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Involvement of Novel Multifunction Steroid Hormone Receptor Coactivator, E6-
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14. ABSTRACT
E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP), is a novel dual function steroid hormone receptor coactivator. E6-AP not only interacts with and enhances the hormone-dependent transcriptional activities of various steroid hormone receptors, including androgen receptor (AR), but also is a member of the E3 class of functionally related ubiquitin-protein ligases. Previously, using E6-AP knockout animals we have shown that E6-AP is required for the proper development and growth of prostate gland. Previously I have shown that E6-AP regulates PI3K-Akt pathway in the prostate gland and as well as LNCaP cells. In this report I have provided evidence that E6-AP plays a vital role in the prostate gland growth and prostate cancer cell proliferation. E6-AP by itself can modulate p53 levels in prostate cancer cells independent of E6. Our data also indicates that over expression of E6-AP could potentially lead to tumor initiation. I have also shown that E6-AP also affects the non-genomic mechanisms of AR. ChIP assays demonstrate that AR is accumulated on its target promoter and enhancer under E6-AP knockdown conditions. Taken together, these studies indicate that E6-AP plays a vital role in both the genomic and non-genomic signaling pathway of AR and their downstream effectors might play critical roles in many biological processes, especially in cell growth.

15. SUBJECT TERMS
E6-AP, Androgen receptor, PI3K-Akt pathway

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Table of Contents

Introduction

Even though prostate cancer is known to be the most common malignancy and the second leading cause of cancer death in American males, the molecular basis of the disease and the mechanisms by which it becomes hormone refractory remains unknown. In order to more fully develop effective prevention and intervention strategies for this prevalent disease, the underlying molecular mechanisms of initiation and progression must be understood. The development, growth and maintenance of the prostate gland is androgen dependent and evidences point out that androgen receptor (AR) and its coactivators are important in prostate cancer. Therefore, it will be important to investigate in what capacity the AR coactivators are involved in the development of prostate tumors and in the progression of hormone resistance. In this context genetically engineered mouse models can provide significant advantages for studying the molecular mechanisms of prostate carcinogenesis.

1) Androgen Receptor (AR) and its Coactivators
The effects of hormones or androgens on the development of the normal prostate gland and prostate tumors are mediated through an intracellular receptor called AR (1, 2). In the absence of hormone, AR is located in the cytoplasm of target cells and is associated with cellular chaperones. In order to activate gene transcription, the AR undergoes a series of well-defined steps. When bound to hormone, the AR undergoes a conformational change, dissociation from cellular chaperones, receptor dimerization, phosphorylation, interaction with coactivators and recruitment of chromatin modifying enzyme activities, DNA-binding at an enhancer element of the target gene, and subsequent recruitment of basal transcription factors to form a stable preinitiation complex (PIC). These events are followed by up- or down-regulation of target gene expression (3-6). However, AR may also be converted into an active form even in the absence of androgen (7-9). The mechanism of hormone-independent activation of AR has not been understood fully yet but it may involve the bypassing of any one of the above mentioned steps of hormone-dependent activation.

Coactivators represent a growing class of proteins, which interact with receptors including the AR in a ligand-specific manner and serve to enhance their transcriptional activity (10-14). A number of coactivators have been cloned to date, including SRC-1 family members (15-19), PGCs (20), SRA (21), CBP (22-24) and **E6-associated protein (E6-AP)** (25) etc. and this list is growing rapidly day by day. In addition to these coactivators, a series of other AR coactivators, ARAs, has also been discovered such as ARA160 (26), ARA70 (27), ARA55 (28), ARA54 (28) and ARA24 (29). Coactivators have been shown to possess enzymatic activities, such as histone acetyltransferase, histone methyltransferase, ubiquitin-conjugation, and ubiquitin-protein ligase. Presumably, the coactivator's *in vivo* functions manifest by congregating their enzymatic activities to the promoter region of the target gene which contribute to their ability to enhance receptor-mediated transcription (10). Because of their ability to enhance receptor mediated gene expression, coactivators are thought to play an important role in regulating the magnitude of the biological response to steroids, vitamin D, and retinoids in different tissues or from individual to individual.

3) E6-associated Protein (E6-AP) as a Coactivator
Our lab has cloned E6-AP as steroid hormone receptor interacting protein. E6-AP enhances the hormone-dependent transcriptional activity of steroid hormone receptors, including that of AR (25). E6-AP was previously identified as a protein of 100 kDa, which mediates the interaction of human papillomaviruses type 16 and 18 E6 proteins with p53 (30). The E6/E6-AP complex specifically interacts with p53 and promotes the degradation of p53 via the ubiquitin-proteasome

pathway. E6-AP also degrades p53 independent of E6 protein. Recent evidences reveal that protein ubiquitination is a multifunctional signaling mechanism whose regulatory significance is comparable to that of phosphorylation. E6-AP is a member of the E3 class of functionally related ubiquitin-protein ligases (31-33). E3 enzymes have been proposed to play a major role in defining substrate specificity of the ubiquitin-proteasome system. The carboxyl-terminal 350 amino acids (aa) of E6-AP contain a "*hect*" (homologous to the E6-AP carboxy terminus) domain, which is conserved among all E3 ubiquitin protein-ligases and E6-AP related proteins characterized to date. We have shown that the ubiquitin-ligase activity of E6-AP is not required for the coactivation function of E6-AP. This finding indicates that E6-AP possesses two independent, separable functions, coactivation and ubiquitin-protein ligase activity (25).

Contribution of coactivators to prostate cancer development and progression has not been well elucidated. Recently, it has been shown that coactivators SRC-1, TIF-2, and ARA 55 were overexpressed in advanced prostate cancer (34). Overexpression of TIF2 and SRC-1 enhanced AR transactivation at the physiological concentrations of adrenal androgen, suggesting a general mechanism for recurrent prostate cancer growth (35, 36). In other steroidal tumors, it has been shown that altered expression of nuclear receptor coactivator, AIB1, contributes to the development of hormone-dependent breast and ovarian cancer (37). Another coactivator SRA is also elevated in breast tumors (38). We have shown that E6-AP is overexpressed in mouse mammary and prostate tumors (39). These findings suggest that enhanced coactivator expression and activity may aberrantly increase steroid hormone receptor activity and give tumors a selective advantage for proliferation, resulting in the development of aggressive tumor. Considering, the influence of E6-AP as a coactivator on transactivation of target genes by AR and its importance in the development of prostate gland (see preliminary data section), we are interested in studying the role of E6-AP in the development and progression of prostate cancer.

4) PI3K-Akt pathway
In addition to androgen signaling, the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway is another important factor that controls the growth and survival of prostate cells. Elevated PI3K-Akt signaling is correlated with prostate cancer progression. It has been suggested that androgen and PI3K/Akt pathways can compensate for each other in growth regulation and prostate development. The PI3K/Akt pathway may up-regulate AR activity by directly phosphorylating AR or through ß-catenin, an AR coactivator. A recent study has shown that the levels of components of PI3K/Akt pathway are elevated and are involved in tumor progression in Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice. Hence, the PI3K-Akt pathway which transduces signals from multiple growth factors and cytokines, apart from regulating cell proliferation, survival and motility, also plays a critical role in modulation of AR activity and prostate cancer.

5) E6-AP knockout mouse line and E6-AP transgenic mouse line
Since E6-AP has been identified as a coactivator of AR in *in-vitro* models, we used E6-AP knockout mice which were generated by targeted gene disruption, to test the effects of loss E6-AP on the normal development of the prostate gland (40, 41). We have also generated E6-AP transgenic mouse line that specifically overexpresses human E6-AP in prostate gland. In this mouse model, E6-AP gene is under the control of rat probasin promoter which specifically targets E6-AP to the prostate epithelial cells. Therefore, these two mice models are excellent tools to study the role of E6-AP in prostate gland development.

Based on our preliminary data, we hypothesized that E6-AP, a coactivator of AR, is a growth promoting protein and is involved in the prostate gland development and tumorigenesis.

Body

Aim 1: To study the role of E6-AP in normal prostate gland development, PI3K-Akt signaling and tumorigenisis.

Aim 2: To study the role of E6-AP in both the genomic and non-genomic mechanisms of AR signaling

I will be summarizing the accomplishments below.

Aim 1: To study the role of E6-AP in normal prostate gland development, PI3K-Akt signaling and tumorigenisis.

In order to study the role of E6-AP in normal prostate gland development, we have created E6-AP transgenic mice. Additionally, we have also created E6-AP over expressing stable cells to study the mechanism by which E6-AP regulates PI3K-Akt signaling.

Role of E6-AP in normal prostate gland development and tumorigenisis

Characterization of E6-AP overexpressing transgenic mice:
We have generated E6-AP overexpressing transgenic mice, which overexpresses wild-type E6-AP under the control of rat probasin promoter, specifically targeting the transgene to the prostate gland. We wanted to see if E6-AP is overexpressed specifically in the prostate glands of transgenic mice, and not in other organs. In addition to the prostate gland, various tissues like brain, testes were isolated from the transgenic mice and also wild-type litter mates, proteins were extracted, Western blot analysis were performed and probed for E6-AP expression. Figure 1 shows that the levels of E6-AP are higher only in the prostate glands of transgenic mice and not in other tissues.

Mice prostate is structurally divided into four pairs of lobes: anterior, dorsal, ventral and lateral. It has been reported that there are lobe specific differences in transgene expression driven by the probasin promoter. In addition to that there are lobe specific differences in certain biochemical and pathological conditions. Hence we wanted to know if there is a lobe specific expression of E6-AP in the transgenic mice. Prosate gland from transgenic and wild-type mice was individually dissected into anterior, ventral and dorsolateral lobes, proteins were isolated and Western blots were performed. Figure 2 shows that E6-AP was over expressed in all the lobes, when compared with wild-type litter mates. Even though, there was a difference in the basal level of expression of E6-AP among the different lobes, there was no difference in the extent of over expression of transgene among the different lobes.

Our preliminary data from the original protocol has shown that the levels of Total Akt (T-Akt) are elevated in our E6-AP transgenic mice. We wanted to examine if there is a lobe specific differences in this phenomenon. Figure 2 shows the expression level of Phosho-Akt in different lobes. Even though there is an over expression of p-Akt in all the lobes compared to the wild-type, there was no significant differences between the extents of p-Akt over expression between the lobes. We also examined the levesl of p-Gskβ, which is a target of activated Akt. We found that p-Gskβ was also elevated in E6-AP transgenic prostate glands.

6

<u>Over expression of E6-AP increases prostate gland size and leads to precancerous lesions:</u>
Our preliminary data from the original proposal had shown that the prostate glands from the E6-AP transgenic mice were ~20% larger when compared to wild-type littermates. We have analyzed more prostate glands and there by expanded the sample size and our results were similar to earlier results with small sample size (Figure 3) showing that E6-AP transgenic prostate glands are ~20% larger than the wild-type litter mates.

Initiation and progression of prostate cancer is a multistage process involving a characteristic lesion termed as prostate intraepithelial neoplasia, which is believed to be the precursor for the formation of prostate cancers. Since E6-AP transgenic mice did not give raise to palpable prostate tumors, we decided to do an histological observation of the prostate glands. Prostate glands from >18 month old transgenic and wild-type litter mate controls were dissected into individual lobes (n=10). These tissues were processed, embedded in paraffin and 5μ sections were made. These sectioned tissues were stained for haematoxylin and eosin to determine the morphological features. Figure 4 shows that E6-AP transgenic mice shows hyperplasic or PIN like characteristics when compared with wild-type litter mate controls. This finding suggests that over expression of E6-AP could result in excessive proliferation and formation of preneoplastic lesions which resembles PIN. The results from these studies are tabulated in Table 1.

Effect of E6-AP on PI3K-Akt signaling in E6-AP over expressing stable cell lines:

<u>Generation of E6-AP stable cell lines:</u>
To investigate the effects of E6-AP overexpression on PI3K-Akt signaling pathway and cell growth, we used tetracycline inducible system, which permits highly sensitive and tightly regulated expression of target gene in response to varying concentrations of doxycycline (Dox). In this report we utilized Tet-off system, to generate E6-AP overexpressing stably transfected LNCaP cell line (E6-AP-LNCaP) in which E6-AP expression is turned on when Dox is removed from the culture medium. Since the stably transfected exogenous E6-AP is FLAG tagged to differentiate it from endogenous E6-AP, we performed Western blot analysis using anti-FLAG antibody to show that various E6-AP stable clones express high levels of exogenous E6-AP (Figure 5). We tested the ability of Dox to regulate the expression of E6-AP in E6-AP-LNCaP stable clones. The parental untransfected LNCaP cells and E6-AP-LNCaP stable clones 2 and 12 were cultured in the presence (2 μg/ml) and absence of Dox. Figure 5 shows that the levels of flag tagged exogenous E6-AP and total E6-AP are less in Dox treated cells when compared to Dox untreated cells, indicating that E6-AP expression is regulated in Dox-dependent manner and furthermore, E6-AP-LNCaP stable clones express high levels of E6-AP compared to control LNCaP cells.

<u>E6-AP modulates PI3K-Akt signaling:</u>
It is known that PI3K-Akt signaling pathway plays a central role in the development and progression of prostate cancer(46-48) . Our previous studies showed that PI3K-Akt signaling is down regulated in the prostate glands of E6-AP knockout mice (14). In order to study the effect of overexpression of E6-AP on PI3K-Akt signaling pathway, we utilized E6-AP-LNCaP cells and examined the expression levels of PI3K, total Akt and p-Akt (active Akt) and compared it with that of control parental LNCaP cells. As shown in Figure 6, the level of PI3K is higher in the E6-AP-LNCaP cells compared to the control parental LNCaP cells. Similarly, the levels of total and p-Akt (Ser-473) are also high in E6-AP-LNCaP cells compared to that of untransfected control LNCaP cells. However, under conditions where the expression of exogenous E6-AP (+Dox) is blocked, the levels of PI3K, total Akt and phospho-Akt are not significantly different from that of control LNCaP cells (Figure 6). The treatment of untransfected LNCaP cells with Dox does not significantly affect the levels of these proteins, which serves as a control. These experiments

suggest that E6-AP is a key regulator of PI3K-Akt signaling pathway where it upregulates the expression of the components of this pathway. These results are consistent with our previously published data from E6-AP knockout animals, suggesting that E6-AP is an important modulator of PI3K-Akt pathway in prostate glands.

E6-AP regulates Akt activity via RhoA:

Our previous studies indicate that small GTPase, RhoA, a negative regulator of Akt activity is increased in E6-AP knockout prostate glands (14). We have also shown that inhibition of RhoA activity in prostate cancer cells increases Akt activity. Here we tested the levels of RhoA in our E6-AP-LNCaP cells, Figure 7A shows that the levels of RhoA are decreased under E6-AP induced conditions (- Dox) in our E6-AP-LNCaP cells. Since RhoA is a known target of the ubiquitin-proteasome pathway and its levels are decreased in E6-AP-LNCaP cells under E6-AP overexpressing conditions, we hypothesized that, E6-AP interacts with RhoA, ubiquitinates it and induces its degradation via the ubiquitin proteasome pathway. To investigate whether RhoA levels are controlled by the ubiquitin-proteasome pathway in prostate cancer cells, we treated LNCaP cells with either vehicle DMSO or proteasome inhibitor, MG132. Figure 7B shows that RhoA protein levels are stabilized by MG132, indicating that RhoA protein levels in prostate cells are regulated by the ubiquitin-proteasome pathway. Since, E6-AP is an E3 ubiquitin-protein ligase enzyme, in order for E6-AP to act as a specific E3 ubiquitin-protein ligase for RhoA it should interact with RhoA. To test this possibility, we utilized glutathione-*S*-transferase (GST) pull down assay. ^{35}S-Methionine-labeled RhoA protein was incubated with either control protein (GST only) or GST-E6-AP protein. Figure 7C depicts a significant interaction of RhoA with E6-AP suggesting that E6-AP may be a putative E3 ubiquitin-protein ligase for RhoA in prostate cells.

Effect of over expression of E6-AP on apoptosis of LNCaP cells:

Our previous data from E6-AP knock-out mice shows that, loss of E6-AP leads to impaired prostate gland development. We also showed that this might be due to elevated apoptosis in E6-AP knockout prostate glands. It is of interest to see if an E6-AP overexpressing condition provides a protective effect against apoptosis. To test this we cultured parental LNCaP cells and E6-AP-LNCaP cells and treated them with 100µM etoposide to induce apoptotic stress. Then cell lysates were probed for pro-apoptotic markers like caspase 3 and Bax using Western immunobloting. As shown in Figure 8, the levels of cleaved caspase 3 and Bax are decreased in E6-AP-LNCaP cells compared to untransfected LNCaP cells indicating that E6-AP overexpression provides the cells with survival advantage by protecting them from apoptosis.

Role of E6-AP in androgen stimulated prostate growth:

Prostate gland growth is androgen dependent and I have previously shown that E6-AP over-expressing transgenic mice are ~20% larger when compared to wild-type littermates. However, to test whether E6-AP plays a role in the ability of prostate gland to respond to androgen signaling, we assessed prostate growth in castrated mice implanted with testosterone slow-release pellets. E6-AP over-expressing transgenic mice and wild-type littermates were castrated for 30 days to deplete the endogenous androgens and testosterone slow release pellets were implanted for additional 21 days. Prostate glands were dissected at 10, 20 and 30 days post-castration and also 21 days after testosterone implantation. Prostate gland involutes after 10, 20 and 30 days post castration (Fig 9), however the rate of involution of E6-AP transgenic mice and wild-type controls remains the same. Also the rate of regeneration of the prostate gland after 21 days of testosterone implantation does not change between E6-AP transgenic mice and wild-type controls. These results indicate that E6-AP has a role in normal prostate gland development, but it does not have any additional effect on prostate gland regeneration under androgen stimulated conditions.

Overexpression of E6-AP increases the cell growth and proliferation:
The members of the PI3K-Akt signaling pathway have been implicated in cell growth and proliferation. Since, over-expression of E6-AP induces the PI3K-Akt pathway, we predicted that E6-AP stable cell lines will exhibit increased cell growth and proliferation compared to that of untransfected control LNCaP cells. As shown in Figure 10A, E6-AP-LNCaP stable cells that overexpress E6-AP exhibit changes in cell shape compared with control LNCaP cells. E6-AP stable cells lacks cell processes and also clump together. Furthermore, E6-AP stable LNCaP cells show increase in cell size compared to that of control LNCaP cells. In addition to cell shape, we also examined the effects of E6-AP over-expression on prostate cell proliferation using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) growth assays. E6-AP-LNCaP stable cells and untransfected control LNCaP cells were grown for a period of 5 days in media containing normal and as well as charcoal stripped serum. Figure 10B shows a significant difference in proliferation of E6-AP over-expressing stable cells compared with untransfected control LNCaP cells both in normal and stripped serum suggesting that over-expression of E6-AP results in increased cell proliferation both in the presence of normal and stripped serum. These data confirms that over-expression of E6-AP leads to prostate cell proliferation and growth, which correlates with our previous finding that loss of E6-AP leads to reduced prostate gland size and increased apoptosis. Since, PI3K-Akt signaling is elevated in our E6-AP-LNCaP stable cells and Akt is involved in both hormone-dependent and independent growth of prostate cells, we also examined the role of Akt in prostate cell proliferation. The E6-AP-LNCaP stable cells along with control untransfected parental LNCaP cells were treated with PI3K inhibitor, LY294002 and cell proliferation was examined. Figure 10C shows that PI3K inhibitor, LY294002 significantly inhibit the proliferation of both control LNCaP and E6-AP-LNCaP stable cells in normal serum conditions. The E6-AP-LNCaP stable cells were more susceptible to the inhibitory effects of LY294002. These data suggest that the increase in proliferation observed in E6-AP-LNCaP stable cells is due to increase in the Akt activation. Since overexpression of E6-AP leads to increased proliferation of LNCaP cells, I also tested if knocking down E6-AP reduces proliferation of LNCaP cells. LNCaP cells were transfected with either scramble siRNA (control) or E6-AP siRNA, starved without hormone for 3 days and then stimulated with DHT for a period of 7 days. E6-AP siRNA treated cells does not proliferate as control cells indicating that E6-AP is required for normal androgen dependent proliferation of LNCaP cells (Figure 10D).

Role of E6-AP in regulating p53 levels in prostate glands and prostate cancer cells:

E6-AP was initially identified as an E3 ligase which promotes the degradation of p53 during HPV infection in cervical carcinoma cells. However, this effect is E6 dependent, as p53 could only be degraded by the formation of E6 and E6-AP complex. It is not known if E6-AP alone affects p53 degradation in the absence of E6 in prostate cancer cells. To test this, E6-AP over-expressing LNCaP cells were probed for p53 expression using Western blot (Figure 11). LNCaP cells under E6-AP over-expression (-dox) demonstrated a decreased level of p53 compared to normal conditions (+dox), indicating that E6-AP regulates p53 levels independent of E6 in prostate cancer cells. The level of p21 is also downregulated under E6-AP over-expressing conditions, indicating that E6-AP just affects the protein level of p53 and not its transcriptional activity (Figure 11).

Aim 2: To study the role of E6-AP in both the genomic and non-genomic mechanisms of AR signaling

Role of E6-AP in regulating the non-genomic mechanism of AR:

AR regulates many cellular processes by acting as a ligand activated transcription factor resulting in the regulation of the transcription of target genes in the nucleus, referred to as classical mechanism or genomic mechanism. Recent data suggests that AR can also mediate non-transcriptional actions outside the nucleus in addition to its ligand-inducible transcription factor function. These activities of AR are collectively called non-genomic actions which primarily involve rapid activation of signal transduction cascades by phosphorylation (42, 43). For example, androgens, by means of AR, are known to activate PI3K-AKT pathway in a transcription independent manner. In order to study the role of E6-AP in non-genomic actions of AR, E6-AP over-expressing stable cells were tested for the activation AKT at 15 minutes of hormone (DHT) treatment (Figure 12A). DHT-BSA, a membrane non permeable form of DHT was used as a control. Under E6-AP over-expressing conditions the levels of pAKT are elevated compared to normal LNCaP cells. However, the total AKT levels (tAKT) levels remain the same. In addition to modulating rapid activation of pAKT, I also tested the rapid hormone dependent in-vivo interaction of E6-AP with AR. Lysates from both vehicle and short-term DHT treated LNCaP cells were immunoprecipitated with E6-AP specific antibody and then immunobloted with AR antibody. Figure 12B shows that E6-AP interacts with AR within 15 minutes of hormone treatment. These data indicate that E6-AP regulates rapid non-genomic actions of AR, which could be mediated through its rapid hormone specific interaction with AR.

Role of E6-AP in regulating the genomic mechanism of AR:

The genomic mechanism of AR includes binding to the hormone in the cytoplasm, translocation to the nucleus, binding to hormone response elements on the promoter of target genes and regulating gene expression. It also recruits several coactivators which bring in several enzymatic activities on to the promoter, which aid in gene expression. E6-AP is one such coactivator which has an E3 ligase function, involved in tagging ubiquitin to target proteins for degradation. We have previously shown that E6-AP is involved in degradation of steroid receptors including estrogen receptor and AR. Additonally, we have also shown that E6-AP modulates AR protein levels both in E6-AP null prostate gland and also in E6-AP over-expressing cells. In order to delineate the role of E6-AP on AR genomic actions on target promoters, Chromatin Immunoprecipitation assays (ChIP) were performed to look at AR recruitment on PSA promoter at different time-points of hormone treatment (Figure 13A and B). These ChIP assays were performed under E6-AP knockdown conditions using siRNA against E6-AP (Figure 13C). Interestingly, the recruitment of AR is increased both in the promoter and enhancer regions in the absence of E6-AP at various time points tested (4h, 8h and 16h). These results indicate that in the absence of E6-AP, AR degradation is attenuated leading to accumulation of AR on the promoter.

Key Research Accomplishments

1. E6-AP is involved in the normal growth of the prostate gland; however it does not have any additional affect on the regeneration of prostate gland after castration.

2. Over expression of E6-AP in the prostate gland leads to the development of PIN like lesions

3. Over-expression of E6-AP increases the cell growth and proliferation, whereas knockdown of E6-AP reduces proliferation

4. Generation of cell lines which stably overexpress E6-AP in LNCaP cells

5. E6-AP regulates PI3K-Akt pathway in the prostate gland and as well as LNCaP cells

6. E6-AP regulation of PI3K-Akt pathway may be due to regulation of RhoA levels by ubiquitin mediated degradation

7. Over-expression of E6-AP increases non-genomic activity of AR

8. AR is accumulated on its target promoters under E6-AP knockdown conditions

Reportable Outcomes

Some of the accomplishments in this report have been accepted for oral presentation at the American Association of Cancer Research (AACR) meeting held at Los Angeles, California, 2007.

Results from this report have been submitted as a manuscript to Biochimica et Biophysica Acta.

Conclusion

In summary, we have demonstrated that E6-AP plays a vital role in the prostate gland growth, PI3K-Akt signaling and RhoA signaling pathways. Here we provide evidence that E3 ubiquitin-protein ligase/steroid hormone receptor coactivator, E6-AP itself can modulate these cellular signaling pathways. Our data also indicates that over expression of E6-AP could potentially lead to tumor initiation. I have also provided evidence that E6-AP by itself can modulate p53 levels in prostate cancer cells independent of E6. I have also shown that E6-AP also affects the non-genomic mechanisms of AR. ChIP assays demonstrate that AR is accumulated on its target promoter and enhancer under E6-AP knockdown conditions. These studies indicate that E6-AP plays a vital role in both the genomic and non-genomic signaling pathway of AR and their downstream effectors might play critical roles in many biological processes, especially in cell growth. Taken together, our studies indicate E6-AP could act as a central protein that can integrate multiple signals into appropriate cellular responses. Furthermore, E6-AP through activation of the PI3K-Akt signaling pathway and their downstream effectors might play critical roles in many biological processes, especially in cell growth.

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Appendices:

1. Figures
2. Abstract
3. Manuscript

Figure 1

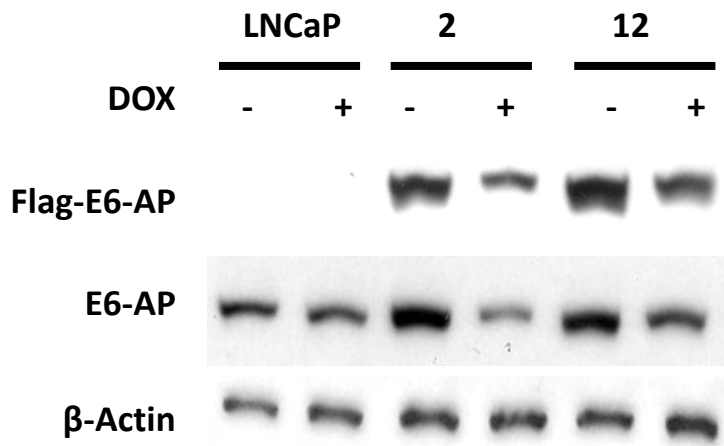**Figure 1:** Representative western blots from two lines of E6-AP over expressing transgenic mice showing the expression levels of E6-AP in different organs. E6-AP is specifically over expressed in the prostate glands of transgenic mice. (WT- Wild-type, Tr- Transgenic)

Figure 2

Figure 2: Western blots showing the expression of E6-AP, p-Akt and p-GSK-β in different lobes of the wild-type and transgenic prostate glands. (WT- Wild-type, Tr- Transgenic)

Figure 3

Figure 3: Wet weights of prostate glands from wild-type and E6-AP over expressing transgenic mice. The wet weights are normalized with body weights. n>20

Figure 4

Wild-type	Transgenic

Figure 4: Representative photomicrographs of H & E staining of wild-type (WT) and transgenic mice (TG) showing PIN like lesions only in the transgenic mice.

Table 1

	Total	Normal	PIN
Wild-type	10	10	0
Transgenic	10	4	6

Figure 5

Figure 5: Representative western blots from two lines of E6-AP over expressing stable LNCaP cells lines (2 and 12) showing the expression levels of Flag-E6-AP and total E6-AP in response to Doxycycline (DOX). E6-AP is specifically over expressed under the absence of (DOX)

Figure 6

Figure 6: Figure 2: Overexpression of E6-AP induces PI3K-Akt signaling pathway. Parental LNCaP cells and E6-AP-LNCaP cells were treated with or without Dox for 48 hrs. Under inducible conditions (- Dox), the expression of E6-AP is increased in E6-AP-LNCaP cells compared to parental LNCaP cells. Increased protein levels of PI3K, total Akt and phosphorylated-Akt (p-Akt, Ser-473) were observed in E6-AP-LNCaP cells under E6-AP overexpressing conditions (-Dox). β-actin was used as a loading control.
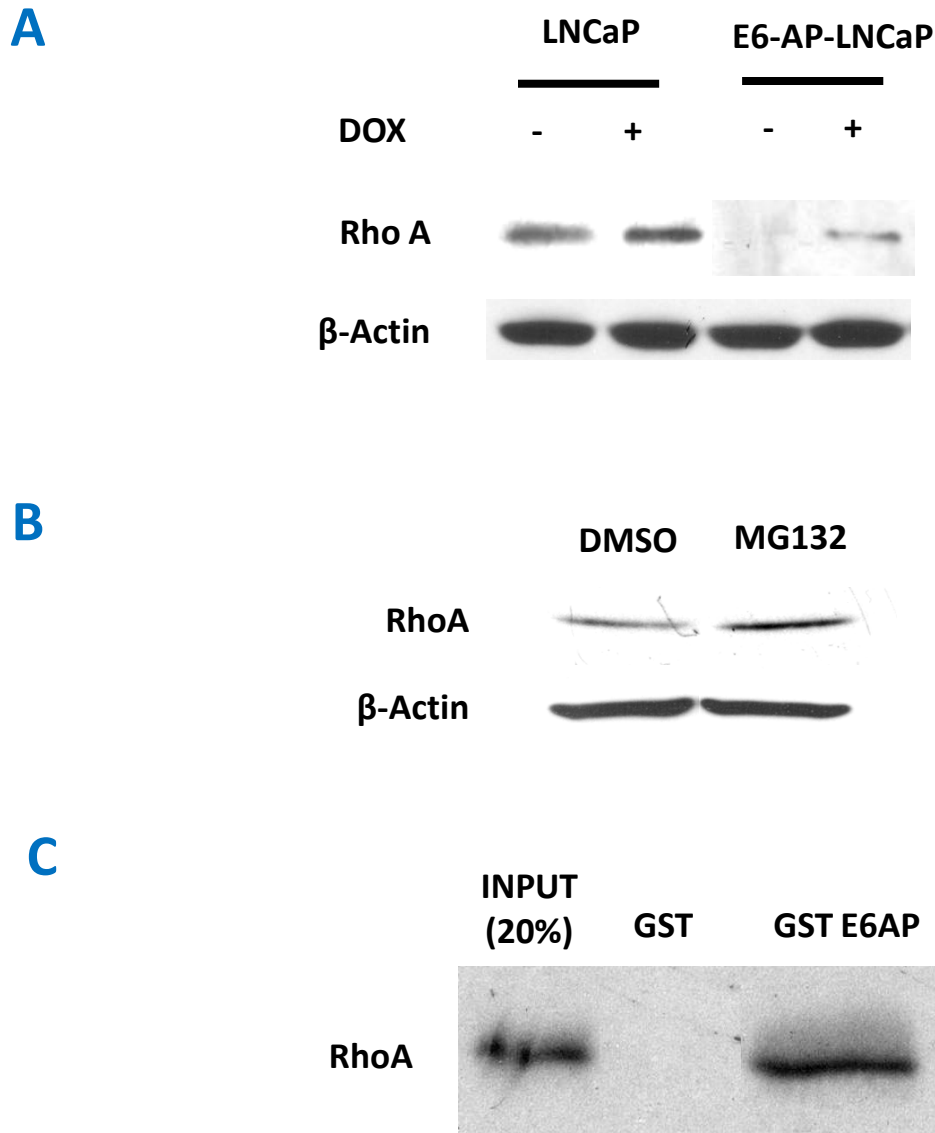
Figure 7

A

B

C

Figure 7: Involvement of RhoA in E6-AP-mediated regulation of Akt activity. (A) Western blots showing that the levels of RhoA are decreased under E6-AP overexpressing conditions (-Dox) in E6-AP-LNCaP stable cells compared to that of parental LNCaP cells. (B) To determine if RhoA is target of the ubiquitin-protesome pathway in prostate cells, LNCaP cells were treated with DMSO or proteasome inhibitor, MG132 and cell lysates were examined for RhoA levels. Western blots showing RhoA stabilization with MG132 treatment. (C) *In vitro* interaction of E6-AP with RhoA. The human RhoA protein was synthesized *in vitro* using the TNT-coupled reticulocyte lysate system. RhoA protein was then incubated with GST-E6-AP fusion protein that was bounded to glutathione-Sepharose beads. The glutathione-bounded proteins were separated on SDS-PAGE, followed by autoradiography. Twenty percent of the TNT reaction was used as input, and GST alone was used as a negative control.
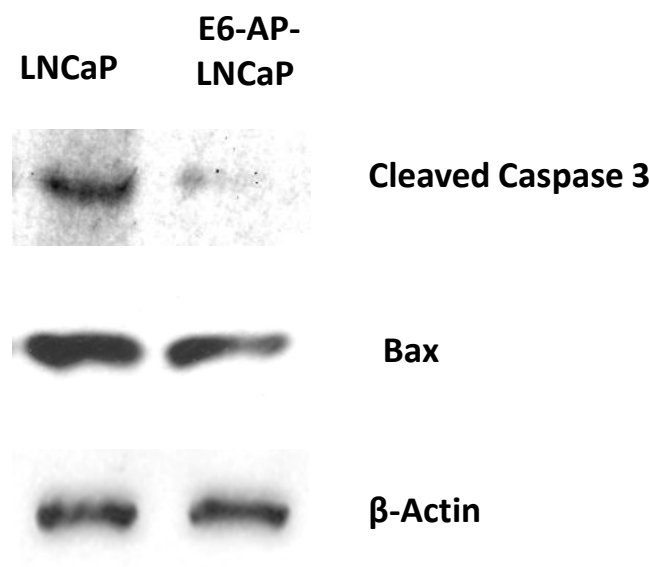
Figure 8

Figure 8: Overexpression of exogenous E6-AP in prostate cells protects cells against apoptosis. Parental LNCaP cells and E6-AP-LNCaP cells were grown in normal serum for twenty four hours and then treated with 100μM etoposide for another 6hrs. The cell lysates were analyzed by Western blots using anti-caspase 3 and anti-bax antibodies. E6-AP-LNCaP cells shows less amounts of proapoptotic proteins than parental LNCaP cells.
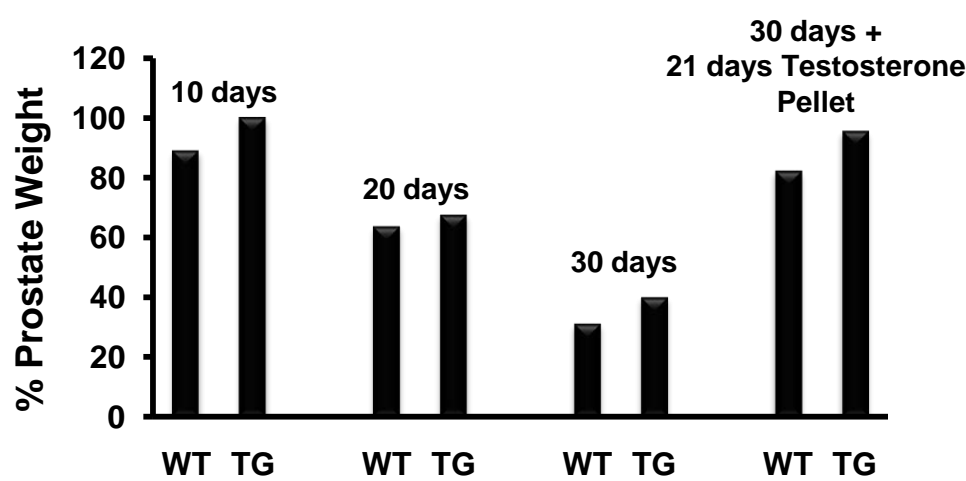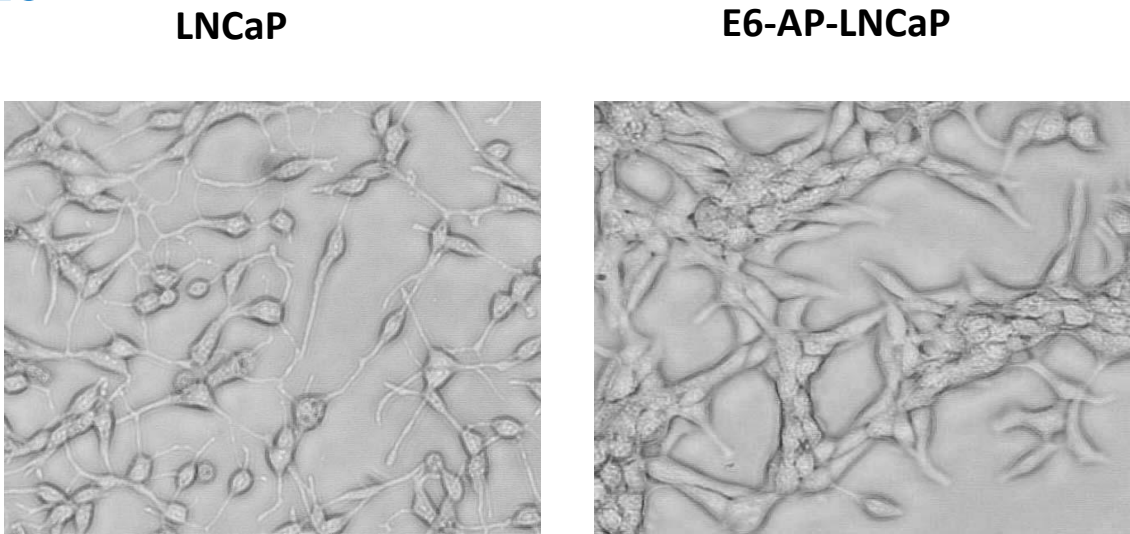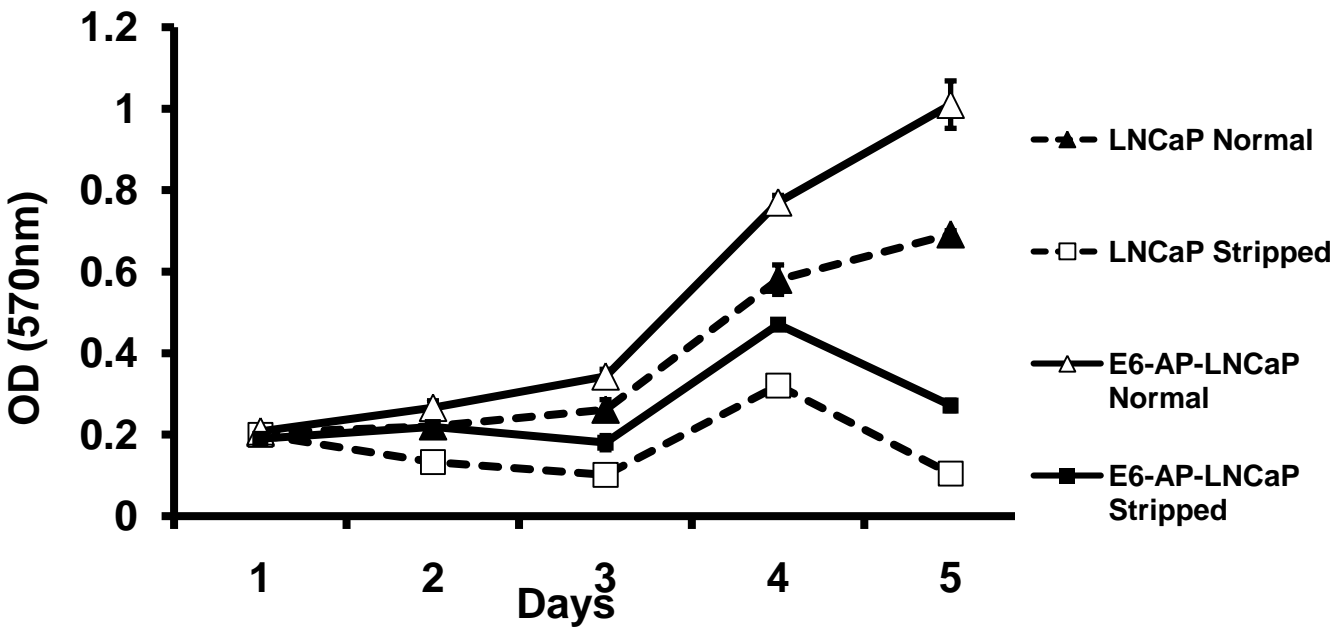
Figure 9

Figure 9: Effect of E6-AP on androgen induced prostate gland growth in vivo. Wild-type and E6-AP transgenic mice were castrated for 30 days and implanted with testosterone pellet for another 21 days. Prostate glands were disected and wet weights were analysed at 10, 20 and 30 days after castration and also after 21 days after testosterone pellet implantation.
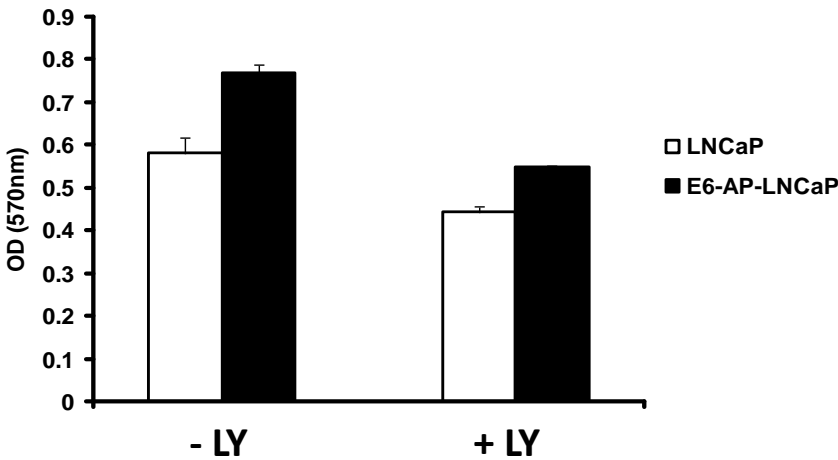
Figure 10

A

LNCaP   E6-AP-LNCaP

B

- --▲-- LNCaP Normal
- --□-- LNCaP Stripped
- --△-- E6-AP-LNCaP Normal
- --■-- E6-AP-LNCaP Stripped

C
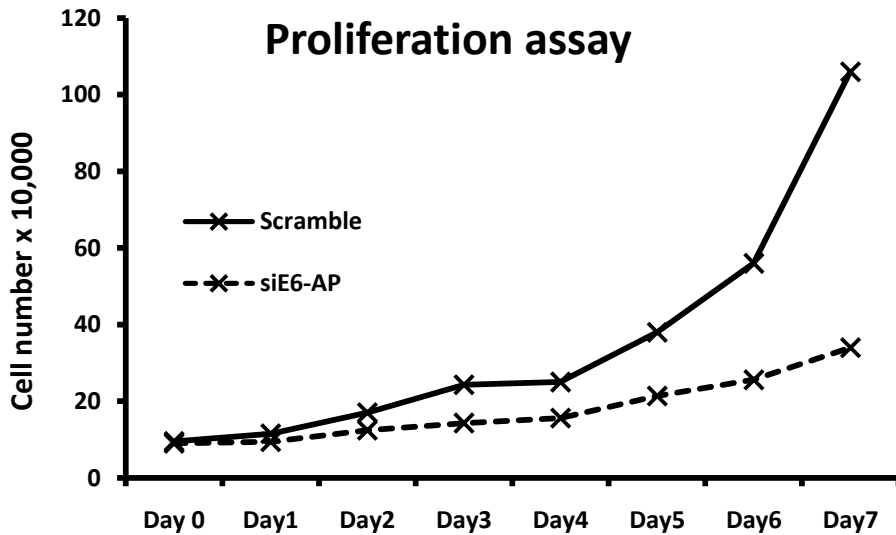

□ LNCaP
■ E6-AP-LNCaP

D

Figure 10: E6-AP regulates prostate cancer cell growth and proliferation. (A) E6-AP stable cells have increased cell size. (B) MTT assay indicating overexpression of E6-AP increases proliferation of LNCaP cells. (C) MTT assay indicating that increased proliferation of E6-AP stable cells is most likely due to increased AKT activity. (D) Loss of E6-AP reduces androgen dependent proliferation of LNCaP cells
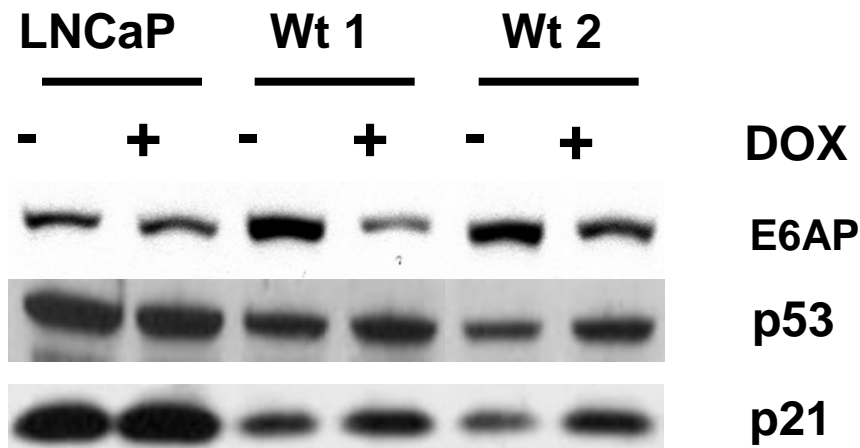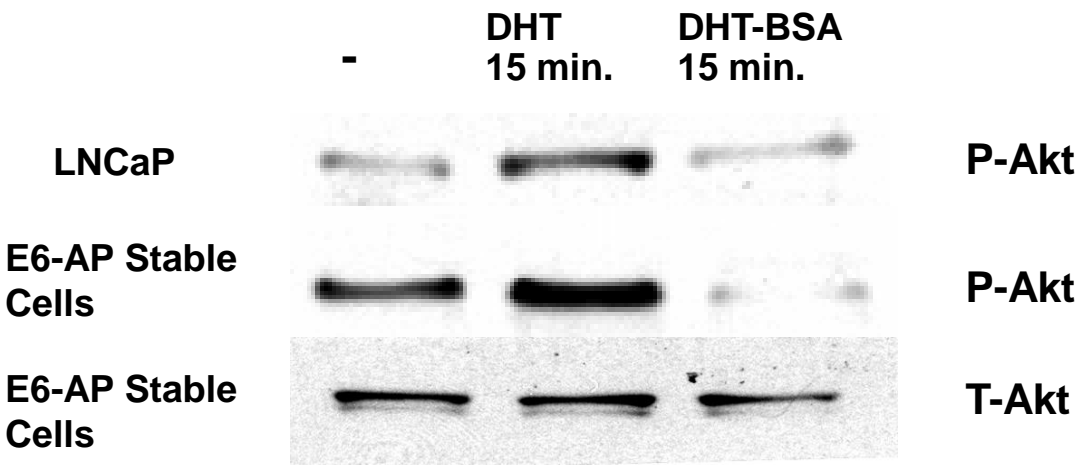
Figure 11

Figure 11: E6-AP regulates p53 levels in LNCaP cells. E6-AP stable cells were treated with or without DOX and probed for E6-AP, p53 and p21. p53 and its target gene p21 levels are lower under E6-AP overexpressing conditions.
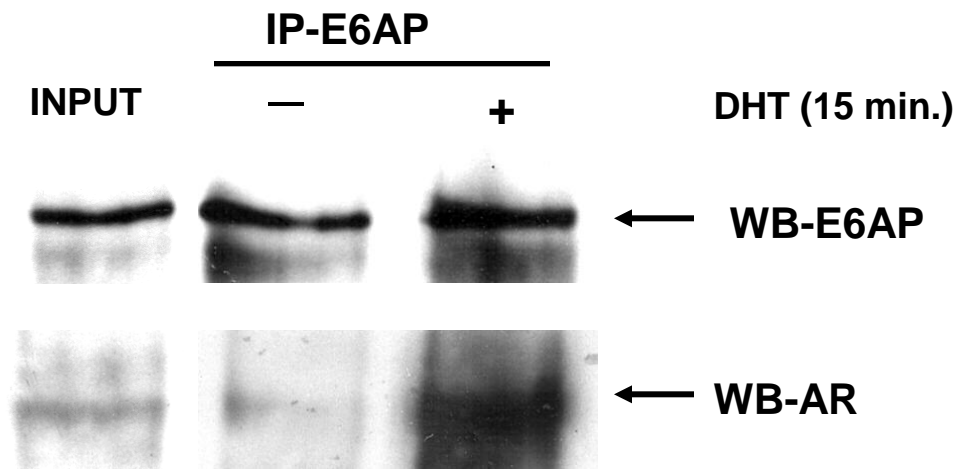
Figure 12

A

B

Figure 12: E6-AP enhances non genomic action of AR. (A) E6-AP stable cells were treated with DHT for 15 min and tested for P-Akt and T-Akt levels by western blot. (B) Co-immunoprecipitation experiments showing E6-AP interacts with AR within 15 mins of DHT treatment
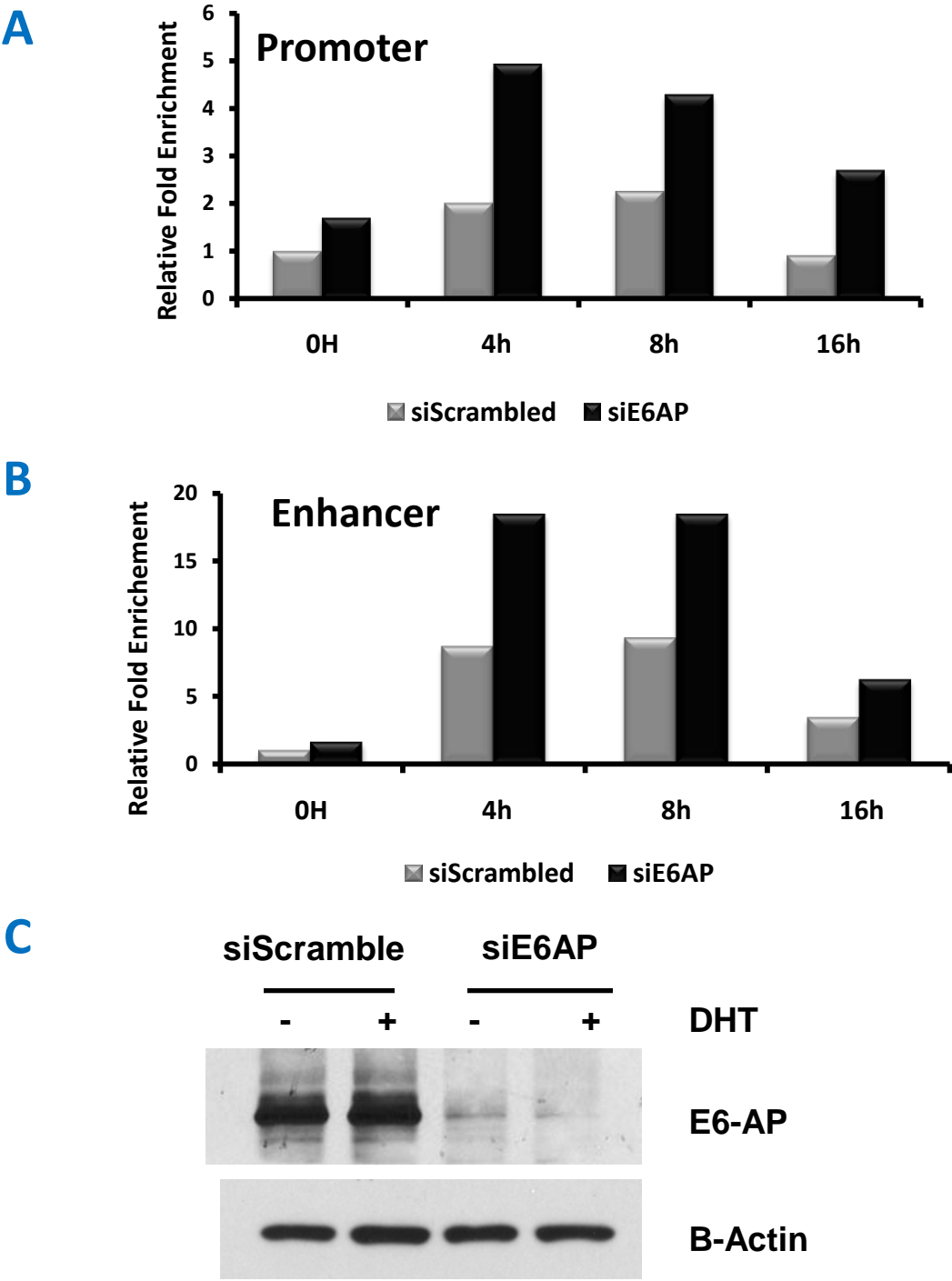
Figure 13

A

B

C

Figure 13: Knockdown of E6-AP increases AR recruitment on target gene promoter. LNCaP cells were transfected with either scramble or E6-AP siRNA, starved for 3 days without hormone and treated with DHT for indicated time points. ChIP assays were performed to test AR recruitment on (A) promoter and (B) enhancer regions of PSA regulatory region. (C) Western blot from ChIP lysates demonstrating the efficiency of knockdown of E6-AP siRNA.

2007 AACR Annual Meeting
April 14-18, 2007
Los Angeles, CA

Abstract Number:	4152
Presentation Title:	**Steroid hormone receptor coactivator, E6-associted protein (E6-AP) regulates PI3K-Akt signaling and prostate cell growth**
Presentation Start/End Time:	Tuesday, Apr 17, 2007, 10:10 AM -10:25 AM
Location:	Room 511 A-C, Los Angeles Convention Center
Author Block:	*Sathish Srinivasan*, *Guilian Fu*, *Sarath Dhananjayan*, *Shazia Zafar*, *Zafar Nawaz*. Univ. of Miami School of Medicine, Miami, FL

E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP), is a novel dual function steroid hormone receptor coactivator. E6-AP not only interacts with and enhances the hormone-dependent transcriptional activities of various steroid hormone receptors, including androgen receptor (AR), but also is a member of the E3 class of functionally related ubiquitin-protein ligases. Previously, using E6-AP knockout animals we have shown that E6-AP is required for the proper development and growth of prostate gland. Furthermore, we also show that protein levels of the components of phosphatidylinositol 3-kinase/protein kinase B (PI3K-Akt) signaling pathway are decreased in E6-AP knockout animals. Furthermore, we also show that E6-AP regulates the protein levels and transcriptional activity of AR in prostate cells suggesting that E6-AP plays important roles in the cytoplasm in addition to acting as a coactivator in the nucleus. Since, the PI3K-Akt pathway has been described as a dominant growth survival pathway in prostate cells and elevated PI3K-Akt signaling is correlated with prostate cancer progression, the main focus of this study is to decipher the mechanism by which E6-AP modulates the components and activity of PI3K-Akt pathway in prostate cells. In this study we report the generation of stable LNCaP cells that stably overexpress exogenous E6-AP protein. Here, we show that the levels of PI3K, total Akt, phosphorylated Akt (active Akt) and its down stream target protein, GSKb are increased in E6-AP overexpressing stable cells suggesting that E6-AP regulates the PI3K-Akt signaling pathway. Furthermore, our data also suggest that E6-AP modulates PI3K-Akt signaling pathway by both androgen-independent and dependent mechanisms. In the androgen-independent mechanism, E6-AP modulates PI3K-Akt signaling by regulating the protein levels of RhoA, a small GTPase, which is a negative regulator of the Akt signaling pathway via the ubiquitin-proteasome pathway. Further, we show that E6-AP, a known coactivator of AR, amplifies the androgen-dependent activation of PI3K-Akt signaling pathway. This amplification of the androgen-dependent activation of the PI3K-Akt signaling by E6-AP is unique and represents the first example of novel roles for coactivators in the regulation of cytoplasmic signaling pathways. In addition, we show that stable overexpression of E6-AP in prostate cancer cells results in increased cell size, proliferation and decreased apoptosis. Overall our data suggests that E6-AP regulates both the positive and negative modulators of the PI3K-Akt pathway in prostate cells which results in increased prostate cell growth, proliferation and decreased apoptosis.

Title Page

A. Full Title:

Steroid hormone receptor coactivator, E6-associted protein (E6-AP) regulates PI3K-Akt signaling and prostate cell growth

B. Running Title:

Regulation of PI3K-Akt signaling by E6-AP

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Abstract:

The purpose of this study is to elucidate the specific roles of E6-associated protein, E6-AP (a dual function steroid hormone receptor coactivator) in the regulation of PI3K-Akt signaling pathway, prostate cell growth and survival. In this study we report the generation of LNCaP cells that stably overexpress exogenous E6-AP protein. Here, we show that the levels of phosphatidylinositol 3-kinase, total Akt, phosphorylated Akt (active Akt) and its downstream target protein, GSKβ are increased in E6-AP overexpressing stable cells suggesting that E6-AP regulates the PI3K-Akt signaling pathway. Our data suggest that E6-AP modulates PI3K-Akt signaling pathway by both androgen-independent and dependent mechanisms. In the androgen-independent mechanism, E6-AP modulates PI3K-Akt signaling by regulating the protein levels of RhoA, a small GTPase, which is a negative regulator of the Akt signaling pathway. Further, we show that E6-AP, a known coactivator of AR, amplifies the androgen-dependent activation of PI3K-Akt signaling pathway. This amplification of the androgen-dependent activation of the PI3K-Akt signaling by E6-AP is unique and represents the first example of novel roles for coactivators in the regulation of cytoplasmic signaling pathways. In addition, we show that stable overexpression of E6-AP in prostate cancer cells results in increased cell size and proliferation. Overall our data suggests that E6-AP regulates both the positive and negative modulators of the PI3K-Akt pathway in prostate cells which results in increased prostate cell growth, proliferation and decreased apoptosis.

Introduction:

E3 ubiquitin-protein ligase enzyme, E6-associated protein (E6-AP), is a novel dual function steroid hormone receptor coactivator (13, 24). E6-AP not only interacts with and enhances the hormone-dependent transcriptional activities of various steroid hormone receptors, including androgen receptor (AR), but also is a member of the E3 class of functionally related ubiquitin-protein ligases (11, 25, 26). E3 ubiquitin-protein ligases have been proposed to play major role in defining the substrate specificity of the ubiquitin-proteasome system. Protein ubiquitination involves two other classes of enzymes, namely the E1 ubiquitin-activating enzyme (UBA) and E2 ubiquitin-conjugating enzymes (UBCs). UBA first activates ubiquitin in an ATP-dependent manner, and the activated ubiquitin then forms a thioester bond between the carboxyl-terminal glycine residue of ubiquitin and a cysteine residue of the UBA. Next, ubiquitin is transferred from the E1 to one of several E2s (UBCs), preserving the high energy thioester bond. In some cases, ubiquitin is transferred directly from E2 to the target protein through an isopeptide bond between the ε-amino group of lysine residues of the target protein and the carboxy terminus of ubiquitin. In other instances, the transfer of ubiquitin from UBCs to target proteins proceeds through an E3 ubiquitin-protein ligase intermediate, such as E6-AP. Finally, the ubiquitin-tagged target proteins undergo degradation via the 26S proteasome pathway. Since, E6-AP acts as a coactivator as well as E3 ubiquitin-protein ligase, E6-AP serves to link two important and opposing activities in a cell, the transcription and the protein degradation, thereby resulting in efficient transcription (5, 12).

Previously, we have shown that the prostate gland is smaller in E6-AP knockout animals implying that E6-AP is required for the proper development and growth of prostate gland (13). Furthermore, we also show that protein levels of the components of phosphatidylinositol 3-kinase/protein kinase B (PI3K-Akt) signaling pathway are decreased in E6-AP knockout animals. In addition, we show that E6-AP regulates the protein levels and transcriptional activity of AR in prostate cells, suggesting that E6-AP plays important roles in the cytoplasm in addition to acting as a coactivator in the nucleus. Since, the PI3K-Akt pathway has been described as a dominant growth survival pathway in prostate cells and elevated PI3K-Akt signaling is correlated with prostate cancer progression (3, 4, 8, 20, 23), the main focus of this study is to decipher the mechanism by which E6-AP modulates the components and activity of PI3K-Akt pathway in prostate cells.

Akt, also known as protein kinase B (PKB), is a proto-oncogene with a pleckstrin homology and serine/threonine kinase domains. The kinase activity of Akt is stimulated by a variety of extracellular stimuli, such as growth factors and cytokines leading to the phosphorylation and regulation of a wide spectrum of its substrates involved in multiple cellular processes, including cell survival, cell growth, cell differentiation, cell cycle progression, cell proliferation and cellular metabolism (15). Akt is activated by PI3K, a heterodimer composed of a p85 regulatory and a p110 catalytic subunit. Activation of PI3K by receptor tyrosine kinases and other cell surface receptors results in the generation of second messenger phospholipids, phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P$_3$) or phosphatidylinositol 3,4-bisphosphate (PI-3,4-P$_2$), which eventually

triggers phosphorylation and activation of the down stream serine/ threonine kinase Akt, which mediate various biological responses. The lipid phosphatase, Phosphatase and tensin homolog (PTEN), a tumor suppressor, dephosphorylates PIP3 back to PIP2 and thus shuts off PI3K-Akt signaling (18, 19, 22). In response to a variety of stimuli, Akt is phosphorylated at Threonone-308 (Thr-308) by 3-phosphoinositide-dependent protein kinase-1 and at Serine-473 (Ser-473) by integrin-linked kinase. This dual phosphorylation of Akt fully activates its kinase activity. A large variety of Akt substrates have been identified and these include among others, BAD, CREB, members of the forkhead family of transcription factors, IkB kinase, procaspase-9, glycogen synthase kinase (GSK-3-Alpha/Beta), mTOR/FRAP, p21, p27 and AR (4, 8, 17). The large variety of Akt substrates identified to date underscores the role of Akt as a key mediator of cell proliferation, differentiation, and survival. Furthermore, increasing evidence indicates that Akt plays an important role in tumorigenesis. Akt expression is frequently elevated in human prostate, ovarian, and breast cancers and that ectopic expression of constitutively active Akt or wild-type Akt2 results in malignant phenotype (28, 29, 31).

The PI3K-Akt pathway which is normally activated by growth factors is also activated by steroid hormones like estrogens and androgens (1, 2, 9, 27). The activation of PI3K-Akt pathway by androgens results in increased cell growth and anti-apoptotic activity (1, 27). Previously published studies indicate that androgen regulates PI3K-Akt pathway via the AR but independent of its transcriptional activity, suggesting an important role of androgen receptors within the cytoplasm. However, the role for coactivator proteins in the regulation of PI3K-Akt pathway was unknown until the recent finding that steroid

receptor coactivator-3 (SRC-3) is able to induce PI3K-Akt pathway in an androgen-independent manner (32). The induction of cell proliferation and growth by SRC-3 has been attributed to its ability to induce Akt activity and expression. Therefore steroid hormone receptor coactivator proteins in addition to their transcriptional activation can also contribute to growth and proliferation by modulating important growth signaling pathways in the cytoplasm, independent of transcription.

In order to elucidate the specific roles of E6-AP (a dual function steroid receptor coactivator) in the regulation of PI3K-Akt pathway, prostate cell growth and survival, we have generated E6-AP stable LNCaP cells (here on referred as E6-AP-LNCaP) that overexpress exogenous E6-AP. Our data from these E6-AP-LNCaP cells suggest that E6-AP modulates PI3K-Akt signaling pathway by androgen-independent and dependent mechanisms. E6-AP modulates PI3K-Akt signaling in an androgen-independent manner by regulating the protein levels of a small GTPase, RhoA which is a negative regulator of the Akt signaling pathway. E6-AP also functions as an androgen-dependent amplifier of the PI3K-Akt pathway. This results in decreased apoptosis, increased cell growth and proliferation of prostate cells.

Materials and Methods:

Plasmid Construction

E6-AP mammalian expression plasmid (pCR3.1-E6-AP) and GST fusion bacterial expression plasmids have been previously reported (29). The pRevTRE-E6-AP vector for

developing stable cell lines was generated as follows. The *Bam*HI-*Hin*dIII fragment of E6-AP isolated from pGEM-E6-AP was inserted into the corresponding sites of the pRevTRE vector.

Cell culture

LNCaP (parental cell line) and E6-AP-stable cell line (E6-AP-LNCaP) were maintained in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2.

Establishment of E6-AP-Stable Cell Line

The Tet-off system was used to develop E6-AP stable cell clones that overexpress exogenous E6-AP (BD Clontech). LNCaP cells were grown to approximately 80% confluence in RPMI 1640 medium and first transfected with pRevTet-off IN vector using the FuGene 6 transfection reagent. After 2 days cells were treated with 200 µg/ml G418 and grown for 2 weeks; every 4th day the medium was changed along with G418. Selected colonies were isolated and grown separately. These clones were screened for the expression of Tet regulator using pTRE2hyg-Luc as a reporter vector. Selected clones that express Tet regulator were used to create double-stable clones.

To develop double-stable clones, the pRevTet-off IN-stable clones were transfected by electroporation with linearized pRevTRE-E6-AP, and after 2 days cells were treated with 150 µg/ml hygromycin and grown for 2 weeks, every 4th day medium was changed along with hygromycin. Selected colonies were isolated and grown separately. Then these clones were tested for E6-AP expression in the presence of 2 µg (+) or absence (-) of Dox.

All double-stable clones were maintained in the presence of 75 µg/ml hygromycin. In order to shut off the exogenous E6-AP expression, cells were treated with Dox (2µg/ml). To induce the expression of exogenous E6-AP cells were grown in the absence of Dox.

Western Blot Analysis

Cells were grown as indicated; afterward cells were harvested and washed in TEN buffer (40 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl). Then cells were lysed in ice-cold RIPA buffer (20mM Tris pH 7.5, 150mM Nacl, 1% NP40, 0.5% Sodium deoxycholate, 1mM EDTA and 0.1% SDS) by pipetting up and down. Thereafter, cell lysates were placed on ice for 30 minutes and cleared by centrifugation at 12,000 x g for 20 minutes at 4°C. The supernatants were collected and frozen at −80°C until used for analysis. The protein concentrations of lysates were measured using the Bio-Rad protein assay kit.

Twenty-five to 50 µg of total protein from each sample was resolved on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, Inc., Keene, NH). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris base (pH 7.5) and 150 mM NaCl) containing 0.05% Tween 20 (TBS-T), then probed with the primary antibody. The primary antibodies were diluted in 1% nonfat dry milk in TBS-T as indicated and used for immunoblotting: Anti-E6-AP (1:1000 dilution; Bethyl laboratories, Inc., Montgomery, TX); anti-total Akt (1:1200 dilution), anti-phosphoAkt (1:1000 dilution), anti-PI3-Kinase and p85 (1:1000 dilution), anti-caspase-3 (8G10; 1:1000 dilution), and anti-Bax (1:750 dilution; Cell Signaling Technology, Beverly, MA); anti-RhoA (26C4; 1:800 dilution;

Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and anti-ß-actin (1:10,000 dilution; Sigma-Aldrich Corp.). After washing in TBS-T, membranes were incubated with their appropriate horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Inc.) and developed using an enhanced chemiluminescence detection system (Amersham Biosciences, Arlington Heights, IL) according to the instructions of the manufacturer and were exposed to X-Ray film (Phenix research products, Hayward, CA).

In Vitro Protein-Protein Interaction Assay

Radiolabeled RhoA protein was synthesized using a rabbit reticulocyte-coupled *in vitro* transcription and translation (TNT) kit in the presence of ^{35}S-methionine according to the manufacturer's recommendations (Promega Corp.). GST-E6-AP and control GST proteins were expressed in *Escherichia coli* DH-5 α cells and immobilized on glutathione-Sepharose beads. The glutathione-bound GST and GST-E6-AP were incubated with *in vitro*-synthesized RhoA G14V protein in NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), and 0.5% Nonidet P-40) for 2–3 hours at room temperature or overnight at 4°C. After washing four times with NETN buffer, E6-AP-bound RhoA protein was isolated by boiling the Sepharose beads in 1x sodium dodecyl sulfate gel loading buffer and were separated on a 10% SDS-PAGE, followed by autoradiograpy.

MTT assay

Cell proliferation was measured using TACS MTT assay (Trevigen, Inc., Gaithersburg, MD). Briefly, LNCaP and E6-AP-LNCaP cells were grown in 96-well cell culture plates

at a density of $2x10^3$ cells/well in 100 µl stripped and normal serum containing medium for 24hrs in 5% CO_2 at 37°C and were treated with vehicle or LY294002 (5µM). Every day, 10 µl of MTT reagent was added to each well, and the cells were further incubated for 4 hours at 37°C to allow the formation of a purple precipitate. The precipitates were dissolved by adding 100 µl of detergent reagent to each well and the plates were incubated in dark over night. The absorbance at 570 nm was measured using a microplate reader (Biorad laboratories, Hercules, CA).

Results:

Generation of E6-AP stable cell lines:

To investigate the effects of E6-AP overexpression on PI3K-Akt signaling pathway and cell growth, we used tetracycline inducible system, which permits highly sensitive and tightly regulated expression of target gene in response to varying concentrations of doxycycline (Dox). In this report we utilized Tet-off system, to generate E6-AP overexpressing stably transfected LNCaP cell line (E6-AP-LNCaP) in which E6-AP expression is turned on when Dox is removed from the culture medium. These stable clones were generated in a two step process; first, we generated a single stable cell line (pRevTet-off IN) containing a regulatory protein (tTA), a chimeric transactivator which regulates the target gene expression. Later, this cell line was retransfected with a response plasmid (pRevTRE-E6-AP) which expresses exogenous E6-AP, ultimately creating double-stable clones containing both the regulatory and the response plasmid, allowing us to induce E6-AP expression only in the absence of Dox. We obtained several clones after selecting against hygromycin (150µg/ml) and we tested the ability of these clones to

overexpress exogenous E6-AP in the absence of Dox. Since the stably transfected exogenous E6-AP is FLAG tagged to differentiate it from endogenous E6-AP, we performed Western blot analysis using anti-FLAG antibody to show that various E6-AP stable clones express high levels of exogenous E6-AP (Figure 1A). We also used anti-E6-AP antibody to detect the total E6-AP levels in the stable clones and found that all the stable clones expressed significantly higher levels of E6-AP when compared to untransfected parental LNCaP cells (Figure 1A). The highest expression of exogenously expressed E6-AP was observed in clone 17 whereas clone 2, 4, 6 and 12 express moderate levels of exogenous E6-AP (Figure 1A). Next we tested the ability of Dox to regulate the expression of E6-AP in E6-AP-LNCaP stable clones. The parental untransfected LNCaP cells and E6-AP-LNCaP stable clones 2 and 12 were cultured in the presence (2 µg/ml) and absence of Dox. Figure 1B shows that the levels of flag tagged exogenous E6-AP and total E6-AP are less in Dox treated cells when compared to Dox untreated cells, indicating that E6-AP expression is regulated in Dox-dependent manner and furthermore, E6-AP-LNCaP stable clones express high levels of E6-AP compared to control LNCaP cells. In addition, we also observed a dose-dependent decrease in the expression of E6-AP in response to increasing concentrations of Dox (0, 1.5 and 2 µg/ml) (Figure 1C).

E6-AP modulates PI3K-Akt signaling:

It is known that PI3K-Akt signaling pathway plays a central role in the development and progression of prostate cancer (28, 29, 31). Our previous studies showed that PI3K-Akt signaling is down regulated in the prostate glands of E6-AP knockout mice (13). In order

to study the effect of overexpression of E6-AP on PI3K-Akt signaling pathway, we utilized E6-AP-LNCaP cells and examined the expression levels of PI3K, total Akt and p-Akt (active Akt) and compared it with that of control parental LNCaP cells. As shown in Figure 2, the level of PI3K is higher in the E6-AP-LNCaP cells compared to the control parental LNCaP cells. Similarly, the levels of total and p-Akt (Ser-473) are also high in E6-AP-LNCaP cells compared to that of untransfected control LNCaP cells. However, under conditions where the expression of exogenous E6-AP (+Dox) is blocked, the levels of PI3K, total Akt and phospho-Akt are not significantly different from that of control LNCaP cells (Figure 2). The treatment of untransfected LNCaP cells with Dox does not significantly affect the levels of these proteins, which serves as a control. These experiments suggest that E6-AP is a key regulator of PI3K-Akt signaling pathway where it upregulates the expression of the components of this pathway. These results are consistent with our previously published data from E6-AP knockout animals, suggesting that E6-AP is an important modulator of PI3K-Akt pathway in prostate glands.

E6-AP-mediated modulation of PI3K-Akt pathway is androgen-dependent:

PI3K-Akt pathway is known to be activated by growth factors acting via membrane bound growth factor receptors. Recent evidences suggest that PI3K-Akt pathway can also be activated by steroid hormones, including androgens, indicating the participation of AR in this pathway (1, 27). Considering the fact that E6-AP is an AR-interacting protein and acts as a coactivator of AR, and also that both E6-AP and AR are found in the cytoplasm before hormone treatment, there is a possibility that E6-AP activates PI3K-Akt pathway in an androgen and AR-dependent manner. To test this, we initially cultured E6-AP-

LNCaP stable cells and untransfected control LNCaP cells in medium containing either 10% normal serum or charcoal stripped serum. Figure 3A shows that cells cultured in medium with normal serum express higher levels of total Akt and p-Akt compared to cells cultured in medium containing charcoal stripped serum. Further more the over all levels of these proteins are higher in E6-AP-LNCaP cells compared to untransfected control LNCaP cells. These data indicate that steroid hormones may be involved in the regulation of PI3K-Akt signaling and this response is further amplified by E6-AP. To further confirm that androgen regulates PI3K-Akt signaling and E6-AP amplifies the hormone-dependent up-regulation of PI3K-Akt signaling, we cultured untransfected control LNCaP cells and E6-AP-LNCaP cells in 10% charcoal stripped medium supplemented with or without synthetic androgen R1881 for 24hrs. As shown in Figure 3B, treatment of control LNCaP cells with R1881 increases the levels of p-Akt and E6-AP further amplifies the hormone-dependent activation of Akt in E6-AP-LNCaP cells (Figure 3B). In order to determine the functional consequences of this increase in p-Akt (active Akt) protein levels, we examined the protein levels of the phosphorylated form of glycogen synthase kinase-3β (GSK-3β), a well known target of Akt. As shown in Figure 3B, the protein levels of p-GSK-3β are increased in E6-AP-LNCaP cells compared to control LNCaP cells, suggesting that E6-AP increases both Akt protein levels and function. Since E6-AP is a coactivator, it is possible that E6-AP may modulate the expression of PI3K and Akt mRNA by activating its transcriptional regulators. To understand whether E6-AP transcriptionally induces PI3K-Akt pathway, we performed real-time PCR analysis under the same conditions in LNCaP cells and E6-AP-LNCaP cells. We found that there was no significant difference in the Akt and PI3K mRNA

levels between LNCaP and E6-AP-LNCaP cells (data not shown). These results indicate that E6-AP modulates PI3K pathway via a non-transcriptional mechanism.

To further confirm the involvement of AR in the E6-AP-mediated activation of PI3K-Akt signaling, we treated control LNCaP cells and E6-AP-LNCaP cells with either vehicle control (-), androgen-R1881 alone (H), anti-androgen-Flutamide (F) or androgen and anti-androgen together (FH) for 24hrs. As shown in Figure 3C, the basal levels of p-Akt is increased in E6-AP-LNCaP stable cells compared to control LNCaP cells and the hormone induced levels of p-Akt are further increased in stable cells that overexpress E6-AP. Furthermore, the anti-androgen, flutamide was able to inhibit this activation, indicating that the E6-AP modulates PI3K-Akt signaling via AR.

E6-AP regulates Akt activity via RhoA:

Our previous studies indicate that small GTPase, RhoA, a negative regulator of Akt activity is increased in E6-AP knockout prostate glands (13). We have also shown that inhibition of RhoA activity in prostate cancer cells increases Akt activity. Here we tested the levels of RhoA in our E6-AP-LNCaP cells, Figure 4A shows that the levels of RhoA are decreased under E6-AP induced conditions (- Dox) in our E6-AP-LNCaP cells. Since RhoA is a known target of the ubiquitin-proteasome pathway and its levels are decreased in E6-AP-LNCaP cells under E6-AP overexpressing conditions, we hypothesized that, E6-AP interacts with RhoA, ubiquitinates it and induces its degradation via the ubiquitin proteasome pathway. To investigate whether RhoA levels are controlled by the ubiquitin-proteasome pathway in prostate cancer cells, we treated LNCaP cells with either vehicle

DMSO or proteasome inhibitor, MG132. Figure 4B shows that RhoA protein levels are stabilized by MG132, indicating that RhoA protein levels in prostate cells are regulated by the ubiquitin-proteasome pathway. Since, E6-AP is an E3 ubiquitin-protein ligase enzyme, in order for E6-AP to act as a specific E3 ubiquitin-protein ligase for RhoA it should interact with RhoA. To test this possibility, we utilized glutathione-*S*-transferase (GST) pull down assay. ^{35}S-Methionine-labeled RhoA protein was incubated with either control protein (GST only) or GST-E6-AP protein. Figure 4C depicts a significant interaction of RhoA with E6-AP suggesting that E6-AP may be a putative E3 ubiquitin-protein ligase for RhoA in prostate cells.

Overexpression of E6-AP increases the cell growth and proliferation:

The members of the PI3K-Akt signaling pathway have been implicated in cell growth and proliferation. Since, overexpression of E6-AP induces the PI3K-Akt pathway, we predicted that E6-AP-LNCaP cells will exhibit increased cell growth and proliferation compared to that of untransfected control LNCaP cells. As shown in Figure 5A, E6-AP-LNCaP cells that overexpress E6-AP exhibit changes in cell shape compared with control LNCaP cells. E6-AP-LNCaP cells lacks cell processes and also clump together. Furthermore, E6-AP stable LNCaP cells show increase in cell size compared to that of control LNCaP cells. In addition to cell shape, we also examined the effects of E6-AP overexpression on prostate cell proliferation using propidium iodide staining of the DNA content of E6-AP-LNCaP cells and control LNCaP cells. As shown in Figure 5B, E6-AP-LNCaP cells have increased number of cells in S phase compared with that of control LNCaP cells. These data suggest that overexpression of E6-AP leads to proliferation,

which may be due to the upregulation of PI3K-Akt pathway. We also confirmed our results using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) growth assays. E6-AP-LNCaP cells and untransfected control LNCaP cells were grown for a period of 5 days in media containing either normal or charcoal stripped serum. Figure 5C shows a significant difference in proliferation of E6-AP-LNCaP cells compared with untransfected control LNCaP cells both in normal and stripped serum suggesting that overexpression of E6-AP results in increased cell proliferation both in the presence of normal and stripped serum. These data confirms that overexpression of E6-AP leads to prostate cell proliferation and growth, which correlates with our previous finding that loss of E6-AP leads to reduced prostate gland size and increased apoptosis. Since, PI3K-Akt signaling is elevated in our E6-AP-LNCaP cells and Akt is involved in both hormone-dependent and independent growth of prostate cells, we also examined the role of Akt in prostate cell proliferation. The E6-AP-LNCaP cells along with control untransfected parental LNCaP cells were treated with PI3K inhibitor, LY294002 and cell proliferation was examined. Figure 5D shows that PI3K inhibitor, LY294002 significantly inhibit the proliferation of both control LNCaP and E6-AP-LNCaP cells in normal serum conditions. The E6-AP-LNCaP cells were more susceptible to the inhibitory effects of LY294002. These data suggest that the increase in proliferation observed in E6-AP-LNCaP cells is due to increase in the Akt activation.

Our previous data from E6-AP knock-out mice shows that, loss of E6-AP leads to impaired prostate gland development. We also showed that this might be due to elevated apoptosis in E6-AP knockout prostate glands. It is of interest to see if an E6-AP

overexpressing condition provides a protective effect against apoptosis. To test this we cultured parental LNCaP cells and E6-AP-LNCaP cells and treated them with 100μM etoposide to induce apoptotic stress. Then cell lysates were probed for pro-apoptotic markers like caspase 3 and Bax using Western immunobloting. As shown in Figure 6, the levels of cleaved caspase 3 and Bax are decreased in E6-AP-LNCaP cells compared to untransfected LNCaP cells indicating that E6-AP overexpression provides the cells with survival advantage by protecting them from apoptosis.

Discussion:

Coactivators enhance the transactivation functions of nuclear hormone receptors by means of their diverse array of enzymatic activities (14). Recent studies have suggested that coactivators can also act as key regulators of signaling cascades, indicating that coactivators also possess functions that are distinct from their transcriptional activation functions (16). E6-AP is an important member of the ubiquitin proteasome pathway and has also been characterized as a coactivator for several steroid hormone receptors including AR (24). In our previous report, we have shown that loss of E6-AP affects the growth of the prostate gland (13). We also identified the mechanisms by which E6-AP affects the prostate gland growth and we showed that E6-AP affects both the protein levels and functions of AR. In addition to that we also demonstrated that the protein levels of PI3K, total Akt and p-Akt are decreased in E6-AP knockout prostate gland, suggesting that apart from its coactivation function, E6-AP may also modulates the functions of PI3K-Akt signaling pathway. In this study, we provide direct evidence which suggests that E6-AP modulates the activity and functions of PI3K-Akt signaling

and regulates prostate cell proliferation, growth and apoptosis. Our results show that overexpression of E6-AP in prostate cancer cells result in increased cell size, increased cell proliferation and decreased apoptosis. Furthermore, our data also indicate that the components of the PI3K-Akt signaling pathway and its downstream effectors that are important mediators of cell growth are induced by E6-AP overexpression.

Based on our data, we postulate that E6-AP regulates PI3K-Akt signaling by both androgen-independent and dependent mechanisms. In the androgen-independent mechanism, E6-AP enhances the basal protein levels and activity of the PI3K-Akt signaling pathway. The hormone-independent activation of PI3K-Akt signaling by E6-AP is consistent with previously published study which suggests that the steroid receptor coactivator, SRC-3 can stimulate cell growth by modulating the Akt signaling pathway in an androgen-independent manner, but it is still unknown how SRC-3 regulates Akt signaling (32, 33). However, in this manuscript, we show the androgen-independent activation of PI3K-Akt signaling by E6-AP is through the down regulation of small GTPase, RhoA in prostate cancer cells. The idea that E6-AP regulates PI3K-Akt signaling and prostate cell growth by modulating the protein levels of RhoA is supported by our data which show that E6-AP interacts with RhoA and may potentially induce its degradation via the ubiquitin-proteasome pathway in prostate cells. Previously, we and others have shown that RhoA negatively regulates PI3K-Akt signaling pathway (7, 13). It has also been shown that RhoA can promote prostate cancer cell apoptosis by inhibiting Akt signaling pathway via the protein kinase C zeta. Additionally, our previously published data also demonstrated that the levels of total RhoA and active RhoA are

increased in E6-AP knockout prostate gland suggesting that RhoA negatively regulates the Akt pathway by inducing the inactivation of Akt in the absence of E6-AP, which subsequently alters prostate cell growth. This possibility was confirmed by the fact that the inhibition of RhoA activity increased the levels of phosphorylated (active) Akt. Consistent with this observation, we now show that the overexpression of E6-AP reduced the levels of RhoA which results in increased basal levels and activation of PI3K-Akt signaling, decreased apoptosis and increased prostate cell growth.

LNCaP cells are known to be PTEN negative which results in the PI3K-Akt pathway being constitutively active in these cells (30). E6-AP not only elevates the activity of constitutively active PI3K-Akt pathway in these cells, here, we show that E6-AP which is a known coactivator of the genomic functions of AR, also amplifies the androgen-dependent activation of PI3K-Akt signaling pathway. It is well-established that the PI3K-Akt signaling pathway is induced by androgens via AR (21) suggesting that in addition to ligand inducible transcriptional activity, AR in the cytoplasm also participate in the activation of a variety of signaling pathways including PI3K-Akt signaling pathway (1, 6, 10, 27). Our data is consistent with and reinforces these findings which suggest that androgens regulate PI3K-Akt signaling pathway. The role of nuclear receptor coactivator proteins in the activation of PI3K-Akt signaling pathway by androgens and AR is largely unknown. Our data suggest that E6-AP amplifies the androgen-dependent activation of PI3K-Akt signaling. This amplification of the androgen-dependent activation of the PI3K-Akt signaling by E6-AP is unique and represents the first example of novel roles for coactivators in the regulation of cytoplasmic signaling cascades. It is also important to

note that this amplification of PI3K-Akt signaling is transcription independent. Together, our data shows that E6-AP regulates prostate cell apoptosis, differentiation and growth by modulating the PI3K-Akt signaling via the regulation of both its positive and negative modulators like androgens and RhoA respectively.

Current studies have revealed that PI3K-Akt pathway regulates a variety of cellular functions including cell survival, cell growth, cell differentiation, cell proliferation, cell cycle progression and cellular metabolism (15). One of the major functions of PI3K-Akt signaling in malignancy is promoting cancer cell survival. Cancer cells develop specific mechanisms to overcome apoptosis, or programmed cell death, which is a normal cellular function involved in the elimination of unnecessary or damaged cells. PI3K-Akt plays a central role in antiapoptotic pathways. Our data from E6-AP overexpressing stable prostate cells also support the notion that PI3K-Akt signaling promotes cell growth and decrease apoptosis. Recently, it has been demonstrated that perturbations in the PI3K-Akt signaling are also significantly correlated with the progression of prostate cancer (28, 29). In addition, it has been demonstrated that there is a direct synergy between AR and Akt signaling which is sufficient to initiate and promote prostate cancer growth and progression (31). It has also been demonstrated that both the nuclear and cytoplasmic functions of AR are required for this biological synergy that exist between AR and PI3K-Akt signaling pathways resulting in prostate cell growth. Since E6-AP acts as a coactivator of AR and modulator of PI3K-Akt and RhoA signaling, it may be a key contributor in the maintenance of this synergy between Akt and AR signaling. This might

explain the increased growth and proliferative rates and decreased apoptosis in cells that overexpress E6-AP.

In summary, we have demonstrated that E6-AP play vital roles in prostate cell proliferation, growth, apoptosis and potential cross talk between PI3K-Akt signaling, RhoA signaling and AR signaling pathways. Here we provide evidence that E3 ubiquitin-protein ligase/steroid hormone receptor coactivator, E6-AP itself can modulate these cellular signaling pathways. Taken together, our studies identify E6-AP as a central protein that can integrate multiple signals into appropriate cellular responses. Furthermore, E6-AP through activation of the PI3K-Akt signaling pathway and their downstream effectors might play critical roles in many biological processes, especially in cell growth.

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Figure Legend:

Figure 1: Expression analysis of E6-AP in E6-AP-LNCaP stable clones. (A) Western blot analysis of exogenous E6-AP in various E6-AP-LNCaP stable clones (2, 4, 6, 12 and 17) using anti-FLAG antibody (top panel) and total E6-AP using anti-E6-AP antibody (bottom panel). Lane LNCaP represents parent LNCaP cell line. (B) Western blots showing inducible expression of exogenous E6-AP in E6-AP-LNCaP stable clones using anti-FLAG antibody (top panel) and total E6-AP using anti-E6-AP antibody (bottom panel). (C) Western blots showing dose-dependent regulation of exogenous E6-AP expression in E6-AP-LNCaP clone with increasing concentration of Dox. In these experiments β-actin was used as a loading control.

Figure 2: Overexpression of E6-AP induces PI3K-Akt signaling pathway. Parental LNCaP cells and E6-AP-LNCaP cells were treated with or without Dox for 48 hrs. Under inducible conditions (- Dox), the expression of E6-AP is increased in E6-AP-LNCaP cells compared to parental LNCaP cells. Increased protein levels of PI3K, total Akt and phosphorylated-Akt (p-Akt, Ser-473) were observed in E6-AP-LNCaP cells under E6-AP overexpressing conditions (-Dox). β-actin was used as a loading control.

Figure 3: Serum and androgen-dependent regulation of Akt activity by E6-AP. (A) LNCaP cells and E6-AP-LNCaP stable cells were grown either in 10% normal or charcoal stripped serum and probed with antibodies against total Akt and p-Akt (Ser-473). (B) Western blot analysis of p-Akt (Ser-473) levels and its down stream target p-GSKβ from LNCaP cells and E6-AP-LNCaP stable cells grown in 10% charcoal stripped serum supplemented with or without synthetic androgen R1881. (C) Western blots showing the involvement of AR in E6-AP-mediated activation of Akt. LNCaP cells and E6-AP-LNCaP stable cells were grown in 10% charcoal stripped serum and treated with either vehicle control (-), androgen-R1881 alone (H), anti-androgen-Flutamide (F) or androgen and anti-androgen together (FH) and the expression of active Akt (Ser-473) was analyzed. In these experiments β-actin was used as a loading control.

Figure 4: Involvement of RhoA in E6-AP-mediated regulation of Akt activity. (A) Western blots showing that the levels of RhoA are decreased under E6-AP overexpressing conditions (-Dox) in E6-AP-LNCaP stable cells compared to that of parental LNCaP cells. (B) To determine if RhoA is target of the ubiquitin-protesome pathway in prostate cells, LNCaP cells were treated with DMSO or proteasome inhibitor, MG132 and cell lysates were examined for RhoA levels. Western blots showing RhoA stabilization with MG132 treatment. (C) *In vitro* interaction of E6-AP with RhoA. The human RhoA protein was synthesized *in vitro* using the TNT-coupled reticulocyte lysate system. RhoA protein was then incubated with GST-E6-AP fusion protein that was bounded to glutathione-Sepharose beads. The glutathione-bounded proteins were

separated on SDS-PAGE, followed by autoradiography. Twenty percent of the TNT reaction was used as input, and GST alone was used as a negative control.
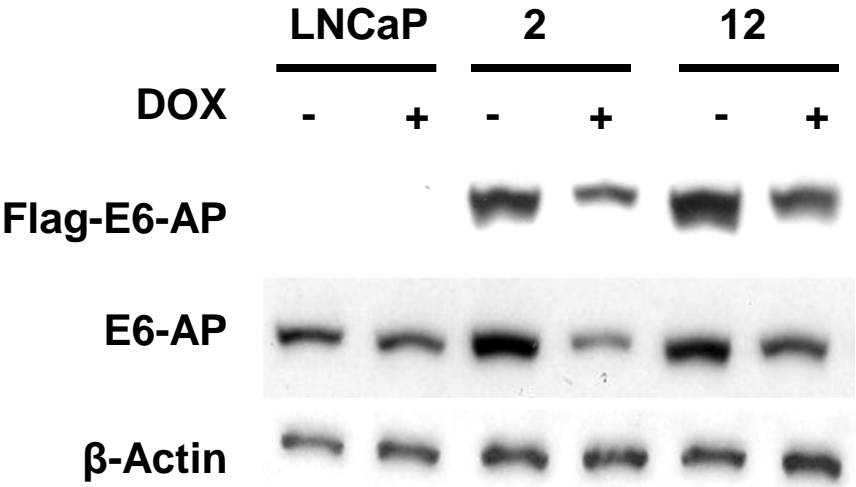
Figure 5: Exogenous overexpression of E6-AP enhances cell growth, proliferation and decreases apoptosis. (A) Morphology of E6-AP-LNCaP cells compared with parental control LNCaP cells, indicating the difference in cell size and shape. (B) E6-AP-LNCaP cells exhibit increased proliferation. Parental LNCaP cells and E6-AP-LNCaP cells were grown for 72 h, harvested, fixed with 70% ethanol, stained with propidium iodide, and processed for FACS analysis. Data are plotted as the percentage of S phase cells. (C) MTT assay to measure cell proliferation. Parental LNCaP cells and E6-AP-LNCaP cells were grown in 96 well plates in the presence of normal and charcoal stripped serum and MTT assay was carried out on each day for 5 days. (D) To examine the role of active Akt on cell proliferation, parental LNCaP cells and E6-AP-LNCaP cells were treated with PI3K inhibitor, LY294002 and MTT assay was performed (E) Overexpression of exogenous E6-AP in prostate cells protects cells against apoptosis. Parental LNCaP cells and E6-AP-LNCaP cells were grown in normal serum for twenty four hours and then treated with 100μM etoposide for another 6hrs. The cell lysates were analyzed by Western blots using anti-caspase 3 and anti-bax antibodies. E6-AP-LNCaP cells shows less amounts of proapoptotic proteins than parental LNCaP cells.
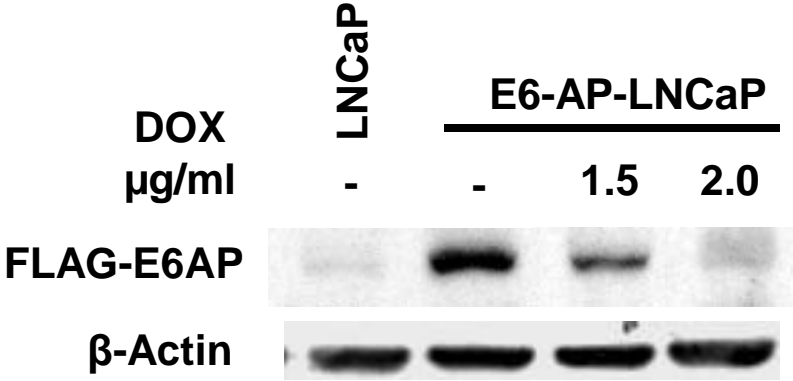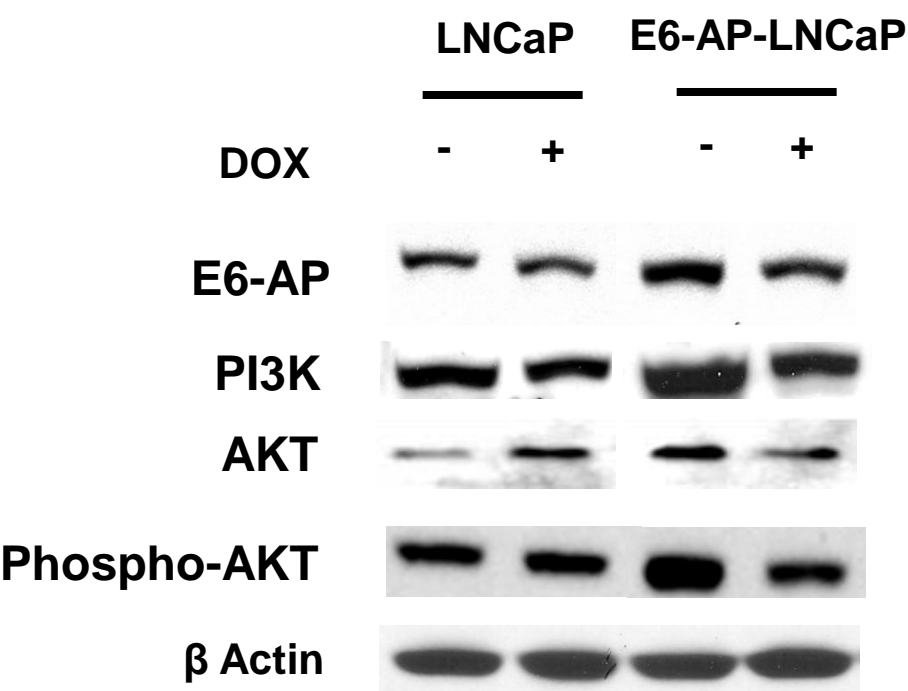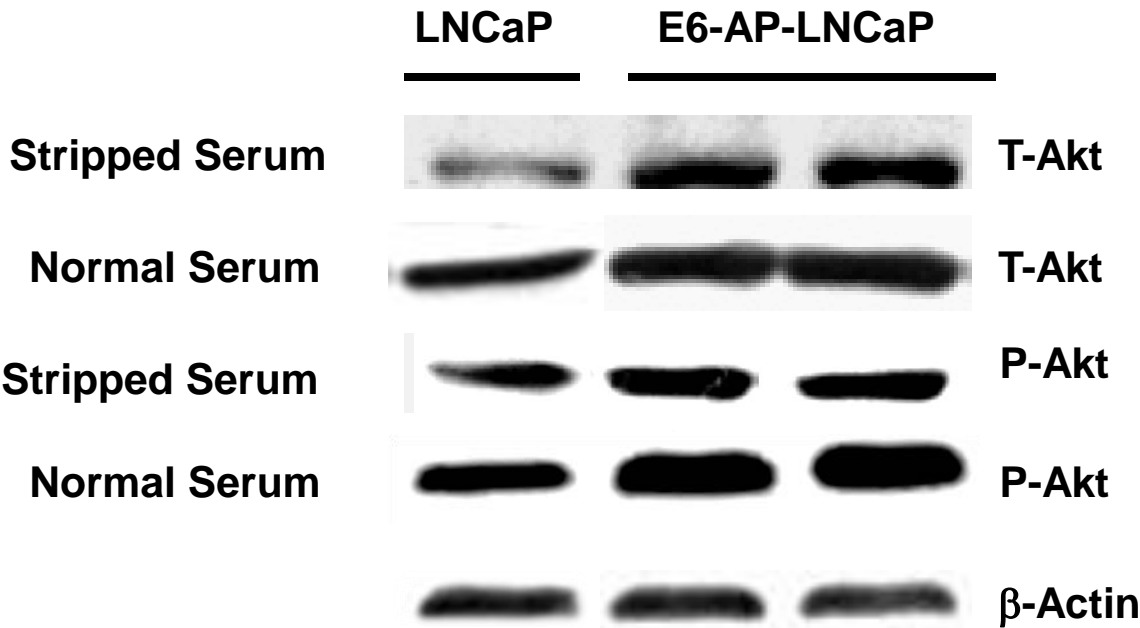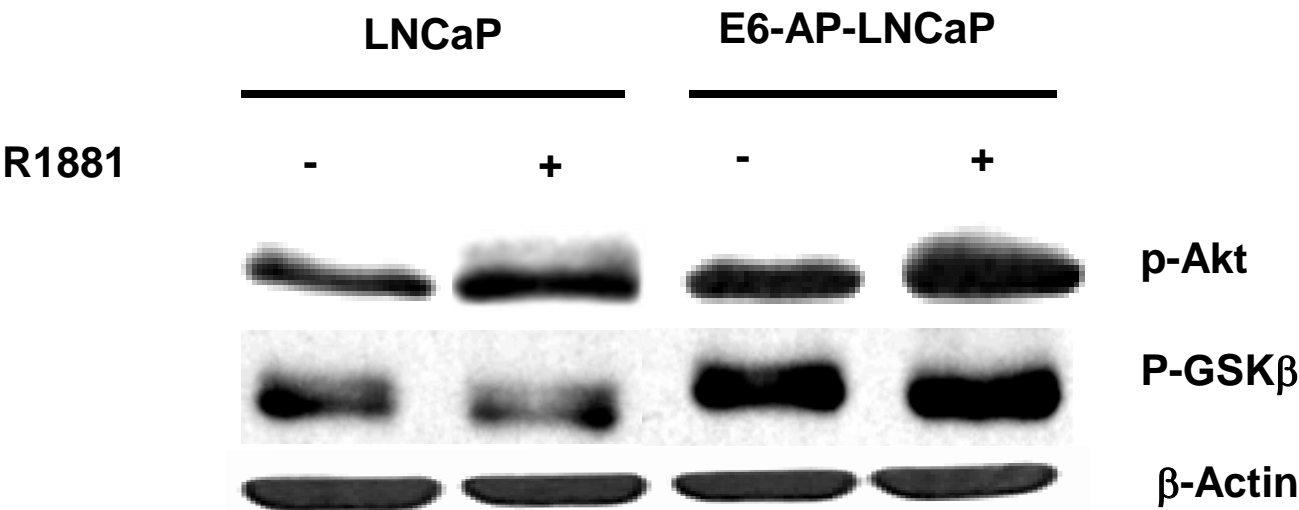
Figure 1

A

B

C

Figure 2

Figure 3

A

	LNCaP	E6-AP-LNCaP	
Stripped Serum			T-Akt
Normal Serum			T-Akt
Stripped Serum			P-Akt
Normal Serum			P-Akt
			β-Actin

B

	LNCaP		E6-AP-LNCaP		
R1881	−	+	−	+	
					p-Akt
					P-GSKβ
					β-Actin

C

	LNCaP				E6-AP-LNCaP			
	−	F	H	FH	−	F	H	FH
P-Akt								
β-Actin								

Figure 4

A

	LNCaP		E6-AP-LNCaP	
DOX	-	+	-	+

E6-AP

Rho A

β-Actin

B

	DMSO	MG132

RhoA

β-Actin

C

	INPUT (20%)	GST	GST E6AP

RhoA

Figure 5

A

LNCaP	E6-AP-LNCaP

B

C

D

E

	LNCaP	E6-AP-LNCaP	
Cleaved Caspase 3			
Bax			
β-Actin			