserved kinases and provided a rationale for selective drug design. Cowan-Jacob *et al*. review these topics and report on mechanisms of selectivity. Particularly interesting is how the energy of different conformational states can have an impact on the binding

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affinity of an inhibitor. The conformational states of target kinases are affected by mutations that can lead to resistance. Structural biology allows the design of molecules that can overcome this. Novel approaches are also being applied in the pursuit of inhibitors that bind primarily to the protein surface or associated domains, instead of the ATP-binding pocket. These processes of rationalized drug design were initiated after the development of Glivec and the study of subsequent resistant-kinase forms. Eck and Manly document the lessons learnt from the Glivec/BCR-ABL interaction. They also discuss the development of assays, ideally in a cellular setting, that rapidly, reliably, and effectively evaluate kinase inhibition, thus gauging the potency of targeted inhibitors.

With the current principles of targeted drug design, the selection of targets driving tumorigenesis is crucial for effective cancer therapies. While much effort has been directed toward ErbB and PI3K pathway inhibition, recent data have revealed that certain tumors can be predominantly driven by other kinases. Two of these kinases, oncogenic BRAF and MAPK, are the focus of the paper by Knauf and Fagin. There is compelling evidence that common oncogenic BRAF mutations activate MAPK pathways and promote the escape from senescence through multiple pathways. Inhibition of this process via downstream MEK has had variable success. There are indications that signaling via other pathways may be initiated or be more susceptible to initiation by oncogenic BRAF. This suggests that further inhibition of these pathways, for example PI3K, may be required for effective treatment. The concept of combinational therapy of cancer is taken up in the review by Stuart and Sellers. They illustrate that while therapies targeted to the driving somatic mutation commonly found in the tumors are initially effective, resistance often arises through the acquisition of mutations in the target, alternate proteins, and feedback activation. Deeper understanding of these responses will be the key to designing truly synergistic therapies that can target multiple nodes of a pathway and multiple activated pathways. Such therapies may allow for more effective cytotoxicity and better tolerance. These principles are perhaps most applicable in the case of glioblastoma (GBM) and this is addressed in the review by Lino and Merlo. They discuss the challenges presented by GBMs, the high mutational load within the tumor, low drug uptake due to the blood-brain barrier, and the outgrowth of more malignant GBM cells after intense chemotherapy regimes. They stress the need for studies into locally applied therapeutics after GBM resection and comment on the contribution of epigenetic abnormalities.

While kinases are commonly associated with cancers, they are also clearly involved in microbial pathogenesis. The pathogens themselves, the resultant inflammatory response, and the residual chronic autoimmune diseases they may initiate, all require kinases to be sustained. As such they present novel, druggable targets. Previous unsuccessful preclinical trials targeting chronic inflammatory and autoimmune disorders inhibited p38αMAPK. Recent data suggest that the adverse effects observed were due not to a lack of inhibition of pro-inflammatory effects like TNFα release but rather to the simultaneous ablation of regulatory anti-inflammatory signaling required for resolution. Accordingly, Cohen reviews data on more selective drug design that targets specific p38αMAPK substrates, supports anti-inflammatory kinases like Tpl2 that are induced by inflammation, or inhibits kinases downstream of Toll-like receptors that initiate innate immune system signaling and may yet bring success. Such pathogenic insults can be minimized by swift and effective antimicrobial treatment. However, with the common use of antimicrobials, pathogens are developing increasing resistance to current drugs and little progress has been made in the development of new classes of drugs. Therefore, the attempts to overcome this by targeting bacterial histidine kinases, described by Schreiber *et al.*, are particularly exciting. They discuss studies of a variety of pathogens including *Mycobacteria tuberculosis*, the causative agent for increasing incidence tuberculosis, and strains of *Staphylococcus* responsible for nosocomially acquired chronic infections and deadly Golden Staph. The results show potential, with the major hurdle being low compound solubility. Although this area of research may be still in its infancy, encouraging progress has been made in combating the challenge of multi-drug resistant bacteria.

In conclusion, from humble beginnings there has emerged a powerful new class of drugs that are slowly but surely taming the most heterogeneous of human diseases, cancer. From a single molecule directed successfully at a single cancer came the challenge to expand the therapeutic spectrum. There now exist numerous therapies targeting a diverse range of cancers. The initial success was also qualified by the challenge of resistance, and there are now numerous new molecules targeting resistant kinase forms. These efforts from around the globe signify a research attitude in kinase-targeted therapy that no challenge is too big, no hurdle too great. As can be gleaned from the articles in this issue of Current Opinion in Cell Biology, this research approach has only grown stronger in the past two years. There still remains the challenge of expanding the therapeutic spectrum, and today we are looking beyond cancer to inflammation and antibacterial therapies. There still remains also the challenge of resistance through acquisition of mutation and alternate pathway activation, and today our research looks beyond new targets in these pathways to new approaches of combinational therapy and tailoring therapeutic

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regimes to cancer types. Thus, finally, given such an approach coupled with the 20–30% of drug discovery programs in the pharmaceutical industry that currently involve protein kinase targets, there are great expectations that the future *will* bring more potent, effective kinase-targeted therapies, in cancer, in the more diverse settings of inflammation and antibacterials, and perhaps beyond.

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CELL SIGNALING

Blocking Akt-ivity

Inhibiting the addition of ubiquitin molecules to the enzyme AKT could improve the effects of anti-cancer drugs.

David F. Restuccia and Brian A. Hemmings

Aberrations in cellular signaling pathways that involve the enzyme Akt (also called protein kinase B) are implicated in diverse diseases, including cancer, diabetes, and neurodegenerative disorders (*1*, *2*). Thus, proteins involved in Akt activation and signaling are potential targets for therapeutic intervention. In fact, drugs directed against some of these targets are now in clinical trials for treating cancers, and the inhibition of Akt activation and signaling remains a major goal of drug discovery (*3*, *4*). On page xxx of this issue, Yang *et al*. (*5*) identify a chemical modification of Akt that controls its activation, identifying another potential means to inhibit this kinase in human cancers.

An important step in Akt activation is its translocation from the cytosol to the plasma membrane, where it becomes activated in response to the stimulation of growth factor receptors at the cell surface. However, the mechanisms that control this membrane localization are not clear. Akt possesses a PH domain, which binds to the molecule phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) in the plasma membrane. Similarly, the enzyme phosphoinositide-dependent protein kinase 1 (PDK-1), which phosphorylates and thereby activates Akt, localizes to the plasma membrane by binding to PIP_3. The membrane localization of PDK-1 triggers the recruitment of Akt to the membrane (*6*), though it is unclear precisely how. Now, Yang *et al*. show that this process is even more complicated (see the figure).

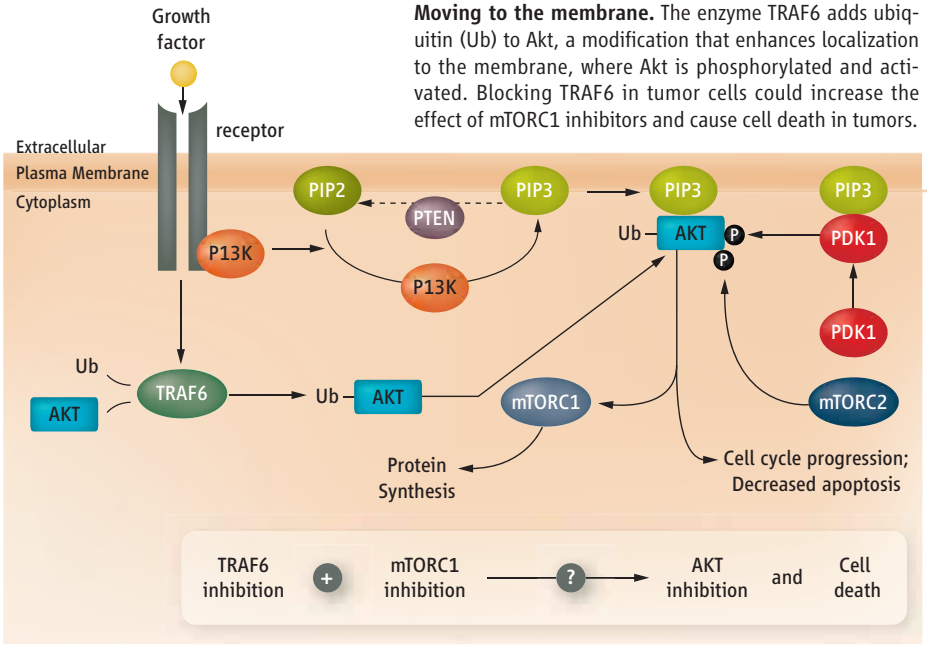
The authors identify Akt as a target of TRAF6, an E3 ubiquitin ligase. Ubiquitin ligases attach a small protein called ubiquitin to target proteins, which induces their degradation or promotes interactions with other proteins to transduce signals. These effects are distinguished by the attachment of single ubiquitin moieties to a protein substrate (monoubiquitination) or chains of ubiquitin proteins (polyubiquitination), as well as by the specific lysine residue that is modified. By ubiquinating Akt, TRAF6 promotes Akt translocation to the plasma membrane, where it becomes phosphorylated. In cells lacking TRAF6, ubiquitination, membrane localization, activation, and signaling of Akt were impaired in response to treatment with growth factors.

The amino acids modified by TRAF6 are lysine residues at positions 8 (K8) and 14 (K14), both of which are monoubiquitinated and lie in the PH domain. Mutation of either lysine residue to arginine impaired Akt activation. The K14R mutation specifically disrupts Akt interaction with PIP_3 (*7*). However, Yang *et al*. found that the K8R mutation did not affect binding to PIP_3. Nevertheless, membrane localization of this mutant was impaired in response to growth factors. Thus, by ubiquitinating Akt on two specific residues, TRAF6 promotes localization of the kinase to the plasma membrane for subsequent activation. Ubiquitination may cause a conformational change that enables Akt to interact with a protein that transports the kinase to the membrane. Ubiquitination of the protein neurotrophin receptor interacting factor (NRIF) by TRAF6 allows NRIF to associate with the protein p62. The resulting complex is then able to translocate to the nucleus (*8*).

To determine whether TRAF6 is an effective target for inhibiting oncogenic Akt hyperactivation, Yang *et al*. examined an activated, mutant form of Akt identified in tumor samples of patients with breast, colorectal, or ovarian cancer (*9*). In this mutant, glutamic acid at position 17 is replaced with lysine (E17K), which increases interaction of its PH domain through a conformational change with PIP_3. Enhanced membrane association of the mutant form of Akt increases its activation, even in the absence of growth factors. The E17K mutant also displayed greater overall ubiquitination—lysine residues at positions 8, 14, and 17 become modified–and its ubiquitination was further potentiated when TRAF6 was overexpressed in cells. Mutating the K8 residue in this mutant decreased Akt activation and downstream signaling. Thus, the Akt mutant uses ubiquitination to attain its hyperactive state.

The authors extended this concept by depleting TRAF6 (by RNA interference) from a human tumor cell line that expresses the hyperactive, mutant form of Akt, and then injecting these cells into "nude" mice (animals that do not mount an immune response to foreign cells). Tumor formation by these cells was severely impaired compared to tumor cells expressing TRAF6 that were injected into animals, consistent with the potential of TRAF6 inhibition to stop tumor growth.

Could inhibiting TRAF6 be an effective clinical therapy for human cancer? Although no inhibitors of TRAF6 are currently available, blocking the function of E3 ligases has shown effective anti-tumor properties in preclinical studies, and such inhibitors are moving toward clinical trials (*10*). Studies of the E3 ligase Mdm2, which targets the tumor sup-



Moving to the membrane. The enzyme TRAF6 adds ubiquitin (Ub) to Akt, a modification that enhances localization to the membrane, where Akt is phosphorylated and activated. Blocking TRAF6 in tumor cells could increase the effect of mTORC1 inhibitors and cause cell death in tumors.

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pressor protein p53 for degredation, show that Mdm2 inhibition can be attained by blocking interaction with its substrate. Yang *et al*. show that a stable complex forms between TRAF6 and Akt, suggesting that this approach may be a good way to block TRAF6-mediated Akt activation. The potential effectiveness of this approach for tumor therapy is highlighted by the point in the signaling cascade at which TRAF6 contributes to Akt activation—downstream of common mutations observed in the clinic that affect phosphatidylinositol 3-kinase (PI3K) or the phosphatase PTEN, either of which cause hyperactivation of Akt. In support of this, the tumor cell line depleted of TRAF6 that was injected into mice by Yang *et al*. did not express PTEN and displayed strong Akt activation.

TRAF6 could be used to augment the effectiveness of rapamycin analogs (rapalogs), drugs that inhibit the mammalian target of rapamycin complex 1 (mTORC1). Rapalogs are approved for limited anti-tumor therapy because they may temporarily stabilize tumors in clinical trials but rarely elicit a full response in terms of tumor ablation. Preclinical studies indicate that rapalogs have a cytostatic effect on tumors, due at least in part to increased Akt activation, because a negative feedback loop that normally prevents PI3K signaling is lost. As Yang *et al*. show, cells lacking TRAF6 displayed increased spontaneous apoptosis (programmed cell death). Thus, TRAF6 inhibition in conjuction with rapalogs could shift the response of tumors to rapalogs from cytostatic to cytotoxic, increasing the efficacy of these drugs in cancer therapy.

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PHYSICS

Coupling Strongly, Discretely

Mechanical systems acting as electronic quantum dots can be tuned at the level of single electrons.

James Hone[1], Vikram V. Deshpande[2]

The fields of electronics and mechanics have made impressive progress toward true quantum mechanical devices. Through improvements in device performance and measurement techniques, nanoelectromechanical systems (NEMS) have enabled high-sensitivity detection of charge, mass, and spin, and have steadily approached the quantum limit of mechanical motion (*1*). Similarly, the ability to manipulate individual electrons in quantum dots has led to developments in solid-state quantum computing (*2*). On pages XXX and YYY of this issue, Lassagne *et al*. (*3*) and Steele *et al*. (*4*) bring together these two fields to study the influence of charge transport on nanomechanical motion in high-performance carbon nanotube mechanical resonators that simultaneously act as quantum dots. They find that the resonant frequency and dissipation in the nanotubes are both highly sensitive to the charge state at the level of single electrons.

Carbon nanotubes are a model system for nanoelectronics. Adding even a single electron to this small system carries a large energetic cost; thus, at low enough temperatures, electrical transport in carbon nanotubes can take place through tunneling of electrons one at a time. This manifests itself in peaks in the current as a function of the voltage on a nearby gate (which modulates the chemical potential of the nanotube), a phenomenon known as Coulomb blockade. Advances in the growth and fabrication of nanotubes (*5*) have enabled the development of clean, freely suspended devices that have been used to observe electronic phenomena such as correlated electron states (*6*, *7*) and spin-orbit coupling (*8*).

Because of their small size, high stiffness, and low density, nanotubes are also excellent materials for NEMS. But, they may also have an additional advantage. They circumvent the surface dissipation mechanism known, in bulk-etched NEMS, to decrease the quality factor Q (which quantifies the sharpness of the resonance peak) with decreasing device size. However, nanotubes have until recently shown Q values of only 100 to 1000 (*9*–*12*), in keeping with the trend for etched devices. Steele *et al*. now show that clean nanotubes do in fact beat the trend, achieving a Q of 100,000 at ultralow temperatures.

The two reports take advantage of these parallel improvements in device performance to examine in detail the coupling

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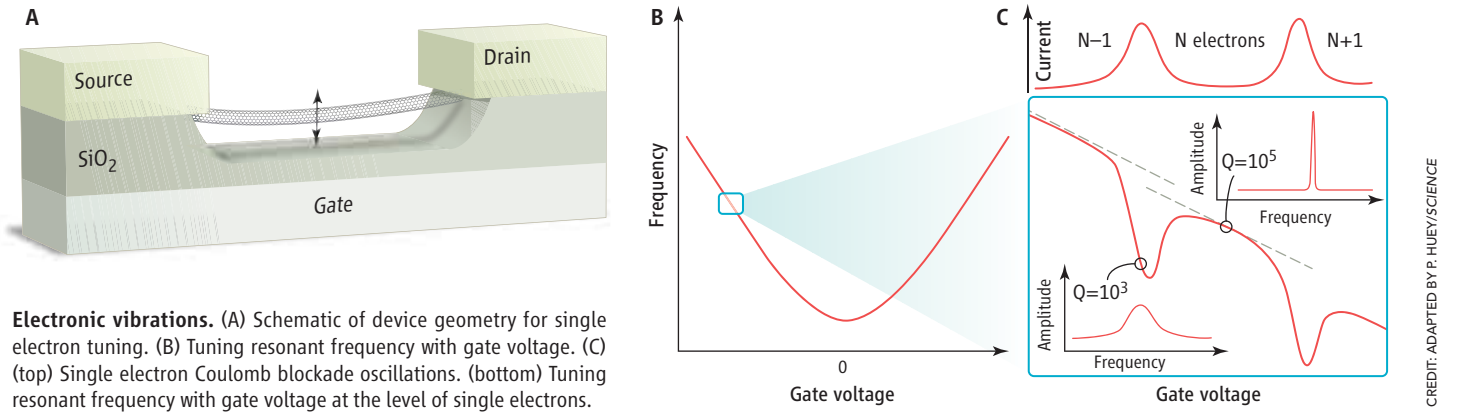

Electronic vibrations. (A) Schematic of device geometry for single electron tuning. (B) Tuning resonant frequency with gate voltage. (C) (top) Single electron Coulomb blockade oscillations. (bottom) Tuning resonant frequency with gate voltage at the level of single electrons.

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ORIGINAL ARTICLE

PKB/AKT phosphorylation of the transcription factor Twist-1 at Ser42 inhibits p53 activity in response to DNA damage

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Protein kinase B (PKB/Akt) is ubiquitously expressed in cells. Phosphorylation of its multiple targets in response to various stimuli, including growth factors or cytokines, promotes cell survival and inhibits apoptosis. PKB is upregulated in many different cancers and a significant amount of the enzyme is present in its activated form. Here we show that PKB phosphorylates one of the anti-apoptotic proteins—transcription factor Twist-1 at Ser42. Cells expressing Twist-1 displayed inefficient p53 upregulation in response to DNA damage induced by γ-irradiation or the genotoxic drug adriamycin. This influenced the activation of p53 target genes such as *p21*^{Waf1} and *Bax* and led to aberrant cell-cycle regulation and the inhibition of apoptosis. The impaired induction of these p53 effector molecules is likely to be mediated by PKB-dependent phosphorylation of Twist-1 because, unlike the wild-type mutant, the Twist-1 S42A mutant did not confer cell resistance to DNA damage. Moreover, phosphorylation of Twist-1 at Ser42 was shown *in vivo* in various human cancer tissues, suggesting that this post-translational modification ensures functional activation of Twist-1 after promotion of survival during carcinogenesis.
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Keywords: PKB/Akt; Twist-1 transcription factor; DNA damage; cancer

Introduction

Twist-1 is a highly conserved member of a family of regulatory basic helix-loop-helix (bHLH) transcription factors (Thisse *et al*., 1987). bHLH proteins form active dimers with E-box proteins and bind to a core sequence (CANNTG, referred to as E-box) in the regulatory elements of many lineage-specific genes in muscle, cartilage and osteogenic cells. Germ-line mutations of the Twist-1 gene that result in haploinsufficiency lead to the development of one of the most commonly inherited craniosynostosis conditions, the Saethre–Chotzen syndrome, which is characterized by premature fusion of cranial sutures and limb abnormalities (Gripp *et al*., 2000; Ghouzzi *et al*., 2001; Yang *et al*., 2004; Cai and Jabs, 2005). Expression of Twist-1 has also been implicated in the inhibition of differentiation of various cell lineages including osteoblasts and myoblasts (Spicer *et al*., 1996; Bialek *et al*., 2004; Hayashi *et al*., 2007).

There are many reports that Twist-1 is involved in oncogenesis in a wide variety of human cancers by inhibiting apoptosis and promoting cell survival after DNA damage or oncogene activation. For example, Twist-1 participates in malignant transformation in neuroblastoma, where it cooperates with the amplified N-Myc oncogene to inhibit p53-mediated apoptosis (Valsesia-Wittmann *et al*., 2004); reviewed by Puisieux *et al*. (2006). Twist-1 can induce an epithelial mesenchymal-like transition, proposed to be an important step in tumorigenesis and metastasis (Yang *et al*., 2004, 2006; Smit *et al*., 2009). A recent study also suggests Twist-1 involvement in tumor progression through direct activation of its transcriptional target YB-1 (Shiota *et al*., 2008). Twist-1 expression can be regulated by hypoxia-induced HIF-1 through direct binding to the hypoxia-response element in the TWIST proximal promoter. This signaling pathway is thought to promote metastasis in response to intratumoral hypoxia (Yang *et al*., 2008).

Elevated Twist-1 expression is correlated with a poor prognosis and high risk of metastasis in breast, prostate, ovarian, cervical and many others human cancers (Elias *et al*., 2005; Kwok *et al*., 2005; Mironchik *et al*., 2005; Kyo *et al*., 2006; Puisieux *et al*., 2006; Hosono *et al*., 2007; Shibata *et al*., 2008). Recent reports suggest that high levels of Twist-1 confer cancer cells resistance to various chemotherapeutic drugs (Pham *et al*., 2007; Zhang *et al*., 2007; Shiota *et al*., 2008).

PKB/Akt protein kinase has a pivotal role in cell signaling in response to a variety of extracellular stimuli, such as growth factors and cytokines, as well as γ-irradiation (Bozulic *et al*., 2008). An intact protein kinase B (PKB) signaling is essential for cell growth and proliferation, whereas loss or gain of the function of this kinase is associated with complex diseases such as type II diabetes and cancer (for review see Fayard *et al*. (2005) and Yoeli-Lerner and Toker (2006)). A somatic mutation (E17K) in the lipid-binding pocket of PKBα was identified recently in human breast, colorectal and ovarian cancers. This mutation resulted in pathological

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localization of the kinase to the plasma membrane, increasing activation and downstream signaling, that can induce oncogenic transformation of mouse lymphocytes (Carpten *et al*., 2007; Restuccia and Hemmings, 2009). Many PKB substrates have been identified in the nucleus. PKB phosphorylation of forkhead transcription factors inhibits their transcriptional activity by promoting their association with 14-3-3 regulatory proteins, retention in the cytoplasm and subsequent ubiquitination (Biggs *et al*., 1999; Kops *et al*., 1999). Phosphorylation of the CDK inhibitor p27 impairs its nuclear import and opposes cell-cycle arrest (Liang *et al*., 2002), whereas phosphorylation of p21 prevents its nuclear localization and interaction with CDK2 (Zhou *et al*., 2001). So far, PKB and Twist-1 have not been identified as members of the same signaling cascade, but several reports have suggested their mutual regulation. Twist-1 transactivates the PKBβ promoter and a positive association between elevated levels of Twist-1 and PKBβ has been found in late-stage breast cancer samples (Cheng *et al*., 2007). PKB in turn might behave as a functional mediator of Twist-1 and is involved in Twist-mediated chemotherapeutic drug resistance (Cheng *et al*., 2007; Zhang *et al*., 2007). Interestingly, Saethre–Chotzen syndrome resulting from Twist-1 haploinsufficiency displays decreased expression of Cbl ubiquitin ligase, resulting in the accumulation of phosphatidylinositol-3-kinase (PI3K) and increased PI3K/PKB signaling (Guenou *et al*., 2006).

Here we show that PKB kinase becomes activated and phosphorylates transcription factor Twist-1 at Ser42 in MCF-7 cells after γ-irradiation and DNA damage induced by adriamycin. This post-translational modification of Twist-1 is necessary for the subsequent decrease in total p53 level and the inhibition of cell-cycle arrest and apoptosis by impaired activation of p53 target genes. Moreover, Twist-1 Ser42 phosphorylation occurs in particular human cancers, especially colorectal and breast, and to a lesser extent lung and prostate. The results presented provide evidence that Twist-1 is a novel PKB nuclear substrate and establish a link between PKB activation and the downregulation of the p53 tumor suppressor.

Results

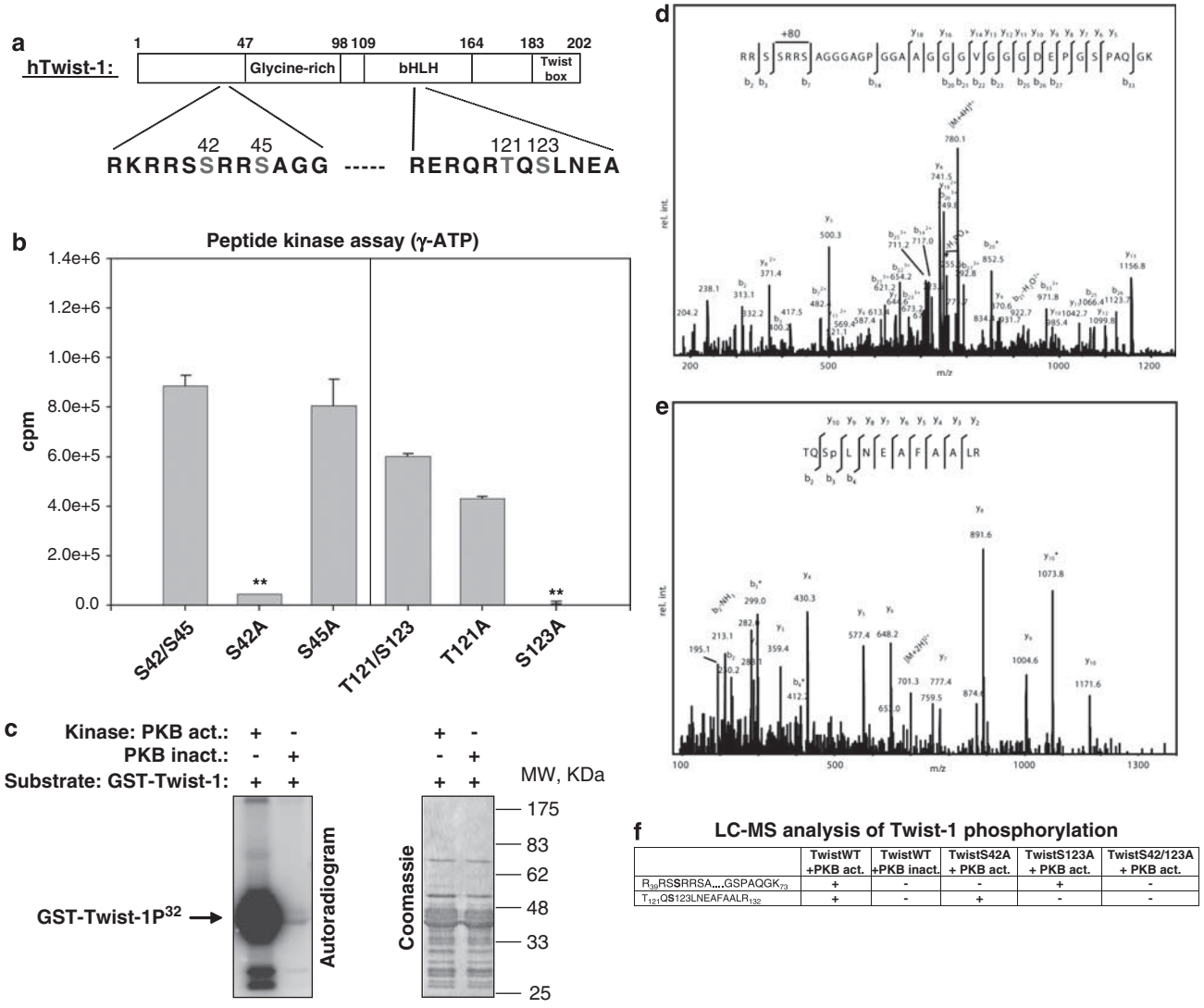
PKB phosphorylates Twist-1 in vitro *at Ser42 and Ser123*

PKB signaling pathway is one of the most frequently altered in human cancer (Yoeli-Lerner and Toker, 2006; Franke, 2008), and yet there are few data directly implicating downstream targets of PKB in an oncogenic switch and cancer progression. As transcription factor Twist-1 was proposed recently to be a potent inducer of malignant transformation, we examined whether this protein is a PKB substrate. Four sites in human Twist-1 (Ser42, Ser45, Thr121 and Ser123) are predicted to be phosphorylated by PKB (Figure 1a). Three of them (S42, T121 and S123) display the canonical PKB substrate consensus motive: K/RXK/RXXS/T (Supplementary Figure S1A). Two N-terminal sites are situated in the low-complexity region of the molecule, whereas Thr121 and Ser123 lie within the bHLH domain, responsible for dimerization and DNA binding activity of Twist-1. We tested the ability of recombinant PKBβ to induce phosphorylation of synthetic peptides comprising PKB recognition motifs and corresponding phosphosites, as well as their mutated analogs Ser42A, Ser45A, Thr121A and Ser125A. Only substitution of serine to alanine at positions 42 and 123 resulted in almost complete loss of phosphorylation of the corresponding peptide by PKB (Figure 1b). Next, we tested the ability of PKB to phosphorylate the full-length Twist-1 protein. To control the specificity of the *in vitro* kinase reaction, we used an inactive PKBβ (Figure 1c). To further investigate which of the potential sites are preferentially phosphorylated in the full-length Twist-1, we performed a series of *in vitro* kinase assays followed by mass spectrometry (MS) analysis. Protein identification was done with MASCOT (Perkins *et al*., 1999) and relative quantification by multiple reaction monitoring (MRM) was performed as described by Hess *et al*. (2008). This identified two phosphopeptides corresponding to Twist-1 amino-acid sequences containing S42 (Figure 1d) and S123 (Figure 1e) phosphosites. A more detailed MS analysis using inactive PKBβ and recombinant Twist-1 mutants (summarized in Figure 1f) confirmed that PKB phosphorylates Twist-1 *in vitro* on two residues S42 and S123. An example of a liquid chromatography (LC)-MS comparison of the LysC digests of GST-Twist-1 WT and GST-Twist-1 S42A mutants phosphorylated *in vitro* by recombinant PKBβ is presented in Supplementary Figure S1.

PKB phosphorylates Twist-1 at Ser42 in vivo

As our results indicated that PKB phosphorylates Twist-1 *in vitro*, we examined whether this is also the case in cell culture. Immunoprecipitated Twist-1 was detected with the pan-PKB phosphosubstrate antibody in serum-stimulated cells but not in starved HEK293 cells (Supplementary Figure S2A). Moreover, pretreatment of the cells with an inhibitor of the PI3K/PKB pathway (LY 294002) resulted in a strong reduction in the phospho-signal, suggesting a specific phosphorylation by PKB (Figure 2a and Supplementary Figure S2B). Treatment of cells with λ-phosphatase almost completely abolished Twist-1 phosphorylation, confirming that Twist-1 exists as a phosphoprotein in cells. Importantly, because phosphorylation of the Twist-1 S42A mutant was not detected but the S123A mutant was phosphorylated as efficiently as the wild-type Twist-1, the results suggested preferential phosphorylation of S42 *in vivo* (Figure 2a and Supplementary Figure S2A). Nevertheless, the pan-PKB phosphosubstrate antibody recognized Twist-1 phosphorylated on S42 or Ser123 *in vitro* equally well (Supplementary Figure S2C), indicating that the antibody is capable of detecting both phosphosites.

To study the function of PKB-mediated Twist-1 phosphorylation in cells, we generated antibodies against the two phosphosites S42 and S123. Thorough characterization (Supplementary Figure S3A) confirmed the phosphospecificity of the antibodies in the *in vitro*



	TwistWT +PKB act.	TwistWT +PKB inact.	TwistS42A + PKB act.	TwistS123A + PKB act.	TwistS42/123A + PKB act.
R_{39}RSSRRSA....GSPAQGK$_{73}$	+	-	-	+	-
T_{121}QS123LNEAFAALR$_{132}$	+	-	+	-	-

Figure 1 Human Twist-1 is phosphorylated by PKB at Ser42 and Ser123 *in vitro*. (**a**) Schematic representation of the potential phosphosites in human Twist-1 predicted by Phosphoscan software (Blom *et al*., 2004). (**b**) Twist-1 wild-type and mutant peptides were synthesized and named as follows: RKRRSSRRSAGG, S42/S45; RKRRSARRSAGG, S42A; RKRRSSRRAAGG, S45A; RERQRTQSLNEA, T121/S123; RERQRAQSLNEA, T121A; RERQRTQALNEA, S123A. The peptides were used in *in vitro* kinase assays with recombinant PKBβ. The results shown come from duplicate assays in three independent experiments. The data are mean ± standard deviation. The *P*-values were as follows: S42A, 0.0083 **; T121A, 0.093 (not significant); T123A, 0.0059 **. (**c**) GST-Twist-1 was *in vitro* phosphorylated in the presence of γ-^{32}P-ATP by recombinant PKBβ. Kinase reactions were resolved by SDS–PAGE. (**d**-**f**) GST-Twist-1 WT and GST-Twist-1 S42A proteins were phosphorylated *in vitro* by recombinant PKBβ and subjected to capillary liquid chromatography–tandem mass spectrometry (LC-MSMS) for identification of phosphopeptides. (**d**) Enhanced product ion spectra of the LysC phosphopeptide of Twist-1. The y- and b-fragments detected are indicated in the sequence. Fragments showing an H_3PO_4 loss are marked with an asterisk. The b_3 and b_7 fragments allow assignment of the phosphorylation to either serine 4 or 7 in the peptide (Ser42 or Ser45). (**e**) Enhanced product ion spectra of the tryptic phosphopeptide of Twist-1. The y- and b-fragments detected are indicated in the sequence. Fragments showing an H_3PO_4 loss are marked with an asterisk. The y_{10}, y_{10}*and b_2 fragments allow assignment of the phosphorylation to the serine in position 3 of the peptide (Ser123). (**f**) Summary of the LC-MSMS analysis of the two phosphopeptides: + peptide phosphorylated, −peptide non-phosphorylated. S in bold shows phospho-Ser mutated to Ala.

kinase assay using wild-type or mutant Twist-1 proteins as substrates (Figure 2b). To verify that Twist-1 can be phosphorylated under physiological conditions, we stimulated starved HEK293 cells with serum to induce PKB activity. Importantly, in these conditions phosphospecific antibodies detected Twist-1 only when phosphorylated at S42 but not at S123 (Figure 2c). Previously, it was shown that S123 can be phosphorylated by protein kinase A (Firulli and Conway, 2008). Indeed, stimulation of cells with forskolin resulted in phosphorylation of Twist-1 at S123, which was also detected by our αTwist-P-Ser123 antibody, thus confirming its specificity (Supplementary Figure S2D). Altogether, these data indicate that PKB preferentially phosphorylates Twist-1 at S42 in cells. To further show specific role for PKB in the regulation of Twist-1 phosphorylation, we decreased the level of endogenous PKB kinase using shRNA. Twist-1 S42 phosphorylation was not induced after serum stimulation of cells with a low PKB content (Figure 2d).

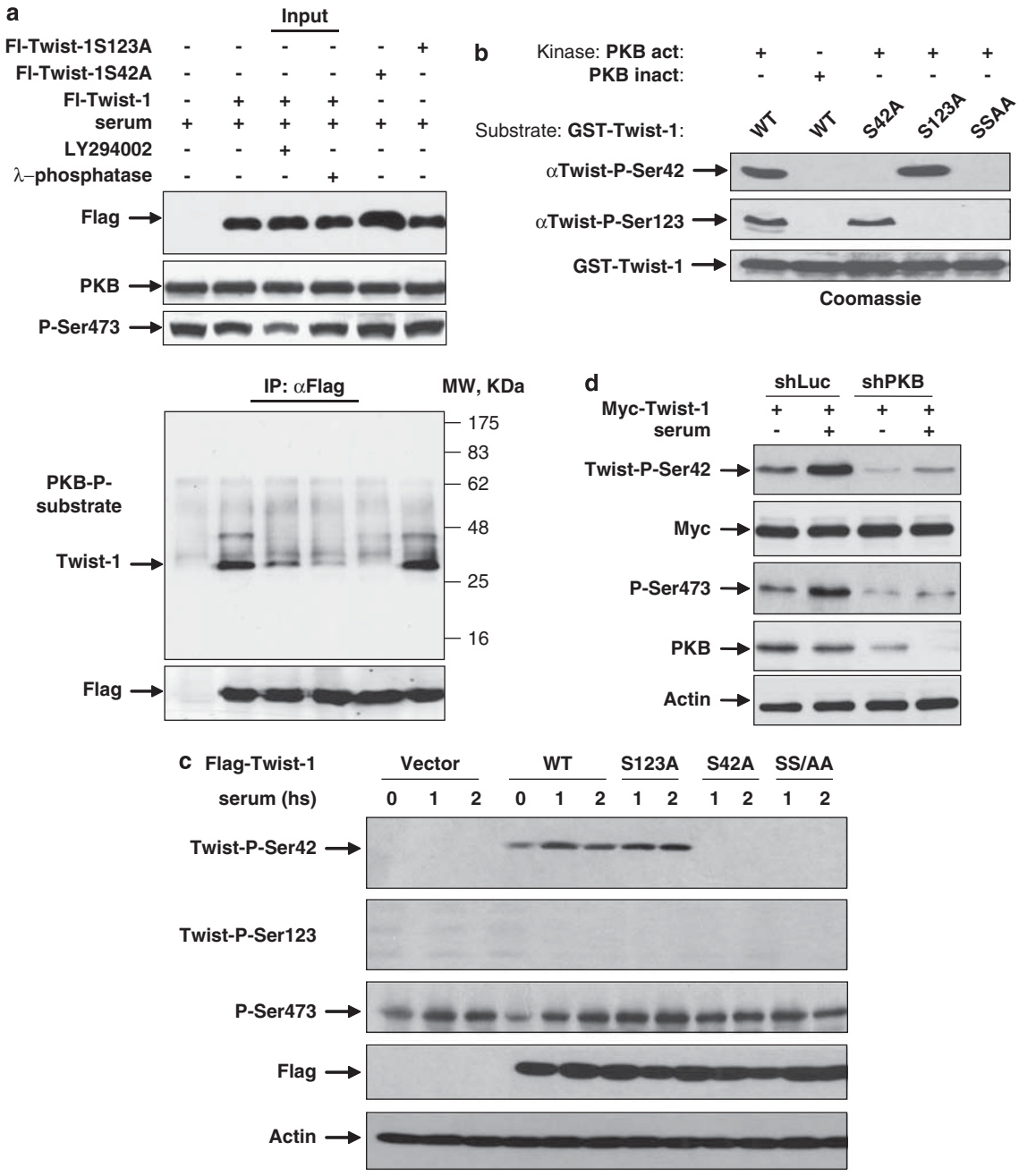


Figure 2 Human Twist-1 is phosphorylated *in vivo* by PKB at Ser42 but not at Ser123. (**a**) Flag-tagged Twist-1 WT, Ser42A and Ser123A expressed in serum-stimulated HEK293 cells were immunoprecipitated with αFlag antibody and phosphorylated proteins detected with the αPKB phosphosubstrate antibody. Two of the precipitates were treated with λ-phosphatase (100 U) and LY294002 (50 μM) added before stimulation, as indicated. (**b**) GST-Twist-1 WT and corresponding mutant proteins were phosphorylated *in vitro* by recombinant PKBβ followed by SDS–PAGE and analysis by western blotting with the specific α-Twist-P-Ser42 and αTwist-P-Ser123 antibodies. (**c**) HEK293 cells expressing Flag-tagged Twist-1 WT, Ser42A, Ser123A or the Ser42,123/AA (SS/AA) double mutant were stimulated with serum for the times indicated and analyzed by western blotting with the αTwist-P-Ser42 and αTwist-P-Ser123 antibodies. (**d**) HEK293 cells expressing shRNA against PKB were stimulated with serum; Twist-1 phosphorylation was detected by western blotting with the αTwist-P-Ser42 antibody.

PKB phosphorylation of Twist-1 at Ser42 regulates Twist-1-mediated inhibition of the p53 response on DNA damage

Taking into account the role of PKB as a pro-survival factor and our recent finding that PKB can be activated in the nucleus in response to DNA double-strand breaks (Bozulic *et al*., 2008), we hypothesized that phosphorylation of Twist-1 at S42 has a role in promoting cell survival after DNA damage-induced stress. To test this hypothesis, we knocked down endogenous PKB in MCF-7 cells (human breast cancer cell line, with a functional p53) expressing Twist-1 and then treated them with γ-irradiation. This resulted in PKB-dependent phosphorylation of Twist-1 at S42 (Figure 3a).

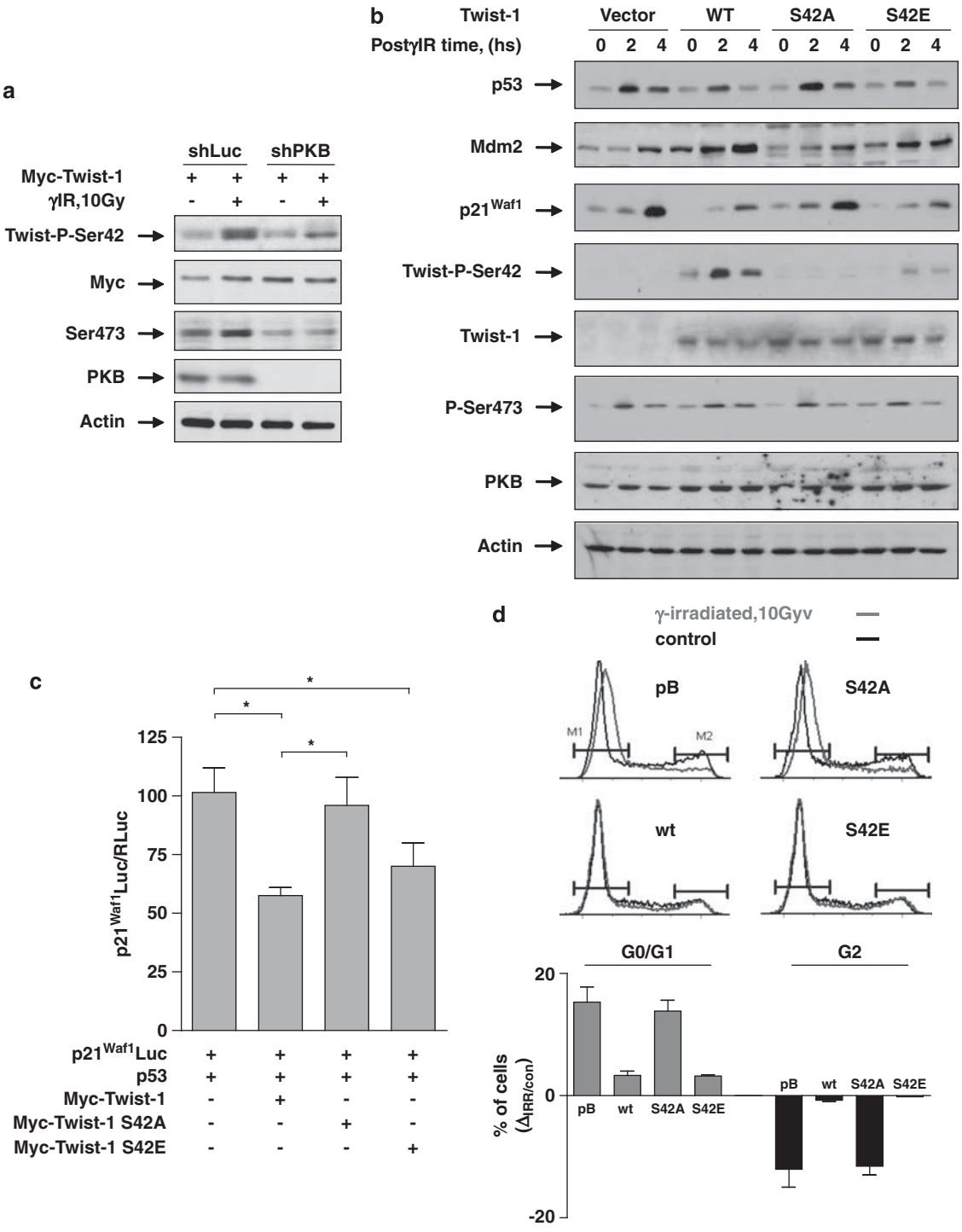


Figure 3 Phosphorylation of Twist-1 at Ser42 by PKB regulates Twist-1-mediated inhibition of p53 on DNA damage. (**a**) MCF-7 cells expressing either control shRNA or shRNA against PKB were γ-irradiated (10 Gy). Cells 2 h after irradiation were harvested and analyzed by western blotting. (**b**) MCF-7 cells transfected with empty vector (pB) as a control or stably expressing WT or mutant Twist-1 proteins Ser42Ala (S42A) and Ser42Glu (S42E) were γ-irradiated (10 Gy) and then harvested after the times indicated. The induction of p53, $p21^{Waf1}$ and Mdm2 as well as the phosphorylation of Ser42 of Twist-1 and Ser473 of PKB were analyzed by western blotting. (**c**) H1299 cells were transfected with a combination of different plasmids as indicated below. At 24 h after transfection, cells were processed and luciferase activity measured. The results are from duplicate assays from three independent experiments showing mean ± standard deviation; asterisk $P<0.005$. (**d**) MCF-7 cells stably expressing Twist-1 or its mutants were γ-irradiated (10 Gy). Cells 24 h after irradiation were fixed and the cell-cycle distribution analyzed by flow cytometry (top). The diagram displays the quantitative differences between the numbers of irradiated and control (non-irradiated) cells in G0/G1 and in G2 (bottom). The results are from three independent experiments expressed as mean ± standard deviation.

Stable MCF-7-Twist-1 cell lines were then generated expressing Twist-1 and its variants at protein levels similar to endogenous (set1 in Supplementary Figure S5A). Notably, the expression of wild-type Twist-1 but not the S42A mutant led to a considerable decline in p53 induction on DNA damage (Figure 3b). The effect of the expression of the S42E mutant did not differ from the wild type. Transient expression of Twist-1 and its mutants in MCF-7 cells had a similar effect (data not shown). After the suppressed p53 response, $p21^{Waf1}$ induction at both transcriptional (Figure 3c) and protein levels (Figure 3b) was decreased in the presence of wild-type Twist-1 but not of S42A Twist-1 mutant, suggesting a potential role of S42 phosphorylation in cell-cycle regulation. Indeed, both wild-type and S42E Twist-1-expressing cells escaped G1 cell-cycle arrest, whereas control cells and cells expressing phospho-deficient S42A Twist-1 accumulated in G1 in response to DNA damage (Figure 3d, top). Quantitative cell-cycle analysis showed a significant rescue effect of Twist-1 S42 phosphorylation on G1 phase arrest after γ-irradiation, indicating that S42 phosphorylation confers the ability to progress through the cell cycle even under genotoxic stress (Figure 3d, bottom).

PKB-dependent phosphorylation of Twist-1 at Ser42 is essential for Twist-1-mediated survival after DNA damage-induced stress

Given that activation of PKB and Twist-1 phosphorylation occurred in response to DNA damage and led to impaired induction of p53, we were prompted to investigate the functional relevance of this phosphorylation in the apoptotic process. For this, we used adriamycin to induce DNA double-strand breaks in MCF-7 cells. Cells expressing wild-type or S42E Twist-1 were less prone to develop morphological signs of apoptosis such as membrane blebbing and cellular shrinkage than control cells or cells expressing the S42A Twist-1 mutant (Figure 4a). Similarly, in the same experimental conditions, wild-type or S42E Twist-1 expression significantly reduced cleavage of PARP (Figure 4b, bottom) and protection of cells from apoptosis was further confirmed by assessing the mitochondrial membrane potential ($\Delta\Psi m$) (Figure 4b). This protection from apoptosis was not observed in the S42A mutant expressing cells. Conversely, shRNA-mediated reduction of the wild-type Twist but not S42A mutant in MCF-7-Twist-1 cell lines resulted in increased sensitivity toward DNA damage (Figure 4c). As expected, S42A Twist-1 was also less potent in downregulating the pro-apoptotic p53 transcriptional target Bax (Figure 4d). Taken together, these data confirm that phosphorylation of S42 is an important part of Twist-1-mediated anti-apoptotic effects.

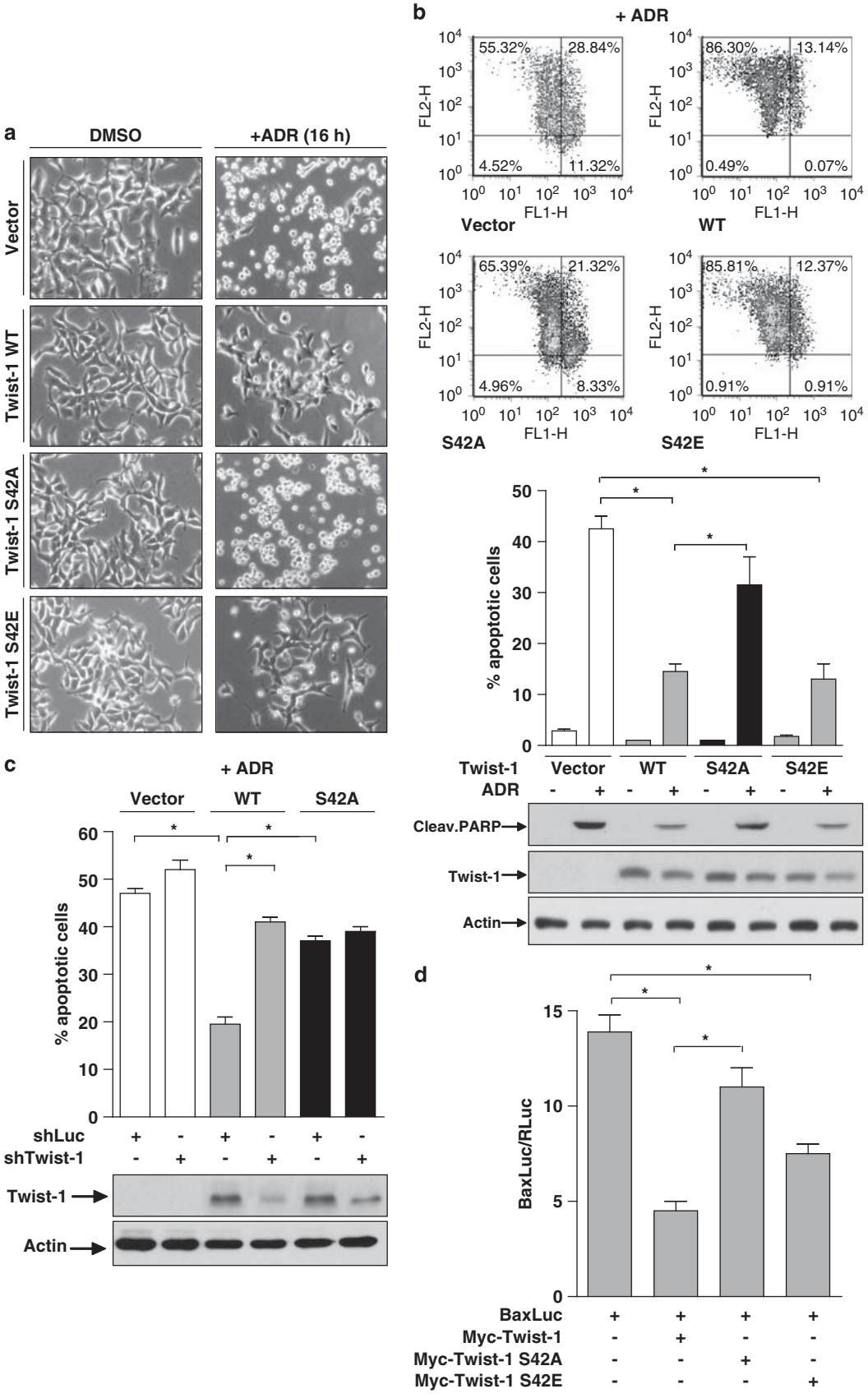
Cancer in various organs is associated with Twist-1 Ser42 phosphorylation

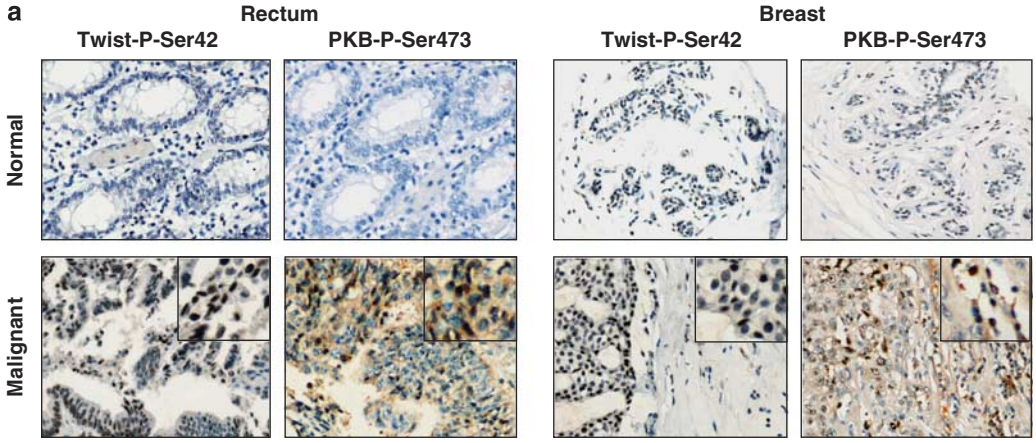
Previous results showed that Twist-1 phosphorylation at Ser42 has a significant role in the overall pro-survival effect of Twist-1. It is also well established that an abnormal cell cycle and resistance to apoptosis are typical hallmarks of cancer. This, together with our finding that S42 phosphorylation of Twist-1 promoted cell survival on genotoxic stress, prompted us to examine S42 phosphorylation in various tumors.

As it has been shown that Twist-1 is transcriptionally active in developing mouse embryos, we tested our αTwist-P-Ser42 antibody on paraffin-embedded sections of mouse embryos (Supplementary Figure S3B). Strong expression was observed in areas known to have active Twist-1 (Gitelman, 1997). We then applied the αTwist-P-Ser42 antibody to stain for phosphorylated Twist-1 on an array of paraffin-embedded primary cancer specimens. Remarkably, prominent S42 phosphorylation of Twist-1 was detected in 50% of 30 colon and 71% of 20 rectal cancers, but not in normal human colorectal tissue. Furthermore, 70% of 39 human breast cancer samples tested positive (Figure 5a), whereas a smaller but still significant number of samples were positive in prostate (24%) and lung (35%) cancers (Figure 5a, bottom). In contrast, Ser42 phosphorylation was not detected in either normal or malignant tissues of human esophagus, stomach, liver or kidney (data not shown). However, it would be of interest to test a larger sample size of this group of negatively stained tumors to determine if Twist-1 S42 phosphorylation is indeed found only in select tissues. The same human tumors were then stained with phospho-PKB (Ser473) antibody to show the status of PKB activation. The analysis of staining for activated PKB displayed a high correlation with phosphorylated Twist-1. Particularly, cancers from rectum (78%) and breast (73%) stained positive for both phosphorylated Ser42 of Twist-1 and Ser473 of PKB. Consistent with this, we observed a strong phospho-Twist signal associated with elevated active PKB levels in neoplastic breast lesions of PTEN+/− mice (Figure 5b).

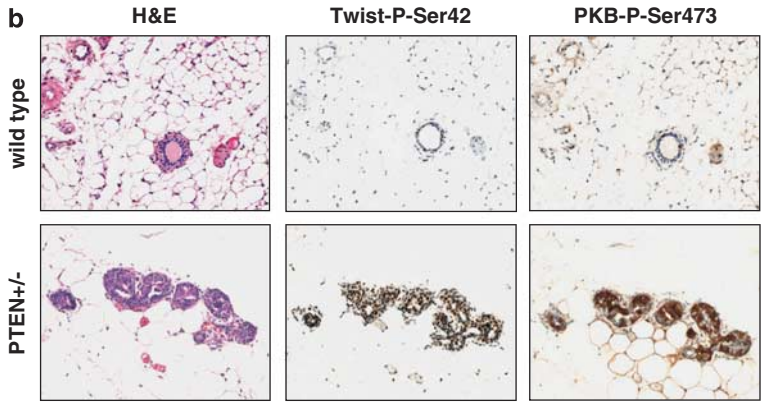
Collectively, our data identify Twist-1 as a novel PKB substrate that becomes phosphorylated by PKB on Ser42 in the N-terminal part of the protein on serum

Figure 4 Phosphorylation at Ser42 is essential for the anti-apoptotic function of Twist-1. (**a**) Images of MCF-7 cells transfected with empty vector (pB) or stably expressing WT or mutant Twist-1 proteins after 16 h stimulation with DMSO (control) or with adriamycin (ADR, 10 μM). (**b**) Cells treated as in **a** were analyzed for depolarization of mitochondrial membrane potential by flow cytometry (top). A quantification summary of three independent experiments is shown at the bottom. Data are mean ± standard deviation; asterisk $P<0.005$. The appearance of cleaved PARP and expression levels of Twist-1 constructs were monitored in parallel by western blotting. (**c**) MCF-7 cells stably expressing Twist-1 or its mutants were transfected with either control shRNA or shRNA against Twist-1, treated with adriamycin and analyzed as in **b**. A quantification summary of two independent experiments performed in duplicates is shown. Data are mean ± standard deviation; asterisk $P<0.005$. (**d**) MCF-7 cells were transfected with a combination of different plasmids as indicated below. At 24 h after transfection, cells were treated as in **a** and then assayed for luciferase activity. The results are from duplicate assays in three independent experiments; the data are mean ± standard deviation; asterisk $P<0.005$.


a
DMSO
+ADR (16 h)
Vector
Twist-1 WT
Twist-1 S42A
Twist-1 S42E
b
+ ADR
55.32%
28.84%
4.52%
11.32%
Vector
86.30%
13.14%
0.49%
0.07%
WT
65.39%
21.32%
4.96%
8.33%
S42A
85.81%
12.37%
0.91%
0.91%
S42E
FL2-H
FL1-H
% apoptotic cells
Twist-1 Vector WT S42A S42E
ADR
Cleav.PARP
Twist-1
Actin
c
+ ADR
Vector WT S42A
% apoptotic cells
shLuc
shTwist-1
Twist-1
Actin
d
BaxLuc/RLuc
BaxLuc
Myc-Twist-1
Myc-Twist-1 S42A
Myc-Twist-1 S42E



Organ	Colon	Rectum	Lung	Breast	Prostate
Twist-P-S42 % of total (pos/total,n)	**50** (15/30)	**71** (14/20)	**35** (16/46)	**70** (23/39)	**24** (8/34)
PKB-P-S473 % of total (pos/total,n)	**64** (16/25)	**89** (16/18)	**85** (33/39)	**88** (35/40)	**44** (16/36)
P-S42/P-S473 % of total (pos/total,n)	**63** (15/24)	**78** (14/18)	**38** (14/37)	**73** (22/30)	**22** (7/32)



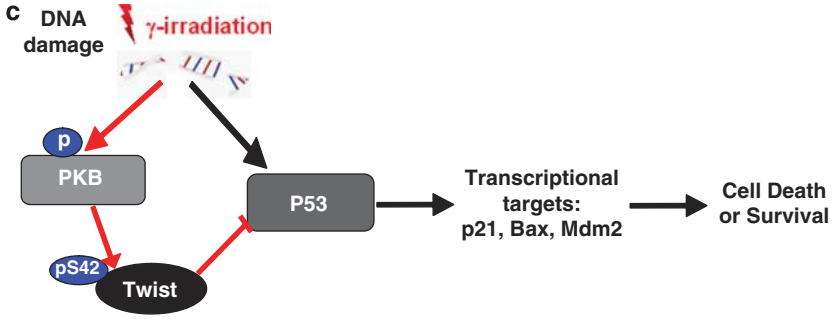


Figure 5 Human cancer in various organs show high levels of Twist-1 Ser42 phosphorylation. (**a**) Sections of paraffin embedded tissue microarray slides were analyzed by IHC for the occurrence of Twist-1 and PKB phosphorylation using αTwist-P-Ser42 and αP-Ser473 antibodies. Images show representative cores from rectum and breast tissues, counterstained with hematoxylin (20 × objective) (top). Summary table of tissue samples analyzed by IHC with the αTwist-P-Ser42 and αP-Ser473 antibodies (bottom). Only patient samples present in both tumor arrays and stained with two antibodies were used for the analysis. (**b**) Sections of paraffin embedded breast tissues from wild type and PTEN +/− mice analyzed with αTwist-P-Ser42 and αP-Ser473 antibodies (**c**) Schematic representation of the signaling pathway triggered by DNA damage with subsequent activation of PKB and induction of a p53 response. PKB-dependent phosphorylation of Twist-1 at Ser42 in turn suppressed p53 induction.

stimulation and genotoxic stress. This phosphorylation appears to have a significant role in the ability of Twist-1 to downregulate the DNA damage-induced p53 response, thus promoting cell survival, which in turn may result in uncontrolled cell overgrowth and cancer (summarized schematically in Figure 5c).

Discussion

The PKB substrate consensus sequence surrounding Ser42 in Twist-1 is evolutionary conserved in vertebrate genomes. In contrast, the Ser42 residue is not conserved in Hand proteins, the closest relatives of Twist-1 in the HLH family, suggesting that this site is phosphorylated in various species exclusively in Twist-1 and not other HLH transcription factors (Supplementary Figure S1A). Moreover, this phosphorylation may have a function distinct from those of other known Twist-1 phosphosites. Protein kinase A phosphorylates two conserved residues within the HLH domain of both Twist-1 and Hand2 (T125/S127 and T112/S114, respectively, in mice and T121/S123 and T112/S114 in human) bringing about their dimerization, which is necessary for the regulation of target genes during limb development. A group of Twist-1 mutations identified in patients with Saethre–Chotzen syndrome was reported to disrupt protein kinase A-mediated phosphorylation, emphasizing the importance of Twist-1 in development (Firulli *et al*., 2005). In contrast to the S42A mutation, most mutations within the bHLH domain of Twist-1 negatively affect its transcriptional repressor function (Sosic *et al*., 2003). Despite Ser42 being located adjacent to a putative NLS of Twist-1 (mutation of Arg39 to Gly present in a patient with mild Saethre–Chotzen syndrome, results in nuclear exclusion of Twist-1 (Funato *et al*., 2005; Singh and Gramolini, 2009), phosphorylation of Twist-1 by PKB did not influence protein localization (Supplementary Figure S4).

Even though the relevance of Twist-1 in cancer development has been studied intensively, there are few reports describing its molecular regulation. We report here that Twist-1 is phosphorylated at Ser42 by PKB (1) in response to serum stimulation of HEK293 cells, (2) in MCF-7 breast cancer cells after γ-irradiation and adriamycin treatment and (3) in human cancer tissues of different origins thereby suggesting that Ser42 phosphorylation is involved in the regulation of cell growth and cell survival on DNA damage.

Focusing on the molecular events triggered by phosphorylation of Twist-1 by PKB in response to DNA damage, one of our key observations is that Ser42 phosphorylation is involved in the downregulation of the p53 tumor suppressor. p53 has a pivotal role in directing cell responses to various stress stimuli, and p53-controlled transactivation of target genes is an essential feature of each stress-response pathway, although some effects of p53 may be independent of transcription (Kruse and Gu, 2009). In our experiments, decrease in p53 stabilization after DNA damage was paralleled by impaired induction of $p21^{Waf1}$, but only in cells with upregulated wild-type Twist-1 and not the S42A Twist-1 mutant. The significant reduction in G0/G1 arrest observed in cells expressing wild-type Twist-1 or the S42E Twist-1 mutant but not in S42A Twist-1 cells provides a functional read out of the inhibitory effect of Twist-1 phosphorylation on the key cell-cycle effector $p21^{Waf1}$. Indeed, Twist-1 was shown to override premature senescence through inhibition of $p16^{INK4A}$ and $p21^{Waf1}$ promoter activation induced by $H\text{-}Ras^{V12}$ and p53 in E1A-immortalized MEFs; however, the molecular mechanisms involved in this effect are still under investigation (Ansieau *et al*., 2008).

Further, our experiments revealed that Ser42 phosphorylation of Twist-1 desensitizes cells to DNA damage induced by adriamycin, with survival markedly decreased in S42A but not wild-type Twist-1-expressing cells (Figure 4 and Supplementary Figure S5B). Thus, it appears that phosphorylation of the Twist-1 transcription factor by PKB in response to DNA damage contributes to an anti-apoptotic mechanism. This is in line with the strong pro-survival signaling mediated by PKB kinase. PKB itself is known to increase p53 degradation by physically associating with MDM2 and phosphorylating it at Ser166 and Ser186. This enhances its stability (Feng *et al*., 2004), as well as its nuclear localization and interaction with p300, and inhibits its association with p19ARF (Zhou *et al*., 2001). Interestingly, expression of the wild-type Twist-1 but not of S42A Twist-1 mutant promoted an increase in MDM2 protein levels (Figure 3b). Therefore, it remains to be addressed whether the effect of Twist-1 Ser42 phosphorylation on p53 and the induction of its target genes are direct or mediated through other molecules. It was described previously that Twist-1 can inhibit a potent p53 transactivator homeobox protein HOXA5, compromising the p53 response to γ-irradiation through suppressed induction of $p21^{Waf1}$ and inhibition of Ser20 phosphorylation (Stasinopoulos *et al*., 2005). Expression of Twist-1 decreases the level of the p53 upstream activator $p14^{ARF}$, presumably by affecting production of its mRNA (Kwok *et al*., 2007). Twist-1 binds to and inactivates histone acetyltransferase CBP/p300, which is required to relieve the suppressive effects of chromatin on p53 target genes (Hamamori *et al*., 1999). Altogether, Twist-1 seems to act through several independent mechanisms that focus on inhibition of the p53 tumor suppressor pathway.

Our hypothesis that Twist-1 Ser42 phosphorylation might be a part of oncogenic signaling during cancer development was further supported by compelling data showing the presence of this post-translational modification in neoplastic tissue displaying high levels of activated PKB in both human and mice. The PKB pathway is often upregulated in human cancers either by overexpression or by activating mutations, which result in increased activity of the kinase.

The continuing identification of PKB substrates adds to the diverse cellular roles of the kinase, including cell growth, proliferation and survival. As phosphorylation of Twist-1 at Ser42 enhances the ability of transformed

cells to circumvent cell-cycle arrest or apoptosis, induced by genotoxic stimuli, it might represent one of the mechanisms used by cancer cells for uncontrolled growth and survival.

Materials and methods

Cell culture, transfection and stimulation
Human HEK293, MCF-7 and H1299 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin. All cells were grown in a humidified incubator at 37°C and 5% CO_2. Cells were plated 24 h before transfection and transiently transfected using jetPEI (Polyplus Transfection, Illkirch, France) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA amounts were standardized by addition of empty expression vector. HEK293 cells were starved in Dulbecco's modified Eagle's medium containing no serum for 24 h before stimulation with 20% fetal calf serum for 1 h; LY 294002 inhibitor was added 30 min before stimulation as indicated. MCF-7 cells were γ-irradiated with the indicated doses 24–36 h after transfection (TORREX 120D; Astrophysics Research Corp., Long Beach, CA, USA).

Antisera, plasmids and reagents
Flag-hTwist-1, Myc-hTwist-1 (both cloned by *Bam*H1/*Xho*1 in pcDNA3) and GST-hTwist-1 (by *Eco*R1 in pGex4T.3) were cloned using standard PCR procedures with the full-length cDNA of the IRAUp969H1277D clone (RZPD, Berlin, Germany) as a template. Point mutations were introduced by PCR using the QuikChange site mutagenesis protocol (Stratagene, Cedar Creek, TX, USA): all primers sequences are available on request. shRNA constructs were cloned into the pTER vector. Targeting sequence used for generating shRNA against human Twist-1 was as follows: 5′-GCTGAGCAA GATTCAGACC-3′. Targeting sequences used for generating shRNA against human PKB and firefly luciferase were as described previously (Bozulic *et al*., 2008; Vichalkovski *et al*., 2008). The reporter plasmids $p21^{Waf1}$-Luc (el-Deiry *et al*., 1993) and Bax-Luc (Fogal *et al*., 2000) were as published, E-cadherin-Luc construct was a kind gift of A. DiFeo (The Mount Sinai School of Medicine). Antibodies recognizing total PKB, phospho-PKB (Ser473), $p21^{Waf1}$ and the phospho-(Ser/Thr) PKB substrate antibody were purchased from Cell Signaling Technology (Beverly, MA, USA); anti-p53 (DO-1) and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A rat monoclonal anti-tubulin (YL1/2) and mouse anti-Myc-9E10 were used as hybridoma supernatants; the antibody against Flag (M2) was from Sigma (St Louis, MO, USA). Anti-Mdm2 antibody was described previously (Feng *et al*., 2004). Anti-Twist-P-Ser42 and anti-Twist-P-Ser123 rabbit polyclonal antibodies were raised against synthetic peptides conjugated to keyhole limpet hemocyanin: CGGRKRRSS(PO3H2)RRSAGG peptide for the Ser42 phosphorylation site and CNVRERQRTQS (PO3H2)LNEA peptide for the Ser123 phosphorylation site. Peptide synthesis, rabbit injection and bleed collection were carried out by NeoMPS (Strasbourg, France). The antibodies were then purified on the corresponding antigenic peptides coupled to cyanogen bromide-activated Sepharose (Amersham Biosciences, Piscataway, NJ, USA). Antibodies were eluted with 0.2 M glycine, pH 2.2. Antibody specificity was confirmed by standard peptide competition. Briefly, an aliquot of the purified antibody was incubated with the phosphopeptide (at 0.5 mg/ml final concentration) in TBS buffer for 2 h on ice with agitation before western blotting. Polyclonal antibody recognizing total Twist-1 was raised in rabbits against full-length GST-fusion Twist-1 (Eurogentec, Liege, Belgium). Antisera were affinity-purified using immobilized antigen and extensively characterized.

In vitro *kinase assays on peptides and GST-fusion proteins*
The peptides for *in vitro* kinase assay were synthesized by NeoMPS and further purified (Franz Fischer, FMI, Basel, Switzerland). For a kinase reaction, we added 2 μl (100 ng) of the activated or inactivated recombinant PKBβ (Yang *et al*., 2002) to a reaction mix containing 70 μM of the corresponding peptide (RKRRSSRRSAGG, S42/S45; RKRRSARRSAGG, S42A; RKRRSSRRAAGG, S45A; RERQRTQSLNEA, T121/S123; RERQRAQSLNEA, T121A; RERQRTQALNEA, S123A), 2 μl (2 μCi) of γ-^{32}P-ATP and 20 μM ATP in 20 μl of kinase reaction buffer (30 mM HEPES/KOH (pH 7.4), 25 mM β-glycerophosphate, 2 mM DTT, 20 mM $MgCl_2$, 0.1 mM sodium vanadate). After incubation for 30 min at 30 °C, kinase reactions were stopped with 50 mM EDTA, transferred to phosphocellulose P11 paper (Whatman, Bottmingen, Switzerland), fixed and washed four times in 1% phosphoric acid and once with acetone, dried and assayed by scintillation counting.

GST-Twist-1 or its point mutants (S42A, S123A and SS42, 123/AA) were purified from bacterial strain BL-21 according to a standard protocol. For *in vitro* kinase assays, we incubated 5–10 μg of GST fusion protein with 100 ng of the recombinant PKBβ in the presence of 20 μM ATP in 25 μl of kinase reaction buffer for 30 min at 30 °C. The reaction was stopped by adding SDS sample buffer and protein phosphorylation was analyzed by SDS–polyacrylamide gel (PAGE) and western blotting with the phospho-(Ser/Thr) PKB substrate, anti-Twist-P-Ser42 and anti-Twist-P-Ser123 antibodies or by capillary liquid chromatography–tandem mass spectrometry (LC-MSMS, see Supplemental Materials and methods).

Mice
The PTEN +/− mutant mice used in this study were supplied by Dr P Pandolfi (Beth Isreal Deconess Mediacl Centre, Harvard) and have been described previously (Di Cristofano *et al*., 1998). Mice were housed in groups with 12-h dark/light cycles and with access to food and water *ad libitum*, in accordance with the Swiss Animal Protection Laws. All procedures were conducted with the appropriate approval of the Swiss authorities. For histological analysis, we dissected organs from 6-month-old wild-type and heterozygous mice, placed them in 4% paraformaldehyde/phosphate-buffered saline (PBS) and allowed to fix overnight (18 h) at 4°C. Tissues were then subjected to a series of washes with PBS, 50% EtOH/PBS and 70% EtOH/PBS before being processed and embedded in paraffin using the Medite Paraffin Processing Unit (Burgdorf, Germany).

Immunohistochemistry
Paraffin-embedded slides of whole mouse embryos (E14.5) and sections of paraffin-embedded tissue microarray slides (MC2081, MCN601) (US Biomax Inc., Rockville, IN, USA) were deparaffinized, blocked by 20% normal goat serum for 1 h and then stained with αTwist-P-Ser42 antibody and phosphor-Ser473 PKB (No. 4060; Cell Signaling Technology) and counterstained with hematoxylin-eosin using standard protocol for the Discovery XT Staining Module (Ventana Medical Systems, Oro Valley, AZ, USA). Images were

processed with a Nikon E600 microscope system (Nikon, Melville, NY, USA).

Luciferase reporter gene assays
Harvested cells were lysed in reporter lysis buffer (25 mM Tris-phosphate, 2 mM DTT, 2 mM CDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100). Luciferase activity was determined in a luminometer (Duo Lumat LB 9507; Berthold, Bad Wildbad, Germany) by injecting 20 µl of assay buffer (40 mM Tricine, 2.14 mM $(MgCO_3)_4Mg(OH)_2 \times 5\ H_2O$, 5.34 mM $MgSO_4$, 0.2 mM EDTA, 66.6 mM DTT, 540 M CoA, 940 µM luciferin, 1.06 mM ATP) and measuring light emission for 10 s.

Cell-cycle analysis and apoptosis measurement
For FACS analysis of DNA content, we trypsinized cells, fixed them in 70% ice-cold ethanol, then treated them with RNase A (10 µg) in propidium iodide solution (sodium citrate (pH 7.5), 69 µM propidium iodide) for 30 min at 37 °C and analyzed them using a FACSCalibur flow cytometer (Becton Dickinson, Bedford, MA, USA). Cells undergoing apoptosis were harvested, washed with PBS and subdivided into two fractions. One fraction was stained with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA) and subjected to flow cytometry for detection of mitochondrial depolarization ($\Delta\Psi$m). Red fluorescence (FL-2 channel) of JC-1 (J-aggregates) indicated intact mitochondria, whereas green fluorescence (FL-1 channel) showed monomeric JC-1 produced by breakdown of $\Delta\Psi$m during apoptosis. The remaining cells were analyzed by western blotting.

Conflict of interest

Part of the results of this study has been used for the patent application EP 09 174 681.

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PERSPECTIVE

From man to mouse and back again: advances in defining tumor AKTivities in vivo

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AKT hyperactivation is a common event in human cancers, and inhibition of oncogenic AKT activation is a major goal of drug discovery programs. Mouse tumor models that replicate AKT activation typical of human cancers provide a powerful means by which to investigate mechanisms of oncogenic signaling, identify potential therapeutic targets and determine treatment regimes with maximal therapeutic efficacy. This Perspective highlights recent advances using in vivo studies that reveal how AKT signaling supports tumor formation, cooperates with other mutations to promote tumor progression and facilitates tumor-cell dissemination, focusing on well-characterized prostate carcinoma mouse models that are highly sensitive to AKT activation. The implications of these findings on the therapeutic targeting of AKT and potential new drug targets are also explored.

Introduction

The AKT [also known as protein kinase B (PKB)] signaling pathway is dysregulated in diverse disease processes, ranging from neurodegenerative disorders to diabetes and cancer. AKT is a protein kinase with three isoforms [AKT1, AKT2 and AKT3 (also known as PKBα, PKBβ and PKBγ, respectively)], which influence cell survival, growth, proliferation and insulin signaling. Hyperactive AKT signaling, in many cases via alterations in phosphoinositol-3 kinase (PI3K) and phosphatase and tensin homolog (PTEN), is common in many pathologies, particularly cancer.

Inhibiting hyperactivated AKT might help to treat cancer, in which the PI3K-PTEN-AKT pathway is one of the most commonly mutated signaling pathways. Therefore, upstream regulators or downstream effectors of AKT are desirable therapeutic targets. For example, humanized monoclonal antibodies specific for the upstream epidermal growth factor receptor family, or inhibitors of the downstream mammalian target of rapamycin complex 1 (mTORC1), are FDA approved, including for the treatment of some cancer types. This suggests the potential for further manipulation of AKT signaling for anti-oncogenic treatments and has promoted extensive research into AKT activation and signaling, as is evident from the growing number of related clinical trials (LoPiccolo et al., 2008; Klein and Levitzki, 2009).

There are several mouse models of cancer that provide a malleable in vivo environment in which to study the role of the AKT pathway in tumorigenesis, and to predict the efficacy, selectivity and side effects that novel therapies will have in patients. In this Perspective article, we review new developments in this field that have enabled important insights into the role of AKT in cancer and, by focusing on AKT mouse models of prostate carcinoma (CaP), explore how these advances should facilitate more effective, tailored cancer treatments for patients in the future.

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PI3K-PTEN-AKT pathway signaling and its activation in human tumors

The PI3K-PTEN-AKT signaling pathway transduces signals from membrane receptors to its major effector molecule, AKT (Fig. 1). This pathway is conserved in lower organisms and is ubiquitous in mammalian cells, in which it promotes cell growth, proliferation and survival, as well as mediates hormone metabolism, immune responses and angiogenesis (for a review, see Alessi, 2001; Brazil and Hemmings, 2001; Altomare and Testa, 2005; Manning and Cantley, 2007; Bozulic and Hemmings, 2009). Receptor tyrosine kinase stimulation activates AKT via a tightly controlled multi-step process (Fig. 1). Activated receptors stimulate class 1A PI3K directly or via adapter molecules such as the insulin receptor substrate (IRS) proteins. Class 1A PI3Ks bind via one of their five regulatory subunits (p85α, p85β, p55α, p55γ or p50α), which in turn binds to one of three catalytic subunits [p110α, p110β or p110δ (in leukocytes)], allowing conversion of phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P_2] lipids to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] at the plasma membrane. AKT binds to PtdIns(3,4,5)P_3 at the plasma membrane, where 3-phosphoinositide-dependent protein kinase 1 (PDK1) can then access the 'activation loop' of AKT to phosphorylate threonine 308 (Thr308), leading to partial AKT activation (Alessi et al., 1997). This AKT modification is sufficient to activate mTORC1 by directly phosphorylating and inactivating proline-rich AKT substrate of 40 kDa (PRAS40) and tuberous sclerosis protein 2 (TSC2). These phosphorylation events release the kinase mammalian target of rapamycin (mTOR) that is bound to PRAS40, prevent TSC2 GTPase activity and allow active, GTP-bound Rheb to activate mTORC1. mTORC1 substrates include the eukaryotic translation initiation factor, 4E, binding protein 1 (4EBP1) and ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (S6K1), which in turn phosphorylates the ribosomal protein S6 (S6; also known as RPS6), promoting protein synthesis and cellular proliferation.

Phosphorylation of AKT at Ser473 in the C-terminal hydrophobic motif, either by mTOR associated with mTOR complex 2 (mTORC2) (Sarbassov et al., 2005) or by DNA-dependent protein kinase (DNA-

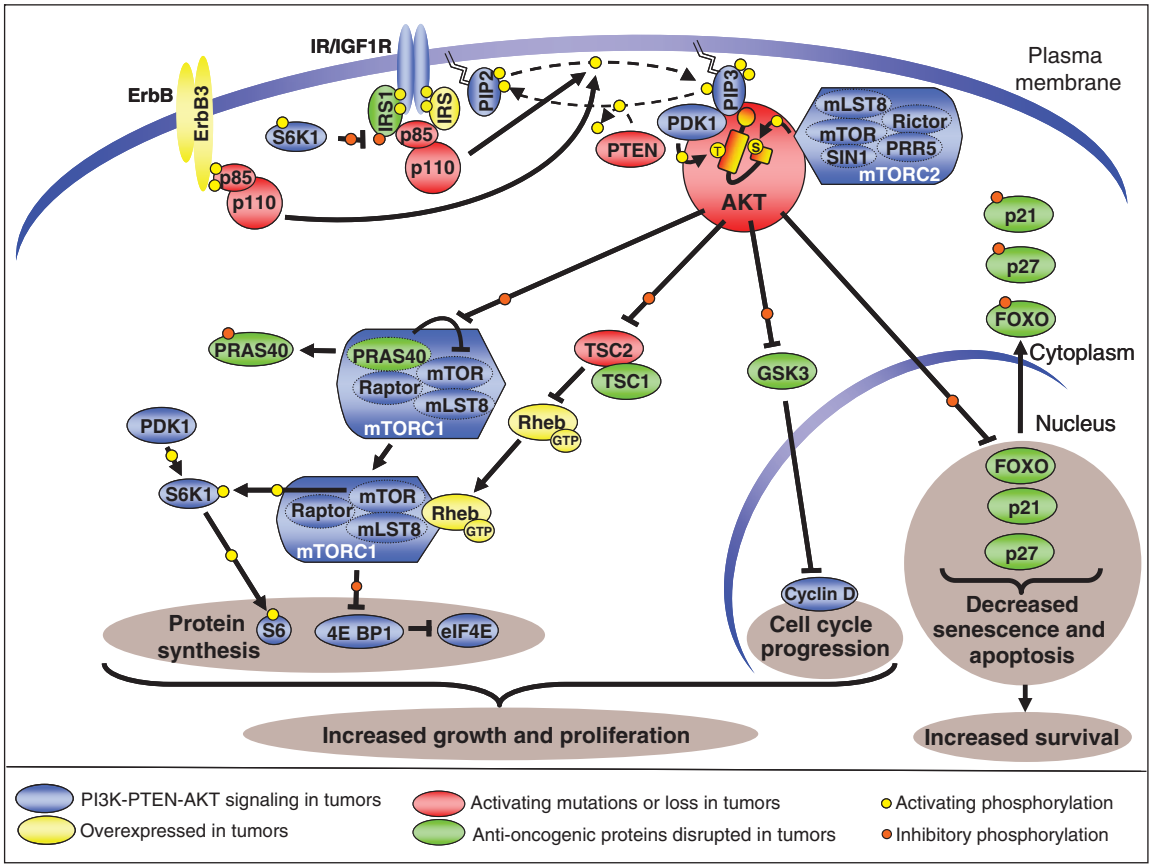

Fig. 1. The PI3K-PTEN-AKT signaling pathway and the causes of its hyperactivation in tumorigenesis. Increased AKT activation can occur through overexpression of pathway components (yellow) or inhibitory mutation or complete loss of components (red). These events can lead to decreased activation of anti-oncogenic proteins (green) and increased growth, proliferation and survival signals to promote tumorigenesis. eIF4E, eukaryotic translation initiation factor 4E; LST8, target of rapamycin complex subunit LST8; PRR5, proline-rich protein 5; SIN1, SAPK-interacting protein 1; PIP2, PtdIns(3,4)P_2; PIP3, PtdIns(3,4,5)P_3.

PK) (Feng et al., 2004) stimulates full AKT activity. Full activation of AKT leads to additional substrate-specific phosphorylation events, including inhibitory phosphorylation of the proapoptotic FOXO proteins. Dephosphorylation of Ser473 by the PH-domain leucine-rich repeat-containing protein phosphatases PHLPP1 and PHLPP2, and the conversion of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 by PTEN, inhibits AKT signaling.

Human tumors commonly display amplification or overexpression of cell-surface receptors or signaling molecules that activate the PI3K-PTEN-AKT pathway, activating mutations of PI3K, loss of expression of the negative regulator PTEN and/or mutation of AKT (Fig. 1). These mutations account for findings that the AKT pathway is activated in a high proportion of tumors, in a wide variety of tissues; a selection of these findings are summarized in Table 1.

From man to mouse: elucidating oncogenic AKT signaling in mice

Mouse models are invaluable tools for understanding how mutations in PI3K-PTEN-AKT signaling contribute to tumorigenesis in human cancer. In humans, mild mutations in PTEN, TSC1 or TSC2 result in familial tumor-susceptibility syndromes, and a similar neoplasia is seen when the mild mutations are modeled in mice. By contrast, human biopsies of spontaneous tumors that display PTEN, TSC1 or TSC2 loss have increased AKT signaling compared with biopsies of tumors from patients with familial syndromes. This increased AKT signaling and the corresponding more severe tumor development are reflected in mouse models that have heterozygous and homozygous loss of PTEN, TSC1 or TSC2. These studies highlight the contribution that mouse models of AKT activation can make in elucidating oncogenic AKT signaling in familial and spontaneous neoplasia.

Human tumor-susceptibility syndromes and neoplasia phenotypes in mice

In humans, mutations in PTEN (which is upstream of AKT), or in TSC1 or TSC2 (which are downstream of AKT), result in complex disease syndromes such as Cowden disease or tuberous sclerosis (Table 2). These diseases display a variety of symptoms (for reviews, see Eng, 2003; Zhou et al., 2003; Crino et al., 2006), because various point mutations or partial deletions in these genes cause diverse effects on the levels of functional protein, thereby affecting AKT-related signaling (Zhou et al., 2003; Trotman et al., 2007). Interestingly, *PTEN*$^{+/-}$, *TSC1*$^{+/-}$ or *TSC2*$^{+/-}$ mice do not show the same spectrum of symptoms as patients with these syndromes, which might reflect the fact that mutated forms of these proteins

Table 1. Common upstream AKT-activating mutations and somatic AKT mutations found in tumors

Gene	Mutation	Affected tissue	Incidence (%) (samples)	References
ErbB2	Point insertions	Breast	4 (4/94)	Stephens et al., 2004; Lee et al., 2006; Forbes et al., 2010
		Lung	4 (5/120)	
		Stomach	5 (9/180)	
		Colorectal	3 (3/104)	
ErbB2	Amplification	Breast	18-40 (19/103, 110/245, 34/86)	Slamon et al., 1987; Slamon et al., 1989; Reichelt et al., 2007; Marx et al., 2009
		Ovary	26 (31/120)	
		Stomach	16 (27/166)	
		Oesophogeal	5-15 (7/145, 16/110)	
IRS2	Amplification	Colon	2 (3/146)	Knobbe and Reifenberger, 2003; Parsons et al., 2005
		Brain	2 (2/103)	
p85 (PI3K)	Deletions	Ovary	4 (3/80)	Philp et al., 2001; Mizoguchi et al., 2004; Parsons et al., 2008
		Colon	2 (1/60)	
		Brain	3-10 (1/30, 9/91)	
p110α (PI3K)	Various (especially point mutants E542K, E545K and H1047R)	Colon	19-32 (6/32, 74/199)	Bachman et al., 2004; Campbell et al., 2004; Samuels et al., 2004; Hartmann et al., 2005; Lee et al., 2005; Levine et al., 2005; Oda et al., 2005; Buttitta et al., 2006; Gallia et al., 2006; Velasco et al., 2006; Parsons et al., 2008
		Brain	7-27 (5/70, 10/105, 11/73, 4/15)	
		Stomach	4-25 (4/94, 12/185, 3/12)	
		Breast	18-40 (13/53, 13/72, 19/92, 25/93, 28/70)	
		Liver	36 (26/73)	
		Lung	4 (1/24)	
		Ovary	6-12 (11/167, 24/198)	
		Uterus	36 (24/66)	
p110α (PI3K)	Amplification	Lung	33 (46/139)	Shayesteh et al., 1999; Campbell et al., 2004; Wu et al., 2005; Yamamoto et al., 2008
		Ovary	25-58 (83/341, 7/12)	
		Breast	9 (9/92)	
K-Ras	Point mutant (especially G12D)	Pancreas	75-95 (5/6, 12/16, 28/30, 21/22)	Almoguera et al., 1988; Smit et al., 1988; Suzuki et al., 1990; Burmer et al., 1991; Boughdady et al., 1992; Lemoine et al., 1992; Rodenhuis and Slebos, 1992
		Colon	30-60 (10/29, 14/40, 37/61)	
		Lung	15-25 (22/129, 43/181)	
PTEN	Promoter methylation	Brain	35-37 (22/60, 27/77)	Salvesen et al., 2001; Baeza et al., 2003; Garcia et al., 2004; Khan et al., 2004; Wiencke et al., 2007
		Breast	34-48 (15/44, 43/90)	
		Uterus	19 (26/138)	
PTEN	Deletions, point mutants, LOH	Most tissues:		Rasheed et al., 1997; Tashiro et al., 1997; Feilotter et al., 1998; Zhou et al., 1999; Kondo et al., 2001; Forbes et al., 2010
		Brain	16-31 (14/91, 13/42)	
		Prostate	49 (25/51)	
		Uterus	50 (16/32)	
		Colon	25 (14/57)	
PDK1	D527E	Colon	<1 (1/204)	Parsons et al., 2005
	T354M	Colon	1 (2/204)	
AKT1	E17K	Breast	4-8 (4/93, 5/61)	Carpten et al., 2007; Davies et al., 2008; Kim et al., 2008; Malanga et al., 2008; Shoji et al., 2009
		Colorectal	6 (3/51)	
		Ovary	2 (1/50)	
		Endometrium	2 (2/89)	
		Skin	<1 (1/137)	
		Lung	6 (2/36)	
AKT1	Amplification	Stomach	20 (1/5)	Staal, 1987; Knobbe and Reifenberger, 2003
		Brain	1 (1/103)	
AKT2	S302G	Colon	<1 (1/204)	Parsons et al., 2005; Soung et al., 2006
	R371H	Colon	<1 (1/204)	
	A377V	Lung	1 (1/79)	
AKT2	Amplification	Colon	1 (2/146)	Cheng et al., 1992; Bellacosa et al., 1995; Ruggeri et al., 1998; Snijders et al., 2003; Parsons et al., 2005; Pedrero et al., 2005; Nakayama et al., 2006; Nakayama et al., 2007; Yu et al., 2009
		Breast	3 (3/106)	
		Ovary	12-18 (16/132, 12/66)	
		Head and neck	30 (12/40)	
		Pancreas	20 (7/35)	
AKT3	E17K	Skin	2 (2/137)	Davies et al., 2008
AKT3	G171R	Brain	11 (1/9)	Hunter et al., 2006
AKT3	Amplification	Brain	4-14 (4/230, 29/206)	Hashimoto et al., 2004; CGARN, 2008; Ichimura et al., 2008
		Liver	30 (6/19)	

PDK1, AKT2 and the AKT3 G171R somatic point mutants were detected in tumor samples and are hypothesized to promote activation due to the mutations occurring in kinase domains; however, their activating potential has yet to be characterized. Genes are listed in the order that their encoded proteins act in the PI3K-PTEN-AKT signaling pathway (from receptor activation to AKT activity). Studies first reporting the indicated mutations, and those with large datasets, are referenced. LOH, loss of heterozygosity.

in the human syndrome can affect regulation of the AKT pathway even without the large decreases in protein levels that are present in the heterozygous mouse models. However, increased neoplasia formation in multiple organs is a feature common to both the human syndromes and mice with the corresponding gene disruptions (Table 2). This suggests that a conserved mechanism underlying the neoplasia phenotype is increased AKT signaling.

Increases in AKT signaling correlate with both the severity of neoplasia and PTEN, TSC1 or TSC2 dysregulation both in neoplasms derived from the human familial syndromes and in the corresponding mouse models, as well as in spontaneous tumor formation. Cowden disease patients with mutations that decrease PTEN levels have a corresponding increase in AKT activity and exhibit increased formation of gastrointestinal polyps (Trotman et

Table 2. Phenotypes of mouse models representing common human familial tumor syndromes

Mutated protein	Human syndrome	Human presentation of syndrome	Mouse phenotype upon deletion of associated gene
PTEN	Cowden disease, Bannayan-Riley-Ruvalcaba syndrome, Proteus syndrome, Proteus-like syndrome	Breast, thyroid and uterine neoplasia, lipomas, macrocephaly, hamartomatous polyps of the gastrointestinal tract, mucocutaneous lesions	Homozygous lethal; conditional deletion in tissues generally results in tumors; heterozygotes develop a range of neoplasms (adrenal, thyroid, uterine, breast, prostate, gastrointestinal tract)
TSC1 or TSC2	Tuberous sclerosis	Hamartomata and cysts in multiple organ systems, polycystic renal disease, renal carcinoma	Homozygous lethal; heterozygotes develop renal cystadenomas, liver hemangiomas, lung adenomas

All data taken from Online Mendelian Inheritance in Man (OMIM), McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), 2009 (http://www.ncbi.nlm.nih.gov/omim/ and http://www.informatics.jax.org/).

al., 2007). In mice, mutations that affect the regulation of PTEN or TSC2 display abnormal activation of AKT signaling and develop a neoplasia phenotype that is reminiscent of the human syndromes (Pollizzi et al., 2009; Alimonti et al., 2010b; Wang et al., 2010), which is milder than that observed in *PTEN*$^{+/-}$ or *TSC1/2*$^{+/-}$ mice. In the case of spontaneous tumor formation, it is homozygous loss of PTEN or the TSC proteins that is seen in tumor progression. These observations suggest that mouse models in which AKT signaling is activated are relevant to both familial and spontaneous neoplasia formation in humans.

Modeling human tumors with activating PI3K-PTEN-AKT pathway mutations

Mutations in components of the PI3K-PTEN-AKT pathway in human tumors (Table 1) lead to the development of tumors that have activated AKT and increased downstream oncogenic signaling. Accordingly, several mouse models have shown that AKT activation is crucial for tumorigenesis. These models demonstrate that, in various tissues, tumor phenotypes are induced by AKT activation and can be reversed by preventing AKT activation through its simultaneous deletion (Table 3).

These models have assisted in elucidating the contribution of AKT signaling in specific tumor tissue settings, as was the case in mammary-specific *ErbB2*-overexpressing mouse models that represent the common *ErbB2* amplification found in human breast tumors. However, owing to the selective activation of AKT, three mouse models of AKT activation – conditional *PTEN*-null, *PTEN*$^{+/-}$ and transgenic mice conditionally expressing a myristoylated form of AKT (myr-AKT) – are the models of choice for studying the contribution of specific AKT signaling to tumorigenesis. myr-AKT expression results in the translocation of constitutively active AKT to the plasma membrane, inducing neoplasia (Staal, 1987). Importantly, although myr-AKT models drive neoplasia development via a non-physiological, modified form of AKT, neoplasia development in these mice mimics the phenotype and AKT activation pattern seen in mice with heterozygous *PTEN* loss, an event that is common in many human tumors (Majumder et al., 2008; Gray et al., 1995). Conditional ablation of *PTEN* results in a more aggressive phenotype, consistent with the observation that homozygous *PTEN* loss is a late event in many human cancers and therefore making it an attractive model for testing therapies for the most refractory tumors (Trotman et al., 2003; Wang et al., 2003; Komiya et al., 1996). *PTEN* loss promotes activation of PDK1, thereby potentially activating multiple signaling pathways via the phosphorylation of over 20 protein kinases, including AKT (Mora et al., 2004). However, *AKT1* ablation on a *PTEN*$^{+/-}$ background inhibits neoplasia formation, indicating that AKT1 and not alternative PDK1 signaling is responsible for neoplasia (Chen et al., 2006). Furthermore, *PTEN*$^{+/-}$ mice with hypomorphic *PDK1* alleles that cause 80-90% reduction in PDK1 expression show reduced tumor formation that is proportional to the loss of PDK1-mediated phosphorylation, which is required for AKT activation (Bayascas et al., 2005). Therefore, neoplasia development correlates with the upregulation of AKT activity in *PTEN* and myr-AKT models, making them particularly useful for determining how alterations in AKT signaling can affect neoplasia development.

Prostate cancer: an example of the involvement of AKT in tumorigenesis

In humans, premalignant proliferation of the epithelium in the prostate gland is commonly referred to as prostatic intraepithelial neoplasia (PIN) and is considered a precursor lesion to CaP. PIN displays decreasing PTEN expression with progression to CaP, and PTEN expression is completely lost in late-stage advanced CaP (McMenamin et al., 1999; Schmitz et al., 2007). PTEN loss correlates with AKT activation and tumor grade, indicating that PTEN contributes to prostate tumorigenesis via loss of its function as a negative regulator of AKT activation (Malik et al., 2002). In mouse models, PTEN loss or AKT activation in the prostate induces PIN and progression to CaP, and increases in the level of phosphorylation of AKT Ser473 parallel the reduction in PTEN levels and correspond with increased incidence, onset and progression to CaP (Di Cristofano et al., 2001; Kwabi-Addo et al., 2001; Trotman et al., 2003; Wang et al., 2003). Mice lacking PTEN expression in the prostate display features that resemble advanced CaP in humans, including local invasion, metastasis and androgen independence. Therefore, in humans and mice, similar molecular pathology seems to underpin CaP development.

The similar pathological features of CaP development in mice and humans, and the importance of AKT in the process, make this an excellent setting in which to dissect how AKT signaling supports tumorigenesis and to determine how it could be therapeutically inhibited to treat cancer. Accordingly, the following sections focus on recent advances in mouse models of CaP that have defined fundamental concepts on how AKT signaling contributes to neoplasia, progression and acquisition of malignancy in CaP (summarized in Fig. 2).

Neoplasia is initiated by AKT signaling to mTORC1

One of the earliest events in human CaP is loss of expression of NK3 transcription factor related, locus 1 (Nkx3.1), which leads to aberrant gene expression (Bethel et al., 2006). This is seen from early PIN, when increased cellular proliferation and moderate activation of AKT is observed (Renner et al., 2007). A connection between

Table 3. Defining mouse models of AKT activation and signaling in tumorigenesis

Gene					
Primary mutation	**Secondary mutation**	**Type of mutation**	**Effect**	**Phenotype**	**References**
ErbB2	–	Tg-MG	O/E	Mammary tumors	Muller et al., 1988
	PTEN	cKO-MG	Loss	Acceleration of tumors	Dourdin et al., 2008
	Myr-AKT1	Tg-MG	O/E	Acceleration of tumors	Young et al., 2008
	AKT1	KO	Loss	Inhibition of tumors and of metastasis	Ju et al., 2007; Maroulakou et al., 2007
	AKT2	KO	Loss	Acceleration of tumors	Maroulakou et al., 2007
	AKT3	KO	Loss	No observable effect on tumorigenesis	Maroulakou et al., 2007
PolyMidT	–	Tg-MG	O/E	Mammary tumors	Guy et al., 1992
	IRS1	KO	Loss	Mammary tumors and metastasis	Ma et al., 2006
	IRS2	KO	Loss	Decreased number of mammary tumors	Nagle et al., 2004
	AKT1	KO	Loss	Inhibition of tumors	Maroulakou et al., 2007
	AKT2	KO	Loss	Acceleration of tumors	Maroulakou et al., 2007
	AKT3	KO	Loss	No observable effect on tumorigenesis	Maroulakou et al., 2007
IRS1	–	KO	Loss	Insulin resistance, reduced growth	Araki et al., 1994; Tamemoto et al., 1994
	–	Tg-MG	O/E	Mammary tumors and metastasis	Dearth et al., 2006
IRS2	–	KO	Loss	Diabetes	Withers et al., 1998
	–	Tg-MG	O/E	Mammary tumors and metastasis	Dearth et al., 2006
K-ras^{G12D}	–	KI-PtMt	G12D	Lung tumors	Johnson et al., 2001
	p85α$^{T208D/K227A}$	KI-PtMt	T208D and K227A	Resistant to Ras binding and Ras-induced lung tumorigenesis	Gupta et al., 2007
Myr-p110α	–	Tg-Pr	O/E	Hyperplasia	Renner et al., 2007
p85α	–	cKO-Pr	Loss	No observable tumor phenotype	Jia et al., 2008
p85β	–	cKO-Pr	Loss	No observable tumor phenotype	Jia et al., 2008
PTEN	–	Tg-Hy	Hy/+	Neoplasia after long latency	Alimonti et al., 2010b
	–	Tg-Hy	Hy/–	Increased neoplasia, decreased latency	Trotman et al., 2003
	–	KO	Ht	MG, adrenal, thyroid, colon, B-cell, uterine, prostate neoplasia	Di Cristofano et al., 1998; Suzuki et al., 1998; Podsypanina et al., 1999
	IRS2	KO	Loss	Decreased number of tumors in multiple tissues	Szabolcs et al., 2009
	p85α$^{+/-}$	KO	Ht	Increased number of GI polyps, PIN unaffected	Luo et al., 2005
	p85β$^{-/-}$	KO	Loss	Decreased PIN	Luo et al., 2005
	p85α$^{+/-}$β$^{-/-}$	KO	Ht/Loss	Increased number of GI polyps, PIN unaffected	Luo et al., 2005
	PDK1	KO	Hy/–	Inhibition of PTEN-driven tumors	Bayascas et al., 2005
	AKT1	KO	Loss	Inhibition of PTEN-driven tumors	Chen et al., 2006
	–	cKO-Sk	Loss	Susceptibility to carcinogens	Inoue-Narita et al., 2008
	B-Raf^{V600E}	Tg-Sk	O/E	Metastatic melanoma	Dankort et al., 2009
	–	cKO-Pr	Loss	Metastatic prostate tumors	Trotman et al., 2003; Wang et al., 2003
	P110α$^{-/-}$	cKO-Pr	Loss	No effect on PTEN tumorigenesis	Jia et al., 2008
	P110β$^{-/-}$	cKO-Pr	Loss	Loss of PTEN tumorigenesis	Jia et al., 2008
	mTOR	cKO-Pr	Loss	Inhibition of tumors	Nardella et al., 2009
	rictor	cKO-Pr	Loss	Inhibition of tumors	Guertin et al., 2009
AKT1	–	KO	Loss	Small, partial lethality	Chen et al., 2001; Cho et al., 2001a
Myr-AKT1	–	Tg-Pr	O/E	High-grade PIN; 100% penetrance	Majumder et al., 2003
	p27	cKO	Loss	Progression to cancer	Majumder et al., 2008
	–	Tg-Lv	O/E	Insulinomas	Alliouachene et al., 2008
	S6K1	KO	Loss	Inhibition of insulinomas	Segrelles et al., 2007
	–	Tg-Sk	O/E	Skin carcinomas, DMBA sensitive	Malstrom et al., 2001
	–	Tg-Tc	O/E	Thymic lymphoma with short latency	Rathmell et al., 2003
	–	Tg-MG	O/E	With DMBA: ER+ mammary tumors	Blanco-Aparicio et al., 2007
Myr-AKT$^{\Delta 11\text{-}60}$	–	Tg-Br	O/E	No tumor phenotype	Holland et al., 2000
	K-Ras^{G12D}	PtMt-Br	O/E	Glioblastoma	Holland et al., 2000
	B-Raf^{V600E}	PtMt-Br	O/E	Gliomas	Robinson et al., 2010
AKT1^{E40K}	–	Tg-Tc	E40K	Peripheral lymphoma with long latency	Malstrom et al., 2001
AKT2	–	KO	Loss	Diabetes	Cho et al., 2001b
Myr-AKT2	–	Tg-Tc	O/E	Thymic lymphoma after long latency	Mende et al., 2001
AKT3	–	KO	Loss	Small brain	Easton et al., 2005; Tschopp et al., 2005
DNAPK$_{cs}$	–	KO	Loss	Thymic lymphomas	Jhappan et al., 1997
	AKT1	KO	Loss	Inhibition of DNAPK$_{cs}$-driven thymic lymphomas	Surucu et al., 2008

Br, brain; cKO, conditional tissue deletion; DMBA, 7,12-dimethylbenz[a]anthracene; DNAPK$_{cs}$, DNA-dependent protein kinase catalytic subunit; ER+, estrogen receptor positive; GI, gastrointestinal; Ht, heterozygous loss of protein; Hy, hypomorphic gene modification; Hy/–, hypomorphic and deleted allele; Hy/+, hypomorphic and wild-type allele; KI, knock-in gene mutation; KO, whole body knockout; Loss, complete protein loss; Lv, liver; MG, mammary gland; O/E, protein overexpression; PolyMidT, polyoma middle T oncoprotein; Pr, prostate; PtMt, genetic point mutant; Sk, skin; Tc, T-cell; Tg, transgenic. Proteins are listed in the order that they act in the PI3K-PTEN-AKT signaling pathway (from receptor activation to AKT activity).

Nkx3.1 and AKT is illustrated by the fact that mice lacking Nkx3.1 expression display cellular proliferation and low-grade PIN, together with increased PI3K signaling to AKT (Abdulkadir et al., 2002; Gary et al., 2004; Song et al., 2009). The onset of PIN also correlates with phosphorylation of the mTORC1 target 4EBP1 (Kremer et al., 2006), indicating that AKT-mediated activation of mTORC1 is involved in this process. In addition, increased AKT activation in myr-AKT or *PTEN*$^{+/-}$ mice leads to the development of high-grade PIN (Di Cristofano et al., 1998; Majumder et al., 2003; Wang et al., 2003; Ratnacaram et al., 2008). Knocking out *AKT1* in *PTEN*$^{+/-}$ mice prevents PIN development, illustrating that this process depends on AKT1 signaling (Chen et al., 2006). Furthermore, AKT signaling to mTORC1 is crucial for PIN development, because inhibition of mTORC1 signaling with a specific inhibitor, RAD001, in myr-AKT1 mice abolished mTOR signaling and cellular proliferation, and restored normal prostatic gland architecture (Majumder et al., 2004).

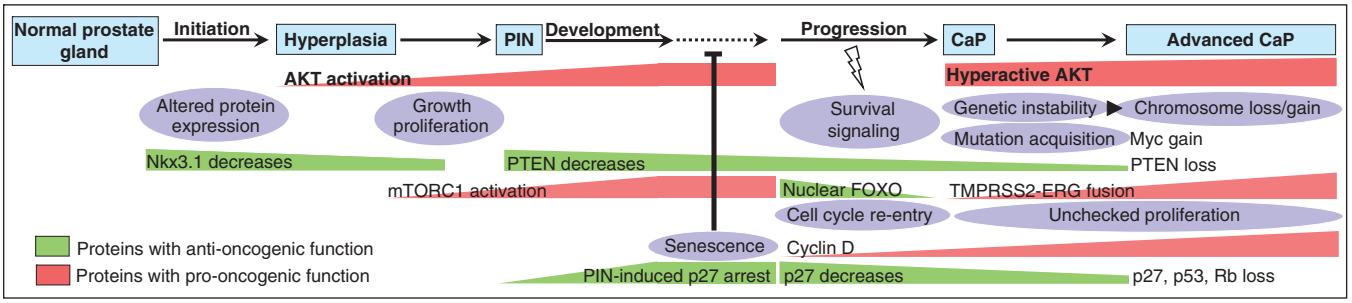

Fig. 2. AKT activation and associated events during tumor development in the prostate. Initiation of tumorigenesis and hyperplasia occur through altered protein expression, which promotes AKT activation, mTORC1 activation and PIN development. p27-induced senescence prevents progression to CaP, which is overcome by AKT signaling combined with changes in the expression and/or activity of other proteins and genes. CaP displays high AKT activation, supporting proliferation, survival and acquisition of mutations with increasing genetic instability, leading to the gross chromosomal losses and gains that are characteristic of advanced malignant CaP.

The role of mTORC1 in proliferation and PIN development is further highlighted by mouse models in which TSC2 (Ma et al., 2005) and Rheb (Nardella et al., 2008) expression is manipulated. Mouse prostates overexpressing Rheb promote activation of mTORC1 and S6K1, and the consequent phosphorylation of their respective targets, 4EBP1 and S6. Prostates in these mice display mild increases in proliferation and low-grade PIN, albeit with long latency (~10 months) and low penetrance (20-30%). Conversely, in *TSC2*$^{+/-}$ mouse prostates, mTOR phosphorylation is insufficient to trigger downstream signaling and phosphorylation of S6. In this case, neither increased proliferation nor PIN development is observed. These studies complement the RAD001 findings, indicating that the activation of mTORC1 and downstream signaling is necessary and sufficient to induce cellular proliferation and initiate PIN. Importantly, PIN develops in the Rheb-overexpressing prostates in the presence of low AKT activation and signaling, owing to a negative feedback loop inhibiting PI3K via S6K1 and IRS1 (Nardella et al., 2008). Thus, independent of other AKT-mediated signaling, activation of mTORC1 signaling seems to be the essential component of PIN development in prostates exhibiting activated AKT.

Senescence responses prevent progression from PIN to CaP

Prostates expressing myr-AKT1 or Rheb express the senescence markers senescence-associated β-galactosidase (SA-βgal) (Majumder et al., 2008; Nardella et al., 2008) and heterochromatin protein 1 (HP1) (Majumder et al., 2008), and exhibit increasing nuclear localization of the cell-cycle inhibitor p27, during PIN development. Cellular growth arrest and reduced incorporation of BrdU (a reagent used to track proliferating cells), indicate a functional and effective senescence checkpoint in affected PIN epithelium (Majumder et al., 2008). Importantly, SA-βgal (Chen et al., 2005; Majumder et al., 2008), HP1 (Majumder et al., 2008) and p27 nuclear accumulation (Di Cristofano et al., 2001; Majumder et al., 2008) are also found in human PIN samples. p27 accumulation is also observed during PIN in *PTEN*$^{+/-}$ mice and in an unrelated mouse model of CaP in which the *Myc* oncogene is expressed in the prostate, suggesting that senescence is a specific response to PIN induction and not to AKT activation or signaling (Majumder et al., 2008).

The relationships between senescence induction, PIN development and mTORC1 activation are illustrated by inhibition of mTORC1 with RAD001 in myr-AKT1 mice. RAD001 does not affect the levels of AKT Ser473 phosphorylation, but does decrease the phosphorylation of the downstream mTORC1 target S6 within 2 days of treatment. However, reduction of p27 nuclear accumulation and expression of HP1 was not observed until after 14 days of treatment, when normal prostatic gland architecture was restored (Majumder et al., 2004). Therefore, senescence is a response to loss of normal prostatic gland architecture rather than to increased mTORC1 signaling, which favors proliferation.

Prostatic glands displaying PIN and senescence have disrupted basement membrane (BM) attachments. E-cadherin mediates crucial attachment to the BM and is reduced in human CaP (Umbas et al., 1992). Knockdown of E-cadherin expression, or culturing isolated myr-AKT mouse prostate epithelial cells or myr-AKT-transfected human prostate epithelial cells in low adherence conditions, compromises BM contacts and induces p27 nuclear accumulation (Majumder et al., 2008). Thus, the loss of BM attachment observed in PIN morphology induces p27-mediated senescence that prevents progression from PIN to CaP in *PTEN*$^{+/-}$ and myr-AKT models.

Overcoming p27-mediated cell-cycle arrest

Loss of p27 expression and cell-cycle dysregulation might be mechanisms by which activated AKT signaling overcomes p27-mediated senescence in the prostate and induce CaP. In human CaP, increasing loss of p27 (Cordon-Cardo et al., 1998; Fernandez et al., 1999; Di Cristofano et al., 2001; Majumder et al., 2008) or activation of the protein that degrades p27, Skp2, is often observed (Yang et al., 2002). In myr-AKT1 or *PTEN*$^{+/-}$ mouse prostates, a gene-dose effect on development of CaP is seen with *p27* loss, with CaP cells exhibiting decreased senescence markers and reactivation of cell cycling (Di Cristofano et al., 2001; Majumder et al., 2008). Reduction of p27 levels is seen when Skp2 is overexpressed in mouse prostate, with low-grade PIN to low-grade CaP lesions developing relative to the levels of Skp2 expressed (Shim et al., 2003). Conversely, loss of Skp2 on a *PTEN*-null background triggers senescence with increased expression of p27 and the other cell-cycle inhibitors p21 and p19^{Arf} (Lin et al., 2010). p27 can inhibit cell cycling by binding to cyclin D, a function also executed by the

cell-cycle inhibitor p18. Similarly to p27, decreased p18 in conjunction with PTEN heterozygosity accelerates the progression to high-grade PIN, whereas complete loss of p18 expression leads to invasive carcinoma that exhibits increased AKT phosphorylation (Bai et al., 2006). Thus, activated AKT can overcome p27-mediated senescence when combined with cellular changes that affect either p27 expression levels or cell-cycle activation.

Alternative signaling inputs can overcome p27-mediated senescence by affecting the interplay between AKT activation, p27 levels, glandular architecture and cell-cycle control. TSC2 inhibits mTORC1 and Wnt signaling via Rheb and β-catenin, respectively. Promoting mTORC1 signaling alone by crossing *PTEN*$^{+/-}$ with Rheb-overexpressing mice results in high-grade PIN with 100% penetrance (Nardella et al., 2008). However, if *PTEN*$^{+/-}$ mice lose a single allele of *TSC2*, PIN develops, similar to when Rheb is overexpressed in *PTEN*$^{+/-}$ mice, but in 75% of mice it progresses to CaP (Ma et al., 2005). This indicates that CaP development can occur via dysregulation of TSC2-mediated control of Wnt signaling. In mice and humans, *TSC2* loss stabilizes β-catenin and increases transcription of the cyclin D gene to promote cell-cycle progression (Mak et al., 2005). However, *TSC2* loss can also affect β-catenin–E-cadherin complexes to impair BM–E-cadherin signaling; this signaling is crucial for prostatic p27-mediated senescence. Indeed, nuclear β-catenin accumulation and decreased E-cadherin is observed in human CaP (Jaggi et al., 2005), and expression of dominant-stabilized nuclear β-catenin in the prostate results in CaP via increased Wnt signaling and disruption of cell contacts (Pearson et al., 2009). Therefore, signaling pathways such as Wnt might promote cell cycling or disrupt senescence signaling to p27 to enable neoplastic cells with activated AKT to progress to human CaP.

AKT antiapoptotic and survival signaling in the progression to CaP

Full activation of AKT occurs via mTORC2-mediated phosphorylation of Ser473, which promotes cell survival by inhibiting the activity of proapoptotic proteins such as the FOXO proteins. Mouse embryonic fibroblasts lacking components of mTORC2 lack AKT Ser473 phosphorylation but exhibit phosphorylation of Thr308 (Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006). mTORC1 activity is unaffected by mTORC2 disruption, but phosphorylation of FOXO1 and FOXO3a are reduced, increasing apoptosis in conditions of stress. Apoptosis is reversed by reconstitution of mTORC2 (Shiota et al., 2006). The nuclear proapoptotic activity of FOXO proteins is inhibited by AKT-mediated phosphorylation, which sequesters them in the cytoplasm. In mice displaying ~60% loss of PTEN expression, cytoplasmic localization of FOXO proteins is observed. Approximately 20% of these animals form CaP lesions with increased Ser473 signaling and decreased p27, but surprisingly with no significant increase in mTORC1 activation compared with *PTEN*$^{+/-}$ PIN lesions (Trotman et al., 2003). Further increases in Ser473 AKT phosphorylation resulting from a complete loss of PTEN expression correlate with additional decreases in p27 expression and nuclear FOXO1 levels without affecting the activation of mTORC1, indicating that these actions on p27 and FOXO1 occur independently of mTORC1 activation (Trotman et al., 2003). Consistent with this, knockout of the mTORC2 component rictor does not affect mTORC1 signaling but does abolish Ser473 phosphorylation, maintaining a strong nuclear accumulation of FOXO1 and preventing the progression to CaP, even in *PTEN*-null prostates (Guertin et al., 2009). This indicates that increased signaling to AKT substrates downstream of Ser473 phosphorylation promotes antiapoptosis and survival to overcome senescence and facilitate the progression of tumors.

Unchecked cell cycling and genetic instability promotes CaP malignancy

Unchecked cell cycling and increased survival signaling in tumor cells promotes the acquisition of mutations that cause genetic instability (GI) and gross genetic aberrations, such as rearrangements and chromosomal loss or gain. GI is detected in ~60% of prostate biopsies from patients with CaP (Thuret et al., 2005), whereas fusions of genes encoding transmembrane protease, serine 2 (TMPRSS2) and ETS-related gene (ERG) transcription factor (*TMPRSS2-ERG*) are seen in up to 65% of cases of human prostate neoplasia (Perner et al., 2006; King et al., 2009). In vitro and in vivo evidence supports the idea that *TMPRSS2-ERG* fusion is an event that occurs in early CaP that promotes malignancy by contributing to migration, invasion and metastasis (Tomlins et al., 2008; Carver et al., 2009; Yu et al., 2010). In mice, TMPRSS2-ERG or ERG overexpression promotes PIN, and the progression from PIN to CaP in both of these cases specifically involves AKT (Furusato et al., 2008; Klezovitch et al., 2008; Carver et al., 2009; King et al., 2009; Zong et al., 2009). In human samples, TMPRSS2-ERG is found in regions of copy-number loss (including of PTEN) (Taylor et al., 2010). Recent data from studies in mice suggest that progression to CaP when ERG is overexpressed and AKT is hyperactivated is supported by increased androgen receptor (AR) signaling (Goldstein et al., 2010). Interestingly, another study, which involved genomic profiling of 218 human prostate tumors, illustrated that, although AR abnormalities were exclusive to metastatic samples, increased signaling via the AR pathway was found in 56% of non-metastatic samples (Taylor et al., 2010).

PTEN heterozygosity is common in human CaP, with complete loss (via deletion of a region of chromosome 10q) occurring only in 30-60% of advanced CaP cases (Gray et al., 1995; Komiya et al., 1996). This suggests that progression to CaP via alternative mechanisms that cooperate with PTEN heterozygosity might be selected for by neoplastic cells. Analysis of *PTEN*-null prostates demonstrated that they exhibit a strong cellular senescence response mediated by p53, with an increase in the expression of cell-cycle inhibitors p19 and p21 (Chen et al., 2005). Maintaining p53 levels in *PTEN*-null prostates by administration of Nutlin-3, the small-molecule inhibitor of p53 degradation by the E3 ubiquitin-protein ligase Mdm2 (Mdm2), results in >50% reduction in tumor volume, with glands showing significantly increased senescence (Alimonti et al., 2010a). Hence, in human tumors in which the loss of a *PTEN* allele occurs in two distinct steps, overcoming a p27- and then p53-mediated senescence might impair tumor survival, suggesting that PTEN loss is observed only in advanced CaP, when additional mutations prevent an effective p53-mediated senescence response.

In advanced CaP, chromosomal regions are frequently lost [such as Ch17q (which encodes p53) and Ch13q (which encodes the tumor suppressor protein Rb)] (Carter et al., 1990; von Knobloch et al., 2004) or amplified [such as Ch8q (which encodes Myc)] (Jenkins et al., 1997; Qian et al., 2002). Similarly to PTEN loss, p53

deletion occurs late in human CaP, promoting malignancy (Qian et al., 2002). In mice, loss of p53 has little effect on the prostate, although *PTEN*-null prostates that mimic the loss of PTEN in late human CaP display invasion and metastasis, which are features of advanced CaP. However, when *p53* is knocked out in *PTEN*$^{-/-}$ prostates, aggressive and lethal CaP develops (Chen et al., 2005). Thus, complete loss of PTEN and p53 is consistent with the concept that, at late stages of CaP, malignancy is promoted by gross chromosomal abnormalities that arise from genetic instability.

From mice to man: targeting AKT in anticancer therapies

The mouse models discussed in the earlier sections illustrate that the alterations in PI3K-PTEN-AKT signaling that are associated with CaP progression in mice are similar to those seen in human biopsies. These studies highlight which members of the pathway might be valid therapeutic targets, and at which stage of the disease current or developing therapies would be most effective (see Table 4). In addition, the studies demonstrate that a crucial aspect of AKT-mediated tumor progression in CaP is the involvement of cooperating mutations (see Table 5), which should direct the development of new combinational therapeutic regimes. Importantly, AKT activation in the prostate affects conserved pro- and anti-oncogenic signaling, which is often disrupted in tumors of other tissues, suggesting that the findings in the prostate are applicable to tissues outside the prostate. The following section explores the potential of current and future strategies by which to control AKT signaling in tumors, including monotherapies and combination therapies.

Inhibiting AKT activation and signaling

PI3K inhibitors

The potential benefits of PI3K inhibition in treating cancer are supported by the finding that PI3K-activating mutations in p110α are common in human tumors, and that the inhibitors LY294002 and wortmannin, which primarily target PI3K, potently inhibit AKT activation in cancer cell lines. Toxicity of these early PI3K inhibitors prompted the development of new, more specific PI3K inhibitors (for reviews, see Brachmann et al., 2009; Maira et al., 2009), including isoform-specific inhibitors that were developed to prevent induction of insulin resistance while retaining anti-tumor efficacy. These might be particularly effective in tumors in non-insulin-sensitive tissues, because deletion of certain isoforms of the p85 or p110 subunits of PI3K has shown that these subunits operate in tumors in a tissue-specific manner (Luo et al., 2005; Jia et al., 2008). Indeed, in the prostate, p110β selectively mediates tumorigenic signaling (Jia et al., 2008). However, pan-PI3K inhibitors and dual PI3K and mTOR inhibitors block tumor growth in mouse models without overt effects on glucose levels (Folkes et al., 2008; Maira et al., 2008; Serra et al., 2008; Liu, T. J. et al., 2009). Interestingly, helical-domain mutations of p110α require Ras binding for AKT activation (Zhao and Vogt, 2010), and Ras binding to p110α is also required for Ras-mediated tumorigenesis (Gupta et al., 2007). Although Ras binding and signaling is unaffected by current ATP-competitive PI3K inhibitors, combination therapy with MEK inhibitors in mice shows strong synergy in inhibiting tumors (Engelman et al., 2008). Thus, PI3K inhibitors might yet prove effective in either single or combinational therapeutic regimes.

AKT inhibitors

Pan and isoform-specific inhibition of AKT isoforms are potential anti-tumor therapies, particularly in tumors that have lost PTEN expression. AKT1 is necessary for tumor progression in *PTEN*$^{+/-}$ mice in multiple organs, including the prostate (Chen et al., 2006), and the loss of AKT1 was found to reduce neoplasia without compensatory AKT2 or AKT3 upregulation. AKT1 is also a promising target because *PTEN*$^{+/-}$ neoplasia development in mice is significantly reduced when AKT1 levels are decreased by 50% (e.g. in heterozygous AKT1 deletions), a decrease in activity that is therapeutically more achievable than complete inhibition. Specific inhibitors of AKT2 or AKT3 could also be effective in the treatment of tumors such as melanomas (AKT3) (Stahl et al., 2004) or ovarian carcinomas (AKT2) (Cheng et al., 1992), in which these

Table 4. Patterns of PI3K-PTEN-AKT signaling in human and mouse CaP, and current therapies

Protein modification	Species	Stage of prostate neoplasia progression				Drug target	References
		BH	PIN	CaP	Metastasis		
PI3K activation	M	√	X	X	X	CT	Renner et al., 2007; Zhu et al., 2008; Brachmann et al., 2009; Maira et al., 2009
	H	X	X	√	√		
PTEN loss	M (+/–)	√	√	X	X	*	Di Cristofano et al., 2001; Kwabi-Addo et al., 2001; Trotman et al., 2003; Wang et al., 2003; Kremer et al., 2006; Ratnacaram et al., 2008
	H (+/–)	X	X/√	√	X/√		
	M (–/–)	√	√	√	√		
	H (–/–)	X	X	√	√		
AKT activation	M	√	√	X	X	CT	Malik et al., 2002; Majumder et al., 2003; Li et al., 2007; Renner et al., 2007
	H	√/X	√	√	√	In dev	
TSC2 loss	M	√	√	X	X	*	Ma et al., 2005
	H	nd	nd	nd	nd		
Rheb activation	M	√	√	X	X	*	Nardella et al., 2008
	H	nd	nd	nd	nd		
mTOR loss	M	X	X	X	X	Avail[a]	Kremer et al., 2006; Apsel et al., 2008; Maira et al., 2008; Guertin et al., 2009; Nardella et al., 2009; Thoreen et al., 2009
	H	X	X	X	X	CT	
mTOR activation	M	nd	nd	nd	nd	In dev	
	H	√/X	√	√	√		
4EBP1 activation	M	nd	nd	nd	nd	*	Kremer et al., 2006; Hsieh et al., 2010
	H	X	√	√	√		

[a]mTORC1 inhibitors (rapalogs). √, observed; X, not observed; *, not currently in development; Avail, approved for use; BH, benign hyperplasia; CT, in clinical trials; H, human samples; In dev, currently under development; M, mouse model; ND, not determined. Proteins are listed in the order that they act in the PI3K-PTEN-AKT signaling pathway (from receptor activation to AKT activity).

Table 5. Oncogenic events in human CaP that have been shown to cooperate with AKT activation in mice and are potential drug targets

Gene			Stage of prostate neoplasia progression					
Primary mutation	Secondary mutation	Species	BH	PIN	CaP	Metastasis	Drug target	References
Nkx3.1 loss	–	M	√	√	X	X	–	Abdulkadir et al., 2002; Kim et al., 2002; Gary et al., 2004; Bethel et al., 2006; Zong et al., 2009
	PTEN$^{+/-}$	M	√	√	√	X		
	–	H	√	√	√	√		
ERG gain	–	M	√	√	X	X	–	Petrovics et al., 2005; Rostad et al., 2007; Klezovitch et al., 2008; Carver et al., 2009
	PTEN$^{+/-}$	M	√	√	√	X		
	–	H	√	√	√	√		
PAR4 loss	–	M	√	X	X	X	–	Fernandez-Marcos et al., 2009
	PTEN$^{+/-}$	M	√	√	√	X		
	–	H	X	X	√	(X)		
FGF8b gain	–	M	√	√	X	X	–	Gnanapragasam et al., 2003; Zhong et al., 2006
	PTEN$^{+/-}$	M	√	√	√	√		
	–	H	X	X	√	√		
TRMSS2-ERG fusion	–	M	√	X	X	X	–	Tomlins et al., 2008; Carver et al., 2009; King et al., 2009
	PTEN$^{+/-}$	M	√	√	√	X		
	–	H	X	√/X	√	√		
p27 loss	–	M	√	X	X	X	CT	Cordon-Cardo et al., 1998; Di Cristofano et al., 2001; Majumder et al., 2008; Dickson and Schwartz, 2009
	PTEN$^{+/-}$	M	√	√	√	X	In dev*	
	–	H	X	X	√	√		
p18 loss	–	M	√	X	X	X	CT	Bai et al., 2006; Dickson and Schwartz, 2009
	PTEN$^{+/-}$	M	√	√	√	X	In dev*	
	–	H	X	X	√	√		
ErbB2 gain	–	M	√	√	X	X	Avail	Kuhn et al., 1993; Morote et al., 1999; Casimiro et al., 2007; Rodriguez et al., 2009
	PTEN$^{+/-}$	M	√	√	√	X		
	–	H	X	X	√	√		

*Cyclin dependant kinase inhibitors; CT, in clinical trials; H, human; M, mouse; (X), Not examined. See Table 4 footnote for abbreviations. Protein modifications are listed in order of reported occurrence in patient biopsies during the development from benign hyperplasia to advanced CaP.

isoforms are specifically increased. However, as with PI3K inhibitors, inhibition of AKT2 activity could promote insulin resistance. In such a case, pan-AKT inhibitors could circumvent off-target effects of AKT2 inhibition. In tumors with supraphysiological levels of AKT activation and a dependency on AKT for tumorigenesis, pan-AKT inhibitors could significantly reduce the levels of activated AKT within the tumor while minimizing adverse drug reactions, such as insulin resistance, in response to complete inhibition of a single AKT isoform in normal tissues. The pan-AKT inhibitor GSK690693 was shown to act in this manner in a mouse xenograph model and is now in clinical trials (Rhodes et al., 2008). These inhibitors could also enable broad-spectrum inhibition and allow targeting of tumors irrespective of the predominant AKT isoform involved.

mTOR inhibitors

The mTORC1 complex was first successfully inhibited by rapamycin, and related 'rapalogs' such as RAD001 that have more favorable pharmacokinetics and tolerance are used in various clinical settings. The ability of RAD001 to reverse PIN in the mouse prostate indicates that rapalogs can effectively target this process, although detection of such early abnormalities in patients is difficult. However, it is worth noting that cellular proliferation mediated by mTORC1 contributes to the development of pre-neoplastic lesions in over 60% of endometrial hyperplasia cases (Milam et al., 2008) and precancerous intestinal polyps from Peutz-Jeghers Syndrome patients (Shackelford et al., 2009). In mouse models of these pathologies, progression can be inhibited with mTORC1 inhibitors (Milam et al., 2007; Shackelford et al., 2009).

In advanced cancers, the rapalog RAD001 is approved as a single agent for renal cell carcinomas that depend on mTORC1-mediated translation of hypoxia-inducible factor 1 (HIF1). Tumor progression after resection has also been shown to be reduced in some gliomas treated with rapamycin in a phase 1 trial (Cloughesy et al., 2008). However, clinical studies suggest that the effectiveness of mTORC1 inhibition is exceptional: in most tumor settings, the anti-tumorigenic effects of mTORC1 inhibition are outweighed by increased AKT-Ser473-mediated pro-survival and antiapoptotic signaling that occurs because of loss of the negative feedback regulation of the PI3K pathway by S6K1 (Shi et al., 2005; O'Reilly et al., 2006; Cloughesy et al., 2008). Indeed, in Rheb-overexpressing mouse prostates, treatment with RAD001 showed loss of phosphorylated S6 (downstream of mTORC1) but increased AKT Ser473 phosphorylation. This suggests that therapeutic efficacy requires rapalogs used in combination with therapies that disrupt the feedback regulation of AKT Ser473 phosphorylation, such as PI3K or mTORC2 inhibitors.

mTORC2 inhibitors should prevent pro-survival and antiapoptotic functions. Indeed, loss of the mTORC2 component rictor prevents CaP in PTEN-deficient mouse prostates (Guertin et al., 2009). Inhibiting mTORC2 activity would be an effective way to target the wide variety of tumors that have high phosphorylation of Ser473, via PI3K activation or reduced activity of PHLPP1 or PHLPP2 (PHLPP1/2; the phosphatases responsible for dephosphorylation of AKT Ser473). In human colon cancer, expression of PHLPP1/2 is lost or reduced in ~75% of tumor samples (Liu, J. et al., 2009). In addition, a significant decrease in the levels of mRNA encoding FKBP51 (the protein that enables docking of PHLPP1/2 to AKT) was reported in pancreatic tumor tissue, and a decrease or loss of FKBP51 protein expression was found in pancreatic and breast cancer cell lines (Pei et al., 2009).

Dual mTORC1 and mTORC2 inhibitors that target the mTOR kinase (Apsel et al., 2008; Maira et al., 2008; Thoreen et al., 2009) are currently in clinical trials for their potential to inhibit tumor

proliferation and survival signals (www.clinicaltrial.gov/). In support of these agents as effective therapies, conditional ablation of *mTOR* in the *PTEN*-null mouse prostate blocks cellular proliferation and the development of PIN and CaP (Nardella et al., 2009). Use of mTOR inhibitors or specific mTORC2 inhibitors could prove particularly useful in settings of advanced cancer, such as CaP, in which PTEN expression has been lost, making AKT refractory to treatment by upstream receptor and PI3K inhibitors.

Combining inhibition of AKT signaling with additional therapeutics

ErbB2 inhibition

Effective inhibition of AKT signaling by ErbB2-specific monoclonal antibodies is the primary strategy in treating ErbB2-expressing breast tumors. Although such treatment can lead to tumor remission (Vogel et al., 2002), resistance occurs in ~50% of patients as a result of downstream mutations in genes encoding oncogenic proteins such as Ras and Src, or via loss of PTEN, leading to AKT activation (Berns et al., 2007). Loss of PTEN or an increase in activated AKT in ErbB2-overexpressing mouse mammary glands accelerates tumor formation in mice, whereas ablation of AKT1 or rapamycin-mediated inhibition of mTORC1 inhibits tumor progression (Mosley et al., 2007). In the prostate, ErbB2 can also cooperate with AKT activation in promoting CaP development (Rodriguez et al., 2009). This supports the use of ErbB2-specific antibodies together with rapalogs or AKT inhibitors to treat ErbB2-expressing tumors, including advanced CaP, in which ErbB2 is associated with androgen independence (Signoretti et al., 2000), increased tumor grade (Ross et al., 1993), anueploidy (Ross et al., 1993) and metastasis (Morote et al., 1999).

Raf, MEK and ERK inhibitors

Activation of the Ras-Raf-MEK-ERK signaling cascade by mutation or overexpression of extracellular receptors such as ErbB2 is common in many cancers. In advanced human CaP, mutations in Ras (7%) or Raf (10%) have been reported (Cho et al., 2006). In mouse models, ERK activation is associated with androgen independence, and simultaneous inhibition of ERK and AKT signaling has shown enhanced tumor inhibition (Gao et al., 2006; Kinkade et al., 2008). A relationship between mTORC1 inhibition and ERK activation has also been observed in the clinic when mTORC1 activity is inhibited with RAD001. In these cases, ERK activation occurs when mTORC1 is blocked (Carracedo et al., 2008), suggesting that blocking AKT signaling might result in compensatory rewiring of proliferation and survival signals through ERK. Positive outcomes after simultaneous ERK and PI3K signaling inhibition were observed in studies of mouse models of hepatocellular carcinoma (HCC) and ErbB2-overexpressing breast tumors; the use of MEK and mTORC1 inhibitors in HCC (Huynh, 2010), or a Raf inhibitor and blocking of PI3K signaling through neutralizing antibodies to ErbB2 in ErbB2-overexpressing breast tumors (Hausherr et al., 2006), improved the extent of tumor inhibition in both cases.

MEK-ERK activation also occurs as a result of the V600E B-Raf mutation in over 60% of pre-malignant melanocytic nevi, leading to increased ERK phosphorylation. Downstream signaling cascades inhibit TSC2 and increase cyclin D levels (Zheng et al., 2009), although melanoma progression is prevented by activation of an oncogene-induced senescence program. This senescence is relieved by increased AKT3 levels and signaling that cooperates with ERK to increase proliferative and survival signaling (Stahl et al., 2004; Cheung et al., 2008). Promotion to malignant melanoma occurs in up to 60% of cases, owing to loss of a portion of chromosome 10 that contains *PTEN* (Herbst et al., 1994; Robertson et al., 1998; Stahl et al., 2003). Highlighting the importance of AKT activation in melanoma, V600E B-Raf expression in *PTEN*-null mice leads to malignant melanoma formation (Dankort et al., 2009). Together, these findings suggest that simultaneous targeting of the ERK and AKT signaling pathways could be an effective way to treat tumors that commonly have Ras and Raf mutations.

Biguanides

Biguanides (AMPK activators) inhibit the activity of mitochondrial respiratory chain complex I, thereby reducing ATP levels and activating AMPK signaling. AMPK negatively regulates the cell cycle and prevents pro-oncogenic signaling by both Wnt and mTORC1 by activating TSC2 (Inoki et al., 2003) and inhibiting the Rag family of GTPases that are required for mTORC1 activation (Kalender et al., 2010). Decreased AMPK activity is observed in human breast cancer samples (Hadad et al., 2009). The AMPK activator metformin is well tolerated as an insulin-sensitizing agent and has also been shown to increase latency and reduce tumors in a mouse ErbB2 mammary model (Anisimov et al., 2005), possibly by inhibiting S6K1 activity and decreasing ErbB2 expression (Vazquez-Martin et al., 2009). Metformin also impairs the ability of p53-negative tumor cells to form tumors in mice (Buzzai et al., 2007) and, combined with doxorubicin, selectively kills cancer stem cells (Hirsch et al., 2009). However, in a mouse estrogen-receptor-negative mammary model of cancer, metformin promoted angiogenesis and supported tumors (Phoenix et al., 2009), suggesting that the drug might be effective only in conjunction with other chemotherapeutic agents. Studies of CaP support this: metformin significantly inhibits tumor growth, but does not induce apoptosis of prostate cancer cells when injected into mice, despite the fact that tumors from treated mice showed a cell cycle block with decreased cyclin D, activation of Rb and increased p27 levels (Ben Sahra et al., 2008). These features of metformin treatment could prove beneficial in conjunction with AKT inhibitors, because increased cyclin D levels (Rodriguez et al., 2009), inactivated Rb or decreasing p27 all cooperate with AKT activation in the mouse prostate to allow PIN progression to CaP. Thus, if metformin can hinder these cooperating alterations, it might prove effective in inhibiting tumor progression and induce apoptosis when used in conjunction with agents that inhibit AKT survival signaling.

Conclusions and future directions

The broad incidence of activating AKT mutations in tumors from diverse tissues indicates a crucial role for AKT signaling in tumor development and progression. In this Perspective, we have discussed recent work on mouse models that has helped to define how AKT signaling contributes to tumorigenesis at different stages, and through different downstream signaling pathways, to facilitate the proliferation, survival and progression of tumors. The findings from mouse models are consistent with analyses of patient tumors, providing validation that mouse experiments are relevant to the

human disease. Although we focused here on CaP, it is clear that the disruption of the PI3K-PTEN-AKT pathway, or of the mTOR complexes, is also associated with tumors in other tissues (Table 3). This suggests that at least the core members of this pathway contribute to oncogenic signaling – and particularly to proliferation – in the prostate, as well as in tumors of other tissues. This is promising from a therapeutic perspective because several of these pathway components are targets of anti-oncogenic therapies that have already been approved for the clinic. Therefore, these therapies might by broadly applicable for treating several different types of cancer.

An interesting finding in studies of CaP is that tumor progression generally requires cooperation of secondary mutations with activated AKT (Table 5). Many mutations that cooperate with AKT hyperactivation in the prostate – such as increased ErbB2 (and ERK activation), loss of p27 or loss of p18 – promote cell cycle progression and are also associated with tumors of a wide variety of tissues. This has important implications for treating tumors of tissues in which mutations in ErbB2 or cell cycle proteins are common because it suggests that the presence of even moderate AKT activation could have significant effects on progression. Inhibition of AKT-mediated survival and antiapoptotic signaling both alone and in conjunction with cell cycle inhibition might be a powerful regime for treating CaP and other tumors with cooperating mutations. Notably, some of these secondary mutations are the target of existing therapies. For example, tumor cells treated with the common anti-diabetic treatment metformin show inhibited cyclin D levels. The cell cycle is also controlled with cyclin-dependent kinase inhibitors that are in clinical trials, raising the possibility that their use together with inhibitors of AKT signaling could provide a well-tolerated therapeutic regime effective for treating a broad spectrum of tumor types. Alternatively, supporting the induction of senescence responses could also prove effective. Proof of this concept was recently shown by the finding that Skp2 inhibition promoted p27-, p21- and $p19^{Arf}$-mediated senescence in a preclinical study (Lin et al., 2010).

A number of areas still remain to be defined with respect to their contribution to AKT signaling in tumors. These include identifying mechanisms of PTEN regulation (Poliseno et al., 2010) and activities independent of its effects on AKT1 (Mounir et al., 2009) that could be compromised during tumorigenesis and therefore be valid therapeutic targets. Similarly, an understanding of how Ser473-specific PHLPP phosphatases are regulated is still to be fully explored. Investigation into both of these areas has the potential to identify novel targets involved in tumor progression.

Finally, with the ongoing progress on strategies to therapeutically inhibit AKT, the compensatory rewiring by tumors will become increasingly relevant in terms of resistance to future AKT signaling inhibitors. As discussed earlier, rewiring to activate ERK signaling is observed upon inhibition of mTORC1 (Carracedo et al., 2008). Similarly, 'PI3K-addicted' cells have been shown to be able to survive AKT inhibition by signaling through PDK1 (Vasudevan et al., 2009). Interestingly, CaP in *ErbB2;PTEN*$^{+/-}$ mice, and PIN in *LKB1*$^{+/-}$ mice, showed signaling downstream of mTORC1 without mTORC1 activation, suggesting alternative activation pathways (Pearson et al., 2008; Rodriguez et al., 2009). Interestingly, in both cases, activated PDK1 was observed and proposed to be mediating this signaling. Understanding whether PDK1 or other proteins can sustain tumors that have inhibited AKT activity, and via which downstream substrates and signaling pathways this can occur, are among the many issues that will be addressed in the next generation of PI3K-PTEN-AKT mouse tumor models.

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COMPETING INTERESTS

The authors declare no competing interests.

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