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PRINCIPAL INVESTIGATOR: Charlene Rivera

CONTRACTING ORGANIZATION: University of North Carolina at Chapel Hill Chapel Hill, NC 27599-0001

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Role of Nonreceptor Tyrosine Kinase Ack1 in Prostate Cancer

Recent studies from our laboratory have shown that constitutively active Ack1 directly binds and tyrosine phosphorylates the androgen receptor (AR), resulting in ligand-independent AR activity. Moreover, Ack1 transforms LNCaP cells into androgen-independent and highly invasive tumors in nude mice. However, the role of Ack-1 in prostate cancer initiation and progression within the context of a complex organ remains poorly understood. To address this question, we generated transgenic mice expressing a myc-tagged constitutively active Ack1 transgene in the prostate epithelium, driven by a modified rat probasin promoter. Furthermore, Pb-Ack1 mice were crossed to genetically engineered mice (PTEN+/− and TgAP-T121) that develop high-grade prostate intraepithelial neoplasias (PIN) or adenocarcinoma. Pb-Ack1 prostates presented focal hyperplasia and nuclear atypia as early as 16 weeks of age. These prostatic lesions progressed to mPIN and microinvasive carcinoma as detected in prostates from 49 weeks old Pb-Ack1 mice. Crossing of Pb-Ack1 mice to TgAPT121 mice resulted in accelerated onset of progressive CaP which was detected as early as 24 weeks of age. Furthermore, the apoptotic index was reduced by 50% in bi-transgenic prostates when compared to single TgAPT121 prostates. An increase in serine-phospho-p65 was also observed in the bi-transgenic when compared to the TgAPT121 prostates. Pb-Ack1;Pten+/− compound mice presented mPIN lesions as early as 16 weeks of age. Phospho-serine Akt was detected exclusively in these mPIN lesions, suggesting PTEN loss of heterozygosity. In summary, these data present evidence that Ack-1's oncogenic activity can promote the initiation and progression of prostate cancer in vivo.

Prostate cancer, transgenic mice, mouse model
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Introduction

In its initial stages prostate cancer depends on androgen signaling, with androgen ablation therapy inducing tumor regression. The disease becomes extremely difficult to treat when it progresses to a hormone refractory state (androgen-independent prostate cancer or AICaP), therefore making it the second leading cause of cancer related death among men in the United States. Dissecting the molecular mechanisms underlying prostate cancer initiation and progression to AICaP will allow us to develop more efficient therapeutics and reduce the mortality rates of patients. Recent studies from our laboratory have shown that a constitutively active form of the intracellular tyrosine kinase Ack1 (activated cdc42-associated tyrosine kinase) directly binds and tyrosine phosphorylates the androgen receptor (AR), resulting in ligand-independent AR activity [1-2]. Constitutive activation was achieved by introducing a mutation that relieves kinase auto-inhibition (L487F) and by COOH-terminal truncation (amino acids 788-1,036) [3]. Moreover, caAck1 transforms poorly tumorigenic LNCaP cells into androgen-independent and highly invasive tumors in nude mice [1]. However, the role of Ack-1 in prostate cancer initiation and progression within the context of a complex organ remains poorly understood. To address this question, transgenic mice were generated expressing a myc-tagged truncated caAck1 transgene in the prostate epithelium, driven by a modified rat probasin promoter which contains two copies of the androgen response region (ARR2PB promoter). Characterization of these transgenic (Pb-Ack1) mice would allow us to understand the mechanisms through which this novel tyrosine kinase maybe drive the progression of prostate cancer in vivo as well as to further understand its potential role in AICaP. Furthermore, Pb-Ack1 mice were crossed to genetically engineered mice (Pten+/− and TgAP-T121) that develop high-
grade prostate intraepithelial neoplasias (PIN) or adenocarcinoma at known rates in order to understand how Ack1 activity, in combination with other lesions, will result in invasive and metastatic adenocarcinomas, thus reflecting the pathophysiology of human AICaP. $Pten^{+/−}$ and $TgAP-T121$ were chosen for the relevance of $PTEN$ and $Rb$ mutations in human prostate cancer as well as for the defined kinetics of cancer initiation and progression in these genetically engineered mice model of prostate cancer [4-5].
The Statement of Work of the present study aimed at: 1) determining if prostate-specific expression of caAck1 resulted in the formation of metastatic adenocarcinomas through increased growth and survival of prostate epithelial cells, 2) determining if prostate-specific expression of caAck1 resulted in androgen-independence growth and survival, 3) examining if the expression of caAck1 in established mouse models of prostate cancer resulted in accelerated tumor formation and metastasis and 4) determining if prostate-specific expression of caAck1 resulted in androgen- independence growth and survival when crossed to \( Pten^{+/−} \) and \( TgAP-T_{121} \) mice. In order to address these tasks, transgenic mice were generated by expressing a myc-tagged constitutively activated Ack1 transgene in the prostate epithelium, driven by a modified rat probasin promoter which contains two copies of the androgen response region (ARR2PB promoter). Furthermore, \( Pb-Ack1 \) mice were crossed to genetically engineered mice \( Pten^{+/−} \) and \( TgAP-T_{121} \). These mice constructs were chosen for the relevance of \( PTEN \) and \( Rb \) mutations in human prostate cancer as well as for the defined kinetics of cancer initiation and progression in these genetically engineered mice model of prostate cancer [4-5].

Task 1

For task 1, prostates from \( Pb-Ack1 \) mice and non-transgenic littermates were collected at multiple time points (12, 16, 24, 36, 48 and 72 weeks of age). Histopathological analysis of hematoxylin and eosin (H&E) stained paraffin sections showed that \( Pb-Ack1 \) prostates presented focal hyperplasia accompanied by nuclear atypia as early as 16 weeks of age. These prostatic lesions appeared to progress as evidenced by the presence of murine prostatic intraepithelial neoplasia (mPIN) and microinvasive carcinoma by 49 weeks of age (Appendix Fig. 1).
later phenotype was detected in one mouse and was determined by the extension of small acini of cytologically atypical cells into the fibromuscular layer (Appendix Fig. 1). mPIN lesions were multifocal in this specimen and displayed a cribiform pattern which resulted from further stratification of the epithelial layer and were accompanied by further cellular atypia (spindle-like shaped cells) and nuclear atypia (nuclear elongation and hyperchromatia) as well as hypercellularity of the stromal layer (Appendix Fig. 1). Desmoplasia (a disruption of the fibromuscular layer often seen in microinvasive adenocarcinoma) was detected by smooth muscle-actin immunohistochemistry on paraffin sections (Appendix Fig. 2). Double immunofluorescence detection of Ki67 (a proliferation marker) and cytokeratin 8 (a luminal epithelial marker) on paraffin sections showed increased cell proliferation in the Pb-Ack1 prostate epithelium when compared to WT (Appendix Fig. 3). The histopathological phenotype of Pb-Ack1 was published in PLoS ONE (see Appendix).

**Task 3**

For task 3, Pb-Ack1 mice were crossed to TgAPT121 mice. These transgenic mice were generated in the laboratory of our collaborator Dr. Terry Van Dyke and are a mouse model of prostate cancer in which T121 (a portion of the large T antigen) exclusively targets and inactivates all members of the pRb family [4]. Transgenic expression of T121 to prostate epithelial cells (TgAP-T121 mice) results in rapid formation of neoplasias that are derived from luminal epithelial cells. mPIN in TgAP-T121 mice develop by 12 weeks of age and progress to micro-invasive adenocarcinomas by 30 weeks of age. Metastasis has not been reported in these mice [4]. Expression of caAck1 in the TgAP-T121 background resulted in accelerated onset of the adeno-progressive phenotype which was detected as early as 24 weeks of age and determined by
H&E staining of paraffin slides (Appendix Fig. 4). Although the adeno-progressive phenotype was also observed some single TgAP-T121 prostates within our cohort, it was not as penetrant (30%) as in the PbAck1;TgAP-T121 bi-transgenic (86%). Furthermore, the apoptotic index was reduced in PbAck1;TgAP-T121 bi-transgenic prostates when compared to single TgAPT121 prostates at 16 and 24 weeks of age (Appendix Fig. 5). Apoptotic cells were detected in situ by TUNEL (Terminal dUTP Nick End Labeling) analysis on paraffin sections from 3 WT, PbAck1, TgAP-T121, and Pb-Ack1;TgAP-T121 prostates at 16 and 24 weeks of age. Apoptotic indexes were calculated by counting TUNEL-positive cells (brown, arrows) as a percentage of total cells (hematoxylin). Data were analyzed by Wilcoxon test (P<0.05 is considered statistically significant). In addition, an increase in NFκB activation was observed in the bi-transgenic prostates as early as 16 weeks of age (p=0.025) when compared to the TgAPT121 prostates (Appendix Fig. 6). NFκB activation was detected in situ by serine-phospho-p65 immunohistochemistry on paraffin slides from at least 3 WT, PbAck1, TgAP-T121, and Pb-Ack1;TgAP-T121 prostates at 16 and 24 weeks of age and analyzed as described above.

The microarray analysis proposed in this task is currently underway. RNA was isolated from anterior prostates of WT, PbAck1, TgAP-T121 and PbAck1;TgAP-T121 mice. RNA from three mice per genotype at 16 and 24 weeks of age were submitted for microarray analysis to the UNC Lineberger Comprehensive Cancer Center Genomics and Bioinformatics Core. A universal mouse reference comprised of total mouse embryonic RNA Universal mouse reference RNA was used as control. Data will be analyzed using the Significance Analysis of Microarrays (SAM) freeware.

Pb-Ack1 mice were also crossed to Pten+/− mice as proposed in task 3. Formation of mPIN from secretory prostate epithelial cells, progression to prostate cancer (CaP), and
formation of metastasis has been observed in mice with prostate-specific PTEN disruption [2]. Heterozygotic loss of PTEN causes prostate cancer at known rates, with most Pten<sup>+-</sup> mice developing LGPIN at around 6 months of age, and homozygote conditional ablation of PTEN in the mouse prostate resulting in mPIN at 6 weeks of age, CaP at 9 weeks of age, and metastasis at 12 weeks of age [2, 5]. This mouse model is highly relevant to the human disease given that the PTEN gene is often subject to loss of heterozygosity (LOH) in human prostate cancer [6]. Our preliminary data showed that Pb-Ack1;Pten<sup>+-</sup> mice present mPIN lesions as early as 16 weeks of age as determined by H&E staining of paraffin sections (Fig. 7). The onset of the mPIN phenotype is accelerated in Pb-Ack1;Pten<sup>+-</sup> prostates by approximately 32 weeks when compared to Pb-Ack1 and Pten<sup>+-</sup> prostates. These mPIN lesions present abnormal tufting glandular patterns with multiple cellular atypia including nuclear enlargement, inversion of nuclear to cytoplasmic ratio and hyperchromatism. The detection of Ki67 showed that these mPIN lesions were highly comprised of proliferating cells (Appendix Fig. 8). Ki67 was detected in situ by Ki67 immunohistochemistry on paraffin slides. Furthermore, abnormal cells within these mPIN lesions were positive for phosphorylated (activated) Akt, suggesting PTEN LOH (Appendix Fig. 9). Activated Akt was detected in situ by serine-phospho-Akt immunohistochemistry on paraffin slides. PTEN LOH is further suggested by the loss of PTEN staining in phospho-Akt positive cells, detected by immunohistochemistry of sequential slides from Pb-Ack1;Pten<sup>+-</sup> prostates (Appendix Fig. 10). Despite the acceleration of the mPIN phenotype in the Pb-Ack1;Pten<sup>+-</sup> as compared to the single Pb-Ack1 or Pten<sup>+-</sup>, progression to adenocarcinoma was not observed in these compound mice by 48 weeks of age as determined by H&E staining of paraffin slides (Appendix Fig. 7). To date, at least 3 Pb-Ack1 and 3 Pb-Ack1;Pten<sup>+-</sup> mice have been characterized.
Task 2, 4

I expect to conduct the androgen-ablation experiments described in Aim 1, Part 3 and Aim 2, Part 5 later in 2010. This will be done on WT, Pb-Ack1, Pten\(^{+/−}\) and Pb-Ack1:Pten\(^{+/−}\) animals and I will follow the methodology previously described in the fellowship.
Key Research Accomplishments

- Provided evidence showing that expression of truncated caAck1 is sufficient to cause to a prostatic phenotype when expressed in the prostate epithelium in vivo.
- Provided evidence showing that truncated caAck1 can lead to accelerated onset of prostatic phenotype when co-expressed in murine models of prostate cancer TgAP-T121 and Pten+/-.
- Provided evidence showing that expression of truncated caAck1 signals a cell survival pathway in vivo which rescues the apoptotic phenotype when co-expressed in TgAP-T121 mice.
- Provided evidence showing that expression of truncated caAck1 leads to increased activation of NFκB when co-expressed in TgAP-T121 mice.

Reportable Outcomes

Conclusions
I assessed the effect of Ack1 activity \textit{in vivo} by directing cell-specific expression of caAck1 to the murine prostate epithelium. The data present evidence for caAck-1 expression to be sufficient to deregulate proper cell cycle of luminal epithelial cells. This deregulation led to a hyperplastic phenotype that progressed to mPIN and in one case to adenocarcinoma. Furthermore, caAck1 activity was shown to accelerate the onset of the prostatic phenotype when crossed to both \textit{Pten}^{+/−} and \textit{TgAP-T121} mice, thus supporting our hypothesis that Ack1 activity in the murine prostate epithelium, in combination with precursor lesions, would result in a more aggressive phenotype.

The \textit{TgAP-T121} mice were generated by our collaborator Dr. Terry Van Dyke. I initially focused on this transgenic construct given that complete inactivation of pRb function was shown to initiate and progress prostate tumorigenesis at well characterized rates. When crossed to the \textit{TgAP-T121} mice, caAck1 was able to accelerate the onset of the prostate adeno-progressive phenotype from 30 weeks in the single \textit{TgAP-T121} transgenic to 24 weeks in the \textit{PbAk1; TgAP-T121} bi-transgenic construct. This acceleration correlated with caAck1’s ability to induce a pro-survival signal in these bi-transgenic prostates, which may explain the reduction in apoptosis observed in the \textit{PbAk1; TgAP-T121} bi-transgenic prostates. A pro-survival role for Ack1 has already been reported in NIH 3T3 cells [7]. Ack1 was shown to be required for the survival of v-Ras-transformed cells, although the cell survival signal remains to be elucidated [7]. Here I showed that the reduction of apoptosis in \textit{PbAk1; TgAP-T121} bi-transgenic directly correlated with an increased in activation and nuclear localization of NFκB, thus providing a molecular mechanism through which Ack1 may be elucidating its cell survival signal.

In addition, \textit{PbAk1} mice were also crossed to \textit{Pten}^{+/−} mice in order to assess the effects of Ack1 activity along with reduction of Pten gene dosage. This construct was chosen given the
high frequency of Pten mutation or reduced gene expression observed in human prostate cancer [6]. It has been previously reported that $Pten^{+/-}$ male mice develop prostate hyperplasia or mPIN at around 9-14 months of age [2, 5]. Here, I observed that the compound $PbAk1; Pten^{+/-}$ male mice develop mPIN as early as 16 weeks, whereas this prostatic phenotype is not seen until approximately 32 weeks in the $PbAk1$ or $Pten^{+/-}$ male mice in the same experimental cohort. Furthermore, the activation of Akt within these mPIN lesions suggests Ack1 activity may be providing the selective pressure needed for inactivation of Pten through LOH. Despite the accelerated mPIN phenotype in $PbAk1; Pten^{+/-}$ male mice, the phenotype fails to progress to adenocarcinoma or metastasis. Addressing the experiments proposed in tasks 2 and 4 will allow me to address whether an additional selective pressure such as lost of androgen will be needed to progress the phenotype.

In summary, I have shown that Ack1 activity is sufficient to cause hyperplasia or mPIN when overexpressed in the murine prostate epithelium in vivo. Furthermore, Ack1 activity is able accelerate the onset of the prostatic phenotype when present in combination with other lesions, thus reflecting the pathophysiology of human prostate cancer.
References

Figure 1. Histopathology of PbAck1 prostates. Normal -vs- hyperplastic epithelia in WT (A,E) and PbAck1 (B,F) prostates. mPIN (C,G) and microinvasive adenocarcinoma (D,H) phenotype in PbAck1 prostate at 49 weeks of age. Hematoxylin and Eosin staining. 200X and 400X magnifications.
Figure 2  **Desmoplastic phenotype in PbAck1 prostate.** Intact fibromuscular layer in normal WT (A) and hyperplastic PbAck1 (B) prostates at 24 weeks of age. (C) Disruption of the fibromuscular layer (star) is evident in 49 weeks old PbAck1 prostate displaying a microinvasive adenocarcinoma phenotype. Smooth muscle actin immunohistochemistry. 400X magnification.

Figure 3  **Hyperproliferation of luminal epithelium in PbAck1 prostates.** 49 weeks old prostate from PbAck1 immunostained for the luminal marker cytokeratin 8 (green) and the proliferation marker Ki67 (red). Double immunofluorescence. 400X magnification.
Figure 4  Histopathology of single and bi-transgenic prostates. Normal WT prostate phenotype compared to the progression from hyperplasia to mPIN to CaP in PbAck1, TgAP-T121 and PbAck1;TgAP-T121 prostates at 12, 16, 24 and 48 weeks of age. Hematoxylin and Eosin. 400X magnification.
Figure 5  Decreased apoptosis in PbAck1;TgAP-T121 prostates. Prostates from 16 weeks old (A,C) and 24 weeks old (B,D) WT, PbAck1, TgAP-T121 and PbAck1;TgAP-T121 animals were analyzed for apoptotic levels. Apoptosis (C,D) was quantified by calculating the number of TUNEL-positive cells (brown, arrows) as a percentage of the total cells. TUNEL assay. 400X magnification. n≥3 for each genotype.
Figure 6  

**Increased NFκB activation in PbAck1;TgAP-T_{121} prostates.** Prostates from 16 weeks old (A,C) and 24 weeks old (B,D) WT, PbAck1, TgAP-T_{121} and PbAck1;TgAP-T_{121} animals were analyzed for phospho-p65 levels. NFκB activation (C,D) was quantified by calculating the number of phosphor-p65-positive cells (brown, arrows) as a percentage of the total cells. Phospho-p65 immunohistochemistry. 400X magnification. n≥3 for each genotype.
Figure 7  **Histopathology of PbAck1, Pten\(^{+/−}\) and PbAck1;Pten\(^{+/−}\) prostates.** Normal WT prostate phenotype is indistinguishable from Pten\(^{+/−}\) at 16 weeks of age. PbAck1 prostate presents hyperplasia at 16 weeks of age while PbAck1, Pten\(^{+/−}\) prostate presents mPIN at same time point. Both Pten\(^{+/−}\) and PbAck1, Pten\(^{+/−}\) prostatic phenotype present mPIN at 48 weeks of age and phenotypes are indistinguishable at this point. Hematoxylin and Eosin. 100X and 400X magnification.

Figure 8  **High localization of proliferating cells within mPIN of PbAck1, Pten\(^{+/−}\) prostates.** Proliferating cells (brown) were visualized by Ki67 immunohistochemistry. Proliferating cells were exclusively localized within the mPIN lesions in 16 week old PbAck1, Pten\(^{+/−}\) prostates. Ki67 immunohistochemistry. 400X magnification.
Figure 9  Akt activation within mPIN lesions of PbAck1, Pten\textsuperscript{+/−} prostates. H&E shows atypical cells mPIN lesion in 16 weeks old PbAck1, Pten\textsuperscript{+/−} prostate. Immunostaining for phosphor-Akt detects the presence of activated Akt (brown) within atypical cells in 16 weeks old PbAck1, Pten\textsuperscript{+/−} prostates. Hematoxylin and Eosin and phosphor-Akt immunohistochemistry. 200X (H&E) and 100X (phosphor-Akt) magnifications.

Figure 10 Correlation between loss of PTEN and activation of Akt in PbAck1, Pten\textsuperscript{+/−} prostate. Sequential slides from 16 weeks old PbAck1, Pten\textsuperscript{+/−} prostates were immunostained for active (phosphorylated) Akt (brown, arrow) and PTEN. PTEN is lost (arrow head) in cells positive for phospho-Akt. PTEN and phospho-Akt immunohistochemistry. 400X magnification.
Ack1 Mediated AKT/PKB Tyrosine 176 Phosphorylation Regulates Its Activation


1 Drug Discovery Program, Moffitt Cancer Center, Tampa, Florida, United States of America; 2 Department of Anatomical Pathology, Moffitt Cancer Center, Tampa, Florida, United States of America; 3 Department of Experimental Therapeutics, Moffitt Cancer Center, Tampa, Florida, United States of America; 4 Proton Beam Facility, Moffitt Cancer Center, Tampa, Florida, United States of America; 5 Radiation Oncology, Moffitt Cancer Center, Tampa, Florida, United States of America; 6 Department of Molecular Oncology, Moffitt Cancer Center, Tampa, Florida, United States of America; 7 Comprehensive Cancer Center, Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina, United States of America

Abstract

The AKT/PKB kinase is a key signaling component of one of the most frequently activated pathways in cancer and is a major target for cancer drug development. Most studies have focused on its activation by Receptor Tyrosine Kinase (RTK) mediated Phosphatidylinositol-3-OH kinase (PI3K) activation or loss of Phosphatase and Tensin homolog (PTEN). We have uncovered that growth factors binding to RTKs lead to activation of a non-receptor tyrosine kinase, Ack1 (also known as Ack or TK2), which directly phosphorylates AKT at an evolutionarily conserved tyrosine 176 in the kinase domain. Tyr176-phosphorylated AKT localizes to the plasma membrane and promotes Thr308/Ser473-phosphorylation leading to AKT activation. Mice expressing activated Ack1 specifically in the prostate exhibit AKT Tyr176 phosphorylation and develop murine prostatic intraepithelial neoplasia (mPINi). Further, expression levels of Tyr176-phosphorylated AKT and Tyr288 phosphorylated Ack1 were positively correlated with the severity of disease progression and inversely correlated with the survival of breast cancer patients. Thus, RTK/Ack1/AKT pathway provides a novel target for drug discovery.


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* Email: nupam.mahajan@moffitt.org

Introduction

Protein kinase AKT plays a central role in growth, proliferation and cell survival [1,2,3]. AKT activation occurs when ligand binding to RTKs facilitates translocation of AKT to the plasma membrane [4,5,6,7], where it is phosphorylated at Thr308 by phosphoinositide-dependent protein kinase 1 (PDK1) and at Ser473 by the PDK2, a class of about 10 different kinases [8] including the mTOR complex [9]. Phosphorylation of AKT at Thr308 and Ser473 leads to its kinase activation [10]. Upon activation, AKT phosphorylates its substrates to transduce survival signals [1,3,11,12]. During AKT activation, the first step is the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3) by PI3K, PDK1, and AKT binds the phospholipid PIP3 via their PH domains and are recruited to the plasma membrane. While RTK/PI3K-mediated recruitment of AKT to the plasma membrane is a well characterized mechanism, mounting evidence indicate that AKT activation can occur in a PDK-independent fashion [13,14,15,16,17,18]. About a third of the breast and prostate tumors and majority of the pancreatic tumors that exhibit AKT activation, retain normal PTEN and PI3K activity [15] [19,20]. Interestingly, normal PTEN expression was also seen in breast, ovarian and prostate tumors that exhibited activated AKT [15].

While RTKs are suggested to be involved [21], the molecular mechanisms regulating RTK mediated AKT activation in cancers with normal PTEN and PI3K activity is poorly understood [22]. Further, PI3KCA activating mutation has recently been shown to be neither necessary nor sufficient for full AKT activation in situ [23]. Thus, collectively these data suggest the existence of additional pathways that regulate AKT activation in response to growth factors.

Ack1, a non-receptor tyrosine kinase has emerged as a critical early enhancer of variety of extracellular growth factor stimuli including heregulin, insulin, EGF and PDGF signaling [24,25,26,27,28]. Ack1 is ubiquitously expressed and primarily phosphorylated at Tyr288 leading to its kinase activation [25,27]. Our earlier studies demonstrated that Ack1 regulates prostate cancer progression in androgen independence by positively regulating androgen receptor (AR) and negatively regulating the tumor suppressor, Wwox [25,26,28]. Ack1 gene is also shown to be amplified in primary lung, ovarian and prostate tumors which correlated with poor prognosis [29]. In this report, we have identified a novel mechanism of Ack1 mediated AKT activation wherein phosphorylation of Tyr176 in the Ack1 kinase domain results in its translocation to the plasma membrane and subsequent kinase activation.
Results

Akt1 Phosphorylates Akt at Evolutionary Conserved Tyr176 Resulting in Akt Activation

We observed that EGF treatment of mouse embryonic fibroblasts (MEFs) resulted in rapid Tyr-phosphorylation of Akt1 as well as Akt1 at S and T sites respectively. Phosphorylation events could be linked (Fig 1A). To test this hypothesis, we examined whether Akt1 could bind and Tyr-phosphorylate Akt following RTK activation. Co-immunoprecipitation of tyrosine derived from Akt1, Akt2, and Akt1+2 knocked out mouse embryonic fibroblasts (MEF1KO, MEF2KO, and MEF12KO, respectively, Fig 1A) that were treated with EGF, either with or without pretreatment with LY294002, a PI3K inhibitor, revealed that endogenous Akt1 (Akt1 here onwards) and Akt1 formed a stable complex which was not abrogated by LY294002 (Fig 1B). The bottom panel shows that upon LY294002 addition there was substantial decrease in Akt Ser173-phosphorylation, suggesting that LY294002 is functional. Akt1 interacted weakly with Akt1, while Akt1 was present at low levels in the MEF12KO cells was not detectable in the complex.

To test whether Akt1 directly phosphorylates Akt1, in vivo binding assay was performed and Akt Tyr-phosphorylation was assessed. Myc-tagged Akt1 and HA-tagged Akt constructs were expressed and purified using respective antibody beads followed by elution, as described in methods section (Fig 1B). In vivo binding assay revealed that purified Akt1 interacted directly with Akt1 resulting in Akt Tyr176-phosphorylation (Fig 1B-D). Further, we generated GST-Akt construct that harbors kinase, SH3 and CRIB domain (schematics shown in Fig 1E) and expressed it in E. coli (Fig 1E) [25, 31]. Androgen-receptor (AR), another Akt1 substrate [25] was expressed as FLAG-tagged construct in HEK293 cells and purified using FLAG-beads (Fig 1E, left panel). GST-tagged Akt1 or GST-AR control bound to glutathione beads were incubated with purified Akt1 or Y176F mutant of Akt1 or AR (shown in Fig 1E and F). GST-Akt1 bound to purified Akt1 and AR but not the Y176F mutant of Akt1 suggesting that Akt1 and AR are direct binding partners of Akt1 (Fig 1F).

Affinity purification of Akt1 complexed with Akt1 (Fig 2A), followed by mass spectrometry analysis revealed that Akt1 was phosphorylated at Tyrostine 176 (Fig 1C-E). Tyr176, located in the kinase domain, is evolutionarily conserved from unicellular eukaryotes to mammals and within all these Akt forms (Fig 1F). Two other phosphorylation events, Ser173 and Thr208 were also identified in the same preparation (Fig 2B-C). In silico analysis revealed that Tyr176 and Ser173 are located in regions with increased conformational stability and phosphorylation at Tyr176 is likely to induce substantial conformational change and thus affect the loop harboring Ser273 (Fig 3A). To determine whether Akt Tyr176-phosphorylation is an upstream event that regulates Akt activation, we performed a phosphorylation analysis of Akt1 in a yeast two-hybrid system. The results revealed that phosphorylation was necessary to generate Akt1 phospho-serosine (Y176F) mutant (Fig 3A). The Y176F mutant interacted poorly with Akt1 in the absence of ligand, and in the presence of ligand failed to interact with Akt1 resulting in decreased Akt Tyr/Ser-phosphorylations (Fig 1G, lane 6). Flow cytometric analysis of EGF treated cells revealed significant reduction in Ser173-phosphorylation in MEF12KO cells expressing Y176F as compared to MEF12KO cells expressing Y176F as compared to Akt1 (Fig 3B, C and D). These results imply that Akt1-mediated Akt1 Tyr-phosphorylation results in subsequent Akt1 activation.

Akt1/AKT Interacting Domains

To identify domains involved in Akt1-AKT interaction, various deletions of Akt1 and AKT were generated (Fig 4A).

MEF12KO cells were co-transfected with HA-tagged AKT deletions and activated Akt1 or c-Myb. Immunoprecipitation using HA antibodies followed by immunoblotting with pTyr antibodies revealed Tyr-phosphorylation of full-length AKT and AKT lacking carboxy terminus (ACT-AKT), however, AKT deletion construct lacking the PH domain (AHF-AKT) exhibited significant decrease in Tyr-phosphorylation (Fig 4D, top panel). The decreased phosphorylation of AKT deletion construct lacking PH domain could be due to poor binding with activated Akt1. To assess this interaction in further detail, co-immunoprecipitation experiment was performed. It revealed that in contrast to Akt1 or ACT-AKT, AHF-AKT weakly binds Akt1 (Fig 4D, top panel). We have demonstrated that Tyr176 resides in AKT kinase domain is necessary for Akt1/AKT interaction, thus, collectively it indicates that the Akt1 needs both the PH domain and tyrosine176 in AKT kinase domain for complex formation. To identify the region in Akt1 that recognize AKT, MEF12KO cells were transfected with Myc-tagged Akt1 deletions (shown in Fig 4A) and HA-tagged AKT. The lysates were immunoprecipitated using Myc antibodies followed by immunoblotting with AKT antibodies. The Akt construct expressing SAM and kinase domains (cAkt) was able to bind AKT, however, construct lacking a part of kinase domain (dAkt) bound poorly to endogenous AKT (Fig 4E, top panel). GST-Akt1 that possess kinase-SH3-CRIB domains but lacking SAM domain was able to bind AKT (Fig 4E). Taken together it indicates that the kinase domain in Akt1 is tyrosine176 in the kinase domain along with AKT PH domain required for efficient Akt1/AKT complex formation.

Somatic Autoactivating Mutation (E346K) in Akt1 Activates AKT

While growth factor binding to RTK or amplification of the Akt1 gene causes Akt1 kinase activation [25,26,30], somatic autoactivating mutations in Akt1 have not yet been identified. Recently, four point mutations in Akt1; i.e. R344E, R349Q, E346K, M401I have been identified in the COSMIC database. Using site-directed mutagenesis, we generated HA-tagged point mutants (Fig 5A). We tested these mutants and observed that E346K mutant undergoes autoactivation and causes AKT Tyr/Ser-Thr-phosphorylation in serum starved cells (Fig 5B and C). Earlier we and others have characterized a point mutant (L487F mutation) that leads to constitutive activation of Akt1, also called cAkt1, (26,32). Both cAkt1(L487F mutant) and E346K autoactivating mutant of Akt1 exhibited Tyr219-phosphorylation in the activation loop (Fig 5D). We also measured the intrinsic kinase activity of the Y176F mutant and the wildtype Akt1 in the absence and presence of activated Akt1. The wildtype Akt1 displays significant increase in the kinase activity as compared to the Y176F mutant when coexpressed with either one of the Akt1 constructs, E346K and cAkt1 (Fig 5E and F). These results demonstrate that the somatic autoactivating mutations in Akt1 are sufficient to activate AKT. Taken together with the earlier evidence indicating direct Akt1-AKT interaction, it opens an intriguing possibility of RTK/Pi3K-independent AKT activation in tumors that is mediated by (auto) activated Akt1.

Tyr176 Phosphorylated AKT Translocates to the Plasma Membrane Leading to AKT Activation

Mechanistically, targeting AKT to the plasma membrane is necessary for AKT activation [1,5,7,3]. Loss of the PH domain resulted in decrease in AKT Tyr-phosphorylation upon coexpression with activated Akt1 (Fig 4A, C and D). Further,
Figure 1. Tyr176 phosphorylation precedes AKT activation. (A) MEF2KO cells were serum-starved (24 h) and treated with EGF (10 ng/ml). The lysates were immunoprecipitated or IP with anti-Akt (top panel), anti-AKT (second panel) and anti-EGFR (fourth panel) antibodies followed by immunoblotting or IB with anti-phospho antibodies. Remaining panel represents IB with antibodies as shown. (B) MEFs were serum-starved (24 h) and...
Ack1 interacts with RTKs which are located in the membrane [25,36,28]. These attributes suggest that activated Ack1 could engage AKT at the plasma membrane. To investigate the role of AKT, Tyr176-phosphorylated on its cellular compartmentalization, we generated phospho-antibodies that specifically recognized Tyr176-phosphorylated AKT or pTyr176-AKT (details in SI methods). The antibodies were extensively validated (Fig. 2A, Fig. S6A, also we top panels of Fig. 2B, C and D, Fig. S6B). Normal prostate epithelial cells, RWPE, exhibited pTyr176-AKT expression upon treatment with EGF and heregulin ligand (Fig. 2A). The pTyr176-AKT was detected when activated Ack1 was coexpressed with AKT but not the Y176F mutant. Further, incubation of the pTyr176-AKT antibody with phosphoAKT-Y176-peptide resulted in loss of binding to Tyr176-phosphorylated AKT (Fig. S6A). Cell fractionation studies revealed that heregulin, insulin and EGF treatment resulted in a time-dependent accumulation of pTyr176-AKT at the plasma membrane that lead to AKT activation (Fig. 2B, C and Fig. S6B, top panels). Optimal AKT Tyr176 phosphorylation and plasma membrane accumulation was observed at 10, 30 and 60 min upon EGF, insulin and heregulin ligand treatments, respectively (Fig. S6B and Fig. 2B, C). To assess whether EGF mediated AKT activation is dependent upon Tyr176 phosphorylation, MEK1/2KO cells expressing AKT or Y176F mutant were treated with EGF ligand. The Y176F mutant failed to translocate to the plasma membrane and become activated by EGF (Fig. 2D). The basal levels of pTyr176-AKT seen in cytokine treated cells (Fig. 2D, panel 2, lanes 4 & 6) is likely to be Tyr176-phosphorylated AKT5. Depletion of Ack1 by siRNA abrogated heregulin mediated AKT Tyr176-phosphorylation, plasma membrane localization and activation in MCF-7 cells (Fig. 2E) and MEFs (unpublished data). Further, GFP-E346K recruited dR-Adenosine receptor but not the dR-Adenosine receptor to the plasma membrane as assessed by immunofluorescence (Fig. 2F, G and H). Taken together, these data suggest that Ack1 is a key intermediate signaling entity necessary for RTK mediated AKT Tyr176-phosphorylation.

ACK1 facilitates AKT plasma membrane localization and activation because Ack1/AKT interaction was unaffected by LY294002 treatment (Fig. 1B) we assessed whether ACK1 Tyr176 phosphorylation could occur upon inhibition of PI3K activity. First, LY294002 treatment neither affected endogenous Ack1 Tyr176 phosphorylation nor its membrane localization (Fig. 3A). Second, in contrast to Ack1 knockdown, depletion of PI3K 110z subunit by siRNA did not inhibit pTyr176-AKT levels in MCF-7 cells treated with insulin (Fig. 3B). However, Ser473 phosphorylation of AKT was reduced upon knockdown of either Ack1 or PI3K, suggesting existence of two distinct pathways of AKT activation. Third, membrane fraction of ACK1 was phosphorylated at Ser473 even in the presence of LY294002 when coexpressed with activated Ack1 in serum starved MEK1/2KO cells (Fig. 5A, panel 2). To determine whether Tyr176-phosphorylated AKT can translocate to the plasma membrane in the absence of PI3, ACK1 pTyr176-AKT binds PI3K inefficiently (4) was generated (Fig. 5B). The Y176F mutant was Tyr-phosphorylated and recruited to membrane when coexpressed with activated Ack1, in the absence of LIF (Fig. 5C and D). Interestingly, in contrast to AKT which binds PI3K, Tyr-phosphorylated AKT bound another membrane phospholipid; phosphatidic acid (PA) (Fig. 5E). Combined together, our data indicates that RTK/Ack1 pathway could directly facilitate AKT plasma membrane localization and activation and a fraction of AKT that is Tyr-phosphorylated can translocate to the membrane and undergo Ser473-phosphorylation even when PI3K is inhibited.

AKT Tyr176 Phosphorylation Suppresses Expression of Apoptotic Genes and Promotes Mitotic Progression

Earlier we have observed that Ack1 translocates to the nucleus upon its pTyr-phenylalanine [26]. We assessed the localization of pTyr176-AKT when Ack1 was activated. Ligated treatment facilitated nuclear translocation of both endogenous pTyr264-Ack1 and pTyr176-AKT (Fig. 5A). FOXO subgroup of transcription factors are phosphorylated by AKT leading to rapid redistribution of FOXO proteins from nucleus to cytoplasm, thus, preventing transcription of target genes [1,11,12]. FOXO proteins regulate genes involved in cell cycle arrest (e.g., p21, p27Kip1), cell death (e.g., Bim-1) and DNA repair (e.g., GADD45) [11]. Real time quantitative RT-PCR analysis revealed that in MEK1/2KO cells coexpressing casein and AKT, expression of p21, p27, Bim-1 and GADD45 is down regulated in comparison to the activated Ack1 and Y176F mutant coexpressing cells (Fig. 5B). Consistent with this observation, depletion of Ack1 protein by siRNA resulted in increased FoxO-responsive gene expression (Fig. 4B).

To further understand the molecular role of Tyr176 in cell growth, we generated a HA-tagged myristoylated Y176F or myr-Y176F (Fig. 4C). As the myristoylated version of AKT is constitutively anchored at the membrane, it exhibits high levels of AKT activation, as seen by Thr308-phosphorylation (Fig. 5B). MEK1/2KO cells expressing myr-Y176F exhibited significant decrease in Thr308-phosphorylation confirming that AKT Tyr176-phosphorylation is an important event for subsequent AKT activation. Further, MEK1/2KO cells expressing myr-AKT expression decreases in growth as observed by an increase in the number of the double-positive HA and phospho-H3 (Ser10) stained cells, indicating of cells undergoing mitosis (Fig. 4D). In contrast, the number of double-positive myr-Y176F expressing cells remained unchanged after 24 hours (Fig. 4D). Thus, AKT Tyr176-phosphorylation can both suppress pro-apoptotic gene transcription and promote mitotic progression.
Prostate-Ack1 Transgenic Mice Display AKT Activation and Develop Prostatic Intracapsular Neoplasia

We generated a transgenic mouse model in which Myc-tagged activated Acll was expressed under the control of modified Probasin (PB) promoter, ACK2PB (Fig. 5A and B). PB-Ack1 transgenic mice (TG) display significant increase in AKT Tyr176-phosphorylation leading to Ser473/Thr308-phosphorylation (Fig. 5C, top 3 panels) and AKT substrate FOXO3a Ser117/318-phosphorylation (Fig. 5B, panel 2) in the prostate. These mice developed intracapsular hyperplasia by 22 weeks (Fig. 5E) and submucosa by 44 weeks (Fig. 5F, G-J). The prostate epithelial of TG mice was crowded with round to polygonal stratified nuclei, forming micropapillary projections and ducts (Fig. 5E). The acini were lined by a rim of basal cells (Fig. 5F). The areas of pPI3K were easily identifiable and were characterized by prostatic acini containing intraductal papillary structures lined by stratified cells with elongated nuclei exhibiting prominent nucleoli. Focally, the papillae merged into each other within the acini generating a coiled pattern of growth (Fig. 5J-L). Donal lobes exhibited an increased number of small acini lined by cells containing nuclei exhibiting prominent nucleoli and the neoplastic acini were devoid of myoepithelial cells (Fig. 5L). We previously demonstrated that Ack1 regulates phosphorylation of androgen receptor [28] and tumor suppressor Wxoxa [25] in human prostate tumors. Neoplasia observed in PB-Ack1 mice could be due to the combined effect of Ack1 mediated AKT, AR and Wxoxa Tyrosine phosphorylation. AR and Wxoxa Tyrosine phosphorylation appear to be involved in late stage progression of prostate cancer to androgen-independence [36]. Ack1 mediated AKT Tyr176-phosphorylation and activation may be more prevalent stage initiating processes in neoplastic progression that mimic or serve as an alternative to those of PTEN loss which has been predominantly emphasized in other mouse models of prostate cancer [35].

pTyr284-Ack1 and pTyr176-AKT Expressions Correlate with Breast Cancer Progression

To examine the role of pTyr284-Ack1 and pTyr176-AKT in breast tumor progression, we performed an extensive tissue microarray analysis (TMA) of clinically annotated breast (n = 476) tumor samples. Tyr284 is the primary autophosphorylation site in Ack1, hence, phospho-Ack1(pTyr284) antibodies were used to assess Ack1 activation [27,29]. Immunohistological analysis revealed that pTyr284-Ack1 and pTyr176-AKT were expressed in both breast carcinoma (Fig. S1A, B). A significant increase in expression of pTyr284-Ack1 and pTyr176-AKT was seen when breast cancer from progressive stage were examined, i.e., normal to hyperplasia, ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC) and lymph node metastatic (LNMM) stages (Fig. 6A-C and Table 1). In contrast to pTyr284-Ack1, the total Ack1 levels remained unchanged between normal and tumor samples (compare Fig. 5H and E with F and G). ANOVA results indicated that both pTyr284-Ack1 and pTyr176-AKT expression differed significantly among progression stages (p<0.0001). When using Tukey-Kramer method to examine all pairwise differences between different stages, the expression levels of pTyr284-Ack1 and pTyr176-AKT

Figure 3. Tyr176-phosphorylation of AKT is PI3K-independent. (A) MCF-7 cells were pre-treated with LY294002 (10 µM, 1 h) followed by heparuln for 40 mins. Cell lysates were fractionated and membrane fraction was subjected to IB with indicated antibodies; (B) MCF-7 cells were mock transfected or transfected with control, AKT and PI3K siRNAs, followed by result obtained for 30 mins. Cell lysates were subjected to IB with indicated antibodies. The experiment was performed with two different Ack1 siRNAs (Immune). doi:10.1371/journal.pone.0009646.g003
Figure 4. Tyr176 phosphorylated AKT suppresses FoxO gene transcription and promotes cell cycle progression. (A) MEF162KO cells were transfected with calciK and HA-tagged AKT or Y176F, serum starved (24 h) and harvested. Total RNA was prepared and quantitative RT-PCR was performed. Data are representative of three independent experiments. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001. (B) MEF/2KO cells were transfected with control or Ack1-specific shRNAs (50 nM) for 48 h and treated with EGF for 30 mins. Total RNA was prepared and quantitative RT-PCR was performed. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001. (C) Schematic representation of myr-AKT and myr-Y176F point mutants. CDM of myr-AKT was performed to generate the Y176F mutation. PH, Pleckstrin homology domain; Kinase, Kinase domain and CT, Carboxy Terminal regulatory region. (D) AKT MEF162 KO cells were transfected with HA-tagged myr-AKT or myr-Y176F mutant and harvested 24 h and 48 h post-transfection. Cells were fixed and stained with anti-HA antibodies conjugated with Alexa 488 and anti-Plk1[H10]H10-IgG conjugated with Alexa 647, a reagent used to distinguish cells in late G2 and early M phase, and analyzed by flow cytometry. HA-myrAKT expressing cells showed 20% increase in the number of cells undergoing mitosis (upper right quadrant), while HA-myrY176F-AKT expressing mitotic cells remain unchanged.

Discussion

Our study indicates that cells employ multiple and possibly mutually exclusive mechanisms to activate AKT (Fig. 7). The reasons why RIKs would employ two distinct modes of AKT activation are not entirely clear. However, a fraction of AKT appears to utilize this alternative mode of activation in normal and prominently in cancerous cells. Our studies showed that even in the presence of PI3K inhibitor, ligand bound ERBB2/ErbB-2 or EGFR activated AKT which in turn Tyr-phosphorylated and

in LNSMM were significantly higher than those of all the earlier tumor stages, the expression levels were significantly lower in the normal samples when compared to those of all the later stages except for hyperplasia (Tables 2 and 3). Kaplan-Meier analysis revealed that patients with high expression of pTyr704-Ack1 and pTyr176-AKT are at a higher risk for cancer-related deaths (Fig. 6D, E and Table 4). Furthermore, expression of pTyr704-Ack1 was significantly correlated with pTyr176-AKT in all Spearman rank correlation coefficient p = 0.43, p = 0.0001; Fig. S10C).
Figure 5. Probasin-Ack1 transgenic mice display pTyr176-AKT and develop mPINs. (A) Transgenic construct (Prob-Ack1) is shown. (B) A 25 wk old Probasin-Ack1 transgenic (TG) and wild type (WT) male mice prostate lysates were subjected to IP using anti-Myc antibodies followed by IB with pTyr antibody (top panel). For bottom panels, lysates were subjected to IB with indicated antibodies. (C) Prostate lysates from 21 and 25 wk old TG and the WT siblings were IB with respective antibodies. The bottom 2 panels represent tail-PCR of these mice. IL-2 was an internal control for PCR. (D-L) Haematoxylin and eosin (H&E) stained WT and TG mice prostates. Histological appearance of the prostate lateral lobe from a 22 wk old WT mouse (D), and corresponding lobes from age-matched TG mice with intraprostatic hyperplasia (L). The lateral prostate from 49 wk old TG mice exhibiting mPIN (F) is shown. Contrasting histological appearance of the lateral, ventral and dorsal lobes of the prostate glands from a WT mouse (G-H), and corresponding lobes from TG mice (49 week old) are shown (J-L). doi:10.1371/journal.pone.0009665.g005
activated AKT. AKT is frequently activated in pancreatic cancer which has been shown to be highly correlated to HER-2/neu overexpression [34]. Moreover, many of the pancreatic cell lines and tumors expressing activated AKT had retained wild-type PTEN [35,36]. We noticed that PanIN, pancreatic adenocarcinoma and breast tumors of MMTV-neo mice exhibit significantly higher levels of pTyr286-Akt and pTyr176-AKT (unpublished data). Taken collectively, our data may explain AKT activation in these tumors that display amplification/activation of RIKs but have normal PI3K/PTEN levels. We propose that other tumors

Figure 6. pTyr286-Akt1 and pTyr176-AKT expression in breast cancer. (A) TMA sections representing different breast cancer stages stained with pTyr286-Akt1 and pTyr176-AKT antibodies. (B) Box plots to summarize distributions of staining intensities for pTyr286-Akt1 in different stages of breast cancer. A significant increasing trend of intensity across progression stages was detected (Mann-Whitney U test, p = 0.02). The box has lines at the lower quartile (25%), median (50%), and upper quartile values (75%) while the red-boxes within the circle marks the mean value. Whiskers extend from each end of the box to the most extreme values within 1.5 times the interquartile range from the ends of the box. The data with values beyond the ends of the whiskers, displayed with black circles, are potential outliers. (C) Box plots to summarize distributions of staining intensities for pTyr176-AKT in different stages of breast cancer. A significant increasing trend of intensity across progression stages was detected (Mann-Whitney U test, p < 0.0001). (D) Kaplan-Meier analysis shows that individuals with breast cancer that have moderate to strong staining (≥3+) of pTyr286-Akt1 have a lower probability of survival (log rank test, p = 0.05). (E) Kaplan-Meier analysis of the breast cancer patients that have moderate to strong staining (≥3+) of pTyr176-AKT have significantly lower probability of survival (log rank test, p = 0.02). doi:10.1371/journal.pone.0009646.g006
that cause somatic activating mutations or amplification in non-receptor tyrosine kinases could use similar mechanisms for AKT activation [37].

Are there conditions when Tyk 76 modification is not needed for AKT activation? Some of the conditions when Tyk 76 phosphorylation of AKT is not required for AKT activation could be: 1) Presence of constitutively active PI3KCA mutations, observed in colorectal, glioblastomas, gastric breast and liver cancers [80]; 2) Loss of tumor suppressor PTEN resulting in increased levels of cellular PIP3, occur commonly in prostate cancer, endometrial cancer, and glioblastomas, among others [5]. 3) A rare somatic activating mutation, E77K in the pH domain which facilitates AKT recruitment to the membrane in PIP3-independent manner [15].

We have used the term AKT 'translocation' to indicate emergence of (cytosolic) AKT in the plasma membrane in response to growth factors. Our data (Fig. 2B and 2D) demonstrate that AKT in the plasma membrane was phosphorylated at Tyr 76 and mutation of this site in AKT abrogates appearance of AKT in the plasma membrane (Fig. 2D). Based on the evidence, our model (Fig. 7) suggests that the AKT signaling pathway is initiated at the plasma membrane by RTKs. AKT is activated with growth factor-bound RTKs (via M69 homology domain in AKT) by transducing signals from the plasma membrane to the nucleus and is activated [5,50,59]. AKT is constitutively bound to AKT (Fig. 1B and G). Activated AKT directly phosphorylates AKT at Tyr77, thus facilitating recruitment of pTyr77-phosphorylated AKT to the plasma membrane. pTyr77-phosphorylated AKT preferentially binds PA, a plasma membrane phospholipid and is opposed to unphosphorylated AKT (see Fig. 5A for details). PH domain in AKT is a lipid binding domain and thus might be involved in the membrane binding of pTyr77-phosphorylated AKT. Collectively, this data suggests that AKT mediated AKT Tyr77 phosphorylation is driving this translocation process. Thus, although AKT Tyr77 phosphorylation and its migration to the plasma mem brane is PI3K independent, the recruitment of Tyr77 AKT in the plasma membrane may require a functional PI3K domain.

In contrast to AKT, pTyr77-AKT specifically binds the plasma membrane anti-angiogenic phospholipid, PA (Fig. 5A). Tyr 77-phosphorylation could induce conformational changes in the AKT PH domain to enable binding to PA. The PH domain of Ser of repressor (SOR) and PA domain of p70S6K have been shown to possess a phosphorylation-binding pocket and a second action binding pocket which either enables them to interact with PA facilitating plasma membrane recruitment [60,61]. We speculate that AKT too might possess a missed action binding pocket, making phosphorylations induced conformational changes could unwind this pocket allowing it to bind PA.

In endogenous systems, AKT1 associates with AKT2 albeit weakly as compared to AKT1 (Fig. 1B). AKT isoforms are differentially distributed among different cellular compartments [42] with majority of AKT1 in the cytoplasm and AKT2 in the mitochondria. Additionally AKT2 protein appears to be not as

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<th>Table 1. The intensities of Tyr284-phosphorylated-Akt1 and Tyr176-phosphorylated-AKT for the trend analysis of breast cancer.</th>
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<th>Table 4. Kaplan-Meier survival estimates by pTyr284- phosphorylated Akt1 and pTyr176-phosphorylated AKT intensities for breast cancer TMA samples.</th>
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AKT Phospho Site Determination Using Mass Spectrometry

293T cells co-expressing activated Akt and HA-tagged AKT were lysed in receptor lytic buffer (RLB) containing 25 mM/L Tris (pH 7.5), 225 mM/L NaCl, 1% Triton X-100, 1 mM/L DTT, 10% glycerol, phosphatase inhibitors (10 mM/L NaF, 1 mM/L Na3VO4), and protease inhibitor mix (Roche). Following immunoprecipitation with HA-beads (E6799, Sigma, St. Louis, MO), purified AKT was subjected to SDS PAGE electrophoresis and the gel was stained Brilliant Blue R250 (BioRad). A prominent band of ~59 kDa was excised, washed once with water and twice with 50 mM ammonium bicarbonate in 50% aqueous methanol. Proteins were reduced and alkylated with 2 mM iodoacetamide (ICLP, Sigma, St. Louis, MO) and 20 mM iodoacetamide (GE Healthcare, Pittsburgh, PA), respectively. Samples were digested overnight with modified sequencing grade tryptic (Promega, Madison, WI), Cleav-C (Worthington, Lakewood, NJ), or chymotrypsin (Roche, Switzerland). Peptides were extracted from the gel slices, phosphopeptides were enriched using IMAC spin column ( Pierce, Rockford, IL) or Mono P (GE Science, Japan). A nanolow liquid chromatograph (Ultimate 3000), LC Packings/Dionex, Sunnyvale, CA) coupled to an electrospray hybrid ion trap mass spectrometer (LTQ Orbitrap, Thermo, San Jose, CA) was used for tandem mass spectrometry peptide sequencing experiments. Peptides were separated with a C18 reverse phase column (LC Packings C18 Pepmap) using a 40 min gradient from 5% B to 50% B (5% acetonitrile/0.1% formic acid). The flow rate on the analytical column was 300 nL/min. For tandem mass spectra were acquired for each MS scan using 60 sec exclusion for previously sampled peptide peaks (Spray voltage 2.3 kV, 30% normalized collision energy, scanning m/z 500-5000). Sequences were assigned using Sequest (Thermo) and Mascot (www.matrixscience.com) database searches against SwissProt protein entries of the appropriate species. Oxidized methionine, deamidation, carbamidomethyl cysteine, and phosphorylated serine, threonine and tyrosine were selected as variable modifications, and as many as 3 missed cleavages were allowed. The precursor mass tolerance was 1.05 Da and MS/MS mass tolerance was 0.8 Da. Assignments were manually verified by inspection of the tandem mass spectra and cocked into Scaffold reports (www.proteomesciences.com).

Cell Fractionation, Immunoprecipitations and Kinase Assay

Membrane and cytosolic fractionation was performed using kit from Biovision. The nuclear/cytoplasmic fractionation was performed using protocol from Alexis. For immunoprecipitations, cells were lysed in receptor lytic buffer (RLB) containing 25 mM/L Tris (pH 7.5), 300 mM/L NaCl, 1% Triton X-100, 10% glycerol, phosphatase inhibitors (10 mM/L NaF, 1 mM/L Na3VO4), and protease inhibitor mix (Roche). For co-immunoprecipitation, cells were lysed in buffer containing 25 mM/L Tris (pH 7.5), 225 mM/L NaCl, 1% Triton X-100, 10% glycerol, phosphatase inhibitors (10 mM/L NaF, 1 mM/L Na3VO4), and protease inhibitor mix (Roche). The kinase assay was performed using kit from Calbiochem.

Purification, In Vitro Binding and Phosphorylation Assay

GST-AKCI was purified using method described earlier [31]. HEK293T cells were transfected with HA-tagged Akl, AKT, Y176F mutant of AKT and FLAG-tagged AR. 40 hours post-transfection cell were lysed in RLB buffer. Lysates were incubated with HA beads (Sigma) for 2 h, followed by washing with RLB buffer and elution in PBS containing HA or FLAG peptide (2 mM) on ice. Purity of preparation was confirmed by cosmoic blue staining of gel. For the in vitro binding assay, 50 mM of purified AKT and AKCI were incubated in modified RLB or RLB containing 25 mM/L Tris (pH 7.5), 175 mM/L NaCl, 1% Triton X-100, 10% glycerol, and protease inhibitor mix at room temperature. After 30 mins, anti-Akt antibodies and Protein-A-sepharose beads were added, incubated with shaking at 4°C for overnight. Beads were washed twice with RLB buffer. Bound protein complex was dissociated from beads by boiling in SDS sample buffer and assessed by gel electrophoresis and detection by immunoblotting with antibody against AKCI. In a control experiment, immunoprecipitation was done using non-specific IgG. For in vitro phosphorylation of AKT by AKCI, 50 mM of purified AKT and AKCI were incubated in kinase buffer containing 20 mL HEPES (pH 7.5), 150 mM/L NaCl, 10 mM/L MgCl2, 0.1 mM/L L- Na3VO4, 0.5 mM/L DTT, 0.25 mM/L ATP for 1 hour at 30°C. The reaction was stopped by adding sample buffer and reaction was assayed by gel electrophoresis and detection by immunoblotting with antibodies as shown.

Quantitative RT-PCR

All RT reactions were done at the same time so that the same reactions could be used for all gene studies. For the construction of standard curves, serial dilutions of pooled sample RNA were used (50, 10, 2, 0.4, 0.08, and 0.016 ng) per reverse transcriptase reaction. One "no RNA" control and one "no Reverse Transciptase" control were included for the standard curve. Three reactions were performed for each sample: 10 ng, 0.8 ng, and a No-RNA (0 ng) control. Real-time quantitative PCR analyses were performed using the ABI PRISM 7700HT Sequence
Detection System (Applied Biosystems). All standards, the no template control (H₂O), the no RNA control, the no Reverse Transcriptase control, and the no amplification control (Bliscept plasmid) were tested in six wells per gene (2 wells/plate × 3 plates/gene). All samples were tested in triplicate wells for each of the 10 ng and 0.8 ng concentrations. The no RT controls were tested in duplicate wells. PCR was carried out with SYBR Green PCR Master Mix (Applied Biosystems) using 2 µl of cDNA and the primers (Table 5) in a 20-µl final reaction mixture: Actb: 300/300 nM; p63: 300/300 nM; p73α: 100/300 nM; p300/300 nM; E2F1: 300/300 nM; HDAC2: 300/300 nM; GADD45α: 390/100 nM; BIRC5: 100/100 nM. After 2 min incubation at 95°C, AmpliTag Gold was activated by a 10-min incubation at 95°C, followed by 40 PCR cycles consisting of 15 s of denaturation at 95°C and hybridization of primers for 1 min at 60°C. Dissection curves were generated for each plate to verify the integrity of the primers. Data were analyzed using SDS software version 2.2.2 and exported into an Excel spreadsheet. The relative levels were normalized to the housekeeping genes by relative quantification method using the 2^-ΔΔCt method. In the second step, the PCR product was sequenced using the Actb primer (Table 5) to confirm the sequence. No other PCR primers were used. The PCR product was sequenced using the Actb primer (Table 5) to confirm the sequence.

Flow Cytometry Analysis

AKT (42×33) and/or DEX-treated cells were stained with 4μg/ml PI and 0.1% Triton X-100. The PI and DEX were washed with PBS and resuspended in 1× PBS. The cells were then incubated for 30 min at room temperature before analysis.

Tissue Microarray (TMA) Analysis

For assessment of p73 in vivo, immunohistochemistry was carried out on sections from the breast cancer specimens. The sections were dewaxed in xylene and rehydrated in a graded series of alcohol and deionized water. The slides were then incubated with rabbit polyclonal p73 antibody for 1 h at room temperature. The slides were then washed with PBS and PBS containing 0.5% BSA. The slides were then incubated with biotinylated goat anti-rabbit secondary antibody, followed by incubation with streptavidin-peroxidase. The slides were then washed with PBS and PBS containing 0.5% BSA. The slides were then incubated with 3,3'-diaminobenzidine substrate (VWR International, Barneby).
ActA1 Activates AKT

Easton, Ontario, Canada) and counterstained with hematoxylin.

Following standard procedures the slides were dehydrated and
sealed with cover slips. Negative controls were included by
omitting pTyr286-Ack1/pTyr76-AKT antibody during primary
antibody incubation. The phospho-AKT/Ack1 antibodies were
testively validated for immunohistochemistry studies. MCF7
cells transfected with human AKT were cells treated with EGFR
ligand (or no ligand) were fixed, paraffin embedded, sectioned
and used for antibody validation. Further, MEFs & 2KO cells
transfected with activated Ack1 and AKT were also used to validate
antibodies. The pTyr286-Ack1 and pTyr76-AKT staining in
paraffin embedded tissues was examined in a blinded fashion by
two independent pathologists (A.L. and D.C.). If needed, a
consensus score was reached for each specimen. The positive
scores were scored according to the intensity of
staining: 0, 1, 2, 3, 4, and 5.* The percentage of pTyr76-AKT
positive cells were also scored into four categories: 0 (0%), 1
(1-33), 2 (34-66), and 3 (more than 66%). The product of the intensity
and percentage scores was used as a final staining score.

Statistical Analysis

The Mantel-Haenszel x2 test was performed to examine if there
is an increasing trend for pTyr286-Ack1 and pTyr76-AKT with
respect to different progression stages of breast or pancreatic
cancer. The ordinal intensity levels of pTyr286-Ack1 and
pTyr76-AKT 0, 1, 2, 3, 4, 5 were pooled into 6 levels (0, 1, 2, 3, 4, and 5) to
accommodate the rare observations in the highest intensity level in most cases. Analysis of variance
was performed to examine whether the expression levels of
pTyr286-Ack1 and pTyr76-AKT differ among different tumor
stages. Repeated measures were used to summarize the intensity distribution
at each progression stage. Furthermore, Tukey-Kramer method
was performed to examine between which pairs of stages
the expression levels are different. This post-hoc procedure adjusts
for all pairwise comparisons and simultaneous inference. When more
than one sample was obtained from a patient, the intensity of the
most progressed stage was used for the analysis. Correlation
between pTyr286-Ack1 and pTyr76-AKT was explored using
Spearman ranked correlation analysis. The association of the
expression levels of pTyr286-Ack1 and pTyr76-AKT to the
overall survival of patients was assessed using the Kaplan-Meier
method. For breast cancer data, there were 144 individuals
with available pTyr286-Ack1 staining and survival information.
Expressed amounts of MEF protein lysates were subjected to IB
as indicated. MCF-7 cell lysate was used as control. (B) Purification of
Ack1 and AKT: HA-tagged Ack1 and AKT were expressed in
HEK293T cells, lysed and incubated with HA-beads. Followed by
estensive washing, proteins were eluted using H2, peptide (2nM, 1 hour) and
assayed by SDS-PAGE and Coomassie Brilliant Blue-
R250/Bind Residual staining. (C) In vitro binding assay. Equinol
amounts of purified Ack1 and AKT proteins were incubated for
30 min, complex was immunoprecipitated with Ack1 (I:50 5)
or IgG (lane-6) antibodies followed by IB with anti-AKT
antibodies (top panel). About 63% of total AKT was in complex
with Ack1 (D). De nutrient ACK1 by Ack1. Equinol amounts of purified Ack1 and AKT proteins were
incubated in kinase buffer for 1 hour at 37°C and reaction mix
was subjected to IB with pTyr176-AKT (top panel), pTyr (2nd
and 3rd panels), AKT (4th panel) and Ack1 (5th panel) antibodies.
(E) Schematic representation of GST-Ack1 construct. FLAG-
tagged AR expressed in HEK293 cells and GST-tagged Ack1 was
expressed in DH5 cells. Purified GST-Ack1 (right panel) and
FLAG-AR (left panel) were assayed by SDS-PAGE followed by
Coomassie staining. (F) In vitro binding assay. Equinol amounts of
purified HA-AKT or FLAG-AR proteins were incubated with
GST-Ack1 bound to beads for overnight. Beads were washed
following IB with anti-GST/HA antibodies (top panel). Lower
panels show IB with FLAG/HA (2nd panel) and GST (bottom
panel) antibodies.

Figure 32 Tyr176-phosphorylated AKT sample also contains
Thr308 and Ser473 phosphorylated AKT. (A) Activated Ack1
(c-Ack1) and HA-tagged AKT were expressed in HEK293T cells
followed by IB with HA-beads. IP: AKT was subjected to SDS-
PAGE electrophoresis and the gel was stained Coomassie. A prominent
band of ~55 kDa corresponding to AKT is seen which was
activated and subjected to mass spectrometry as described in
methods section. The upper ~115 kDa band corresponds to
(c-Ack1) that bound to AKT. (B) Purified AKT peptide
peptide that lead to the identification of pTyr176-AKT was assayed for
other phosphorylation events. A peptide was detected at
21.15 mins in the total ion chromatogram with mass-to-charge
ratio 938.45, which represents an error of 1.0 ppm (C). The
tandem mass spectrum matched the sequence, FVLKCEGKDG-
GATMRyTYC indicating that Thr308 in AKT was phosphoryl-
atated; the detection of the phosphoserine y3 is consistent
with this localization. (F) Another peptide was detected at 21.72 mins in the
total ion chromatogram with mass-to-charge ratio 944.93,
which represents an error of 0.59 ppm (D). (E) The tandem mass spectrum matched the sequence, ERRPHPFQyISYyASGTA
that lead to the detection of b6, b7, y7 and y8 is consistent with this localization.

Figure 33 ACK Tyr176-phosphorylation affects the loop
humping Ser173. (A) Residues Tyr176 and Ser173 are located in
regions with increased conformational flexibility. The backbone of
AKT is color-traced according to crystallographic B-factors
from blue (20 Angstrom, less flexible) to red (76 Angstrom, highly
flexible). (B) B-factor plot of all C-alpha atoms. The average main
chain B-factor is 36 Angstrom (dashed horizontal line). (C) ACK
Tyr176-phosphorylation induces substantial conformational
changes of residues in its vicinity. Electronic interactions could
be established with Arg174 and/or Tyr214 while electronic
repulsion and/or steric hindrance (due to the bulky phosphate
migrating the trimodal portion of the loop
humping Ser173, in turn causing structural alterations of this
residue.

Figure 34 Kinase domains of Ack1 interact with AKT FK
domain/Tyr176 in kinase domain. (A) Schematic representation of
wild type AKT, Ty176 point mutant and deletion constructs.
Site-directed mutagenesis of AKT was performed to generate the tyrosine to phenylalanine, Y176F, point mutant. PH, Pleckstrin homology domain; Kinase, Kinase domain and CT, Carboxy terminal regulatory region. Schematic representation of Ack1 and deletion constructs. SAM, Sterile alpha motif; Kinase, kinase domain; SH3, Src homology domain 3; C, Cdc42 Rac interactive binding domain. (A) Flow cytometry of AKT (E36K) and/or HA-Y176F, expressing HA-AKT and/or HA-Y176F. Top left panel indicates mock transfected cells stained with AKT-Ser473 antibody conjugated to Alexa 647 (untreated; 0.1%). Bottom left panel shows percentage of cells with AKT Ser473 phosphorylation upon EGF stimulation (15.2%). Right top and bottom panels show percentage of cells expressing HA-AKT (23%) or HA-Y176F (51%), respectively, in cells stained with anti-HA antibody conjugated to Alexa 488. (B) MEF/36KO cells were co-transfected with HA-tagged AKT deletions and cAck1. The lysates were IP using HA antibodies followed by IB with pTyr antibodies (top panel). Lower panel shows IP using HA antibodies followed by IB with AKT antibodies. Bottom panel shows IB of the lysates with Ack1 antibodies. (C) HEK293T cells were co-transfected with HA-tagged AKT deletions and HA-tagged Ack1. The lysates were IP using Ack1 antibodies followed by IB with HA antibodies (top panel). Lower panels are as described above. (D) MEF/36KO cells were transfected with mutant Ack1 deletions and HA-tagged AKT. The lysates were IP using Ack1 antibodies followed by IB with HA antibodies (top panel). Lower panels show IB with Ack1 and AKT antibodies. Found at: doi:10.1371/journal.pone.0009564.s004 (0.03 MB PDF)

Figure 55: Somatic autoactivation of Ack1. (A) Schematic representation of Ack1 and various point mutants identified in the COSMIC database. Site-directed mutagenesis of Ack1 was performed to generate four HA-tagged point mutants. SAM, Sterile alpha motif; Kinase, kinase domain; SH3, Src homology domain 3; C, Cdc42 Rac interactive binding domain; Proline, Proline rich domain; UBA, Ubiquitin binding domain. (B) E364K mutation results in Ack1 autoactivation leading to AKT activation. MEF/362KO cells were transfected with Ack1 mutants and the lysates were IP using anti-HA antibodies followed by IB with pTyr antibodies (top panel). Lower panels show IB with indicated antibodies. (C) E364K mutant Ack1 interacts with and Tyr phosphorylates AKT. 293T cells were co-transfected with HA-tagged Ack1 point mutants. Equal amounts of protein lysates were subjected to IP using HA antibodies. IB with AKT antibodies revealed formation of activated Ack1/E364K/endoogenous AKT complex (top panel). (D) HEK293T cells were transfected with HA-tagged E364K, cAck1 or cAck1 (K53R) mutant. Lysates were subjected to IP using anti-HA (top panel) antibodies followed by IB with pTyr antibodies (bottom panel). The same lysates were probed for kinase activity shown in S6F. (E) Ack1 autoactivation leads to AKT kinase activation. As described in S6E, lysates were IP with HA antibodies, followed by AKT kinase assay. Low levels of Ack1 kinase activity in vector transfected cells was treated as zero and increased kinase activity (in percentage) over the vector expressing cells is shown. Found at: doi:10.1371/journal.pone.0009564.s005 (0.03 MB PDF)

Figure 56: Generation and validation of pTyr76-ACK phospho-bodies. (A) EGFR and heregulin treatment results in AKT Tyr76 phosphorylation. RWPE-1 normal prostate epithelial cells were treated with EGF (10 ng/ml, 10 min) and heregulin (10 ng/ml, 35 min) ligand, equal amounts of protein lysates were subjected to immunoblotting as indicated. pTyr76-antibodies specifically recognize endogenous Tyr phosphorylated AKT following treatment with ligands. (B) Ack1 autoactivation lead to AKT Tyr76-phosphorylation. 293T cells were co-transfected with myc-tagged cAck and dAck and AKT and Y176F mutant. Equal amounts of protein lysates were subjected to immunoblotting with pTyr76-ACK antibodies. The pTyr76-antibodies recognize only the pTyrACK (lane 3), but not the Y176F protein (lane 4). (C) Tyr76-phosphorylated AKT localizes to plasma membrane. NIH3T3 cells were co-transfected with EGFP/E364K mutant of Ack1 and dIhRagN1-ACK (D-F) or dIhRagN-1/Y176F-ACK (G-J) DNAs overnight. Cells were serum starved, fixed and visualized by fluorescence microscopy. AKT but not Y176F mutant was localized to the plasma membrane in activated Ack1/E364K expressing cells. Found at: doi:10.1371/journal.pone.0009564.s006 (0.07 MB PDF)

Figure 57: Tyr76-phosphorylation of mutant AKT (R25C) that inefficiently binds phosphatidylinositol 3,4,5-trisphosphate. (A) MEF/18/2KO cells were transfected with activated Ack1 and AKT followed by LY294002 (30 μM) for 1 h. Cell lysates were fractionated and subjected to immunoblotting with indicated antibodies. AKT Ser473 phosphorylation in membrane fraction was unaffected by LY294002 treatment suggesting Ack1 mediated AKT activation is not dependent upon PI3K activity. (B) Schematic representation of wild type AKT and R25C point mutant constructs. Site-directed mutagenesis of AKT was performed to generate the R25C point mutant. PH, Pleckstrin homology domain; Kinase, kinase domain and CT, Carboxy terminal regulatory region. (C) MEF/362KO cells were transfected with empty vector or cAck and HA-tagged AKT or R25C mutant DNAs. Serum starved (18 h) cells were treated with EGF (10 ng/ml, 15 min). The lysates were subjected to immunoprecipitation with anti-HA (top panel) or anti-Ack1 (second panel) antibodies followed by immunoblotting with pTyr antibodies. (D) MEF/362KO cells were transfected with empty vector or cAck and HA-tagged AKT or R25C mutant DNAs. Serum starved (18 h) cells were treated with EGF (10 ng/ml, 15 min). Cell lysates were fractionated and subjected to immunoblotting. Found at: doi:10.1371/journal.pone.0009564.s007 (0.05 MB PDF)

Figure 58: Tyr-phosphorylated AKT binds to phosphatidic acid. Protein-phospholipid overlay assay was performed using phosphatidylcholine membranes spotted with 100 pmol of different phospholipids (A-C, F-G). Cells transfected with vector or activated Ack1 and AKT or Y176F were lysed and immunoprecipitated with pTyr beads followed by elution with phosphatidylcholine. The eluted Tyr-phosphorylated proteins were incubated with phosphatidylcholine blots overnight at 4°C. Blots were extensively washed and bound proteins were detected with (A, B and F) pTyr76-ACK and (C and G) AKT antibodies. (D and E) Cells expressing HA-tagged AKT (D) and Y176F mutant (E) were lysed and immunoprecipitated with HA beads followed by elution with HA peptide. The eluate was incubated with phosphatidylcholine and bound protein was detected with AKT antibodies. The pTyr76-ACK bound to phosphatidic acid, in contrast, AKT protein primarily binds to phosphatidyl-myo-inositol 3,4,5-trisphosphate (PIP3). (H) HA-peptide
and phosphorylated AKT. (B) Akt translocate to the nucleus. (C) Expression levels of p-AKT and Akt are shown in Western blots. (D) Breast cancer and normal breast tissue samples were stained with anti-AKT and p-AKT antibodies. Equal amounts of protein from these two fractions were subjected to Western blotting with indicated Abs. Akt phosphorylation was detected in Western blots. (E) Akt translocate to the nucleus. (F) Breast cancer and normal breast tissue samples were stained with anti-AKT and p-AKT antibodies. Equal amounts of protein from these two fractions were subjected to Western blotting with indicated Abs. Anti-AKT stained high levels of Akt activation, as seen by Western blotting.

References
