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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 hormonedependent 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. In this report we show that over expression of E6-AP in the prostate gland leads to increased prostate gland size and also showed PIN like precancerous lesions. We also found that PI3K-Akt pathway is elevated in E6-AP transgenic prostate gland. In addition to that, stable LNCaP cells that stably overexpress exogenous E6-AP protein have elevated levels of PI3K, total Akt, phosphorylated Akt (active Akt) suggesting that E6-AP regulates the PI3K-Akt signaling pathway. This report also suggest that E6-AP may regulate 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 ubiquitinproteasome pathway. In addition, we show that stable overexpression of E6-AP in prostate cancer cells results in decreased apoptosis. Overall our data suggests that E6-AP regulates the PI3K-Akt pathway in prostate cells which results in increased prostate gland growth, proliferation and decreased apoptosis.					
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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 β -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 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.

Additionally, the small G protein, RhoA, which belongs to the Rho family of guanosine triphosphatases (GTPases), is an important intracellular signaling protein that controls diverse cellular functions related to prostate gland development. Recently, it has been demonstrated that activated RhoA can negatively modulate Akt signaling pathway via protein kinase C ϵ in TRAMP cell lines. Inhibition of RhoA activation results in the activation of Akt, leading to enhanced cell survival of TRAMP cell lines. TRAMP cell lines were derived from TRAMP mice, which I will use as a mice model in this proposal to identify how E6-AP modulates the level of these important signaling proteins during prostate cancer progression.

5) TRAMP mouse model

In recent years, TRansgenic Adenocarcinoma of the Mouse Prostate (TRAMP) has emerged as an excellent mouse model of prostate cancer. TRAMP mouse model was generated by specifically targeting expression of SV40 Tag in prostate epithelial cells. This model recapitulates many salient aspects of human prostate cancer. The major advantages of this prostate cancer mouse model includes: 1) the tumor tissue histologically resembles the human

disease appearing as early as 10 weeks; 2) the tumors arise with a short latency period and with 100% frequency; 3) the mice exhibit progressive stages of prostate cancer ranging from mild to severe prostatic hyperplasia with focal adenocarcinoma and seminal vesicle invasion; 4) metastatic spread of the disease to the lymph nodes, lung and bone has been observed; and 5) the mouse model can be used to follow the progression/multi-stage development of disease all within a 10-20 week time period (40-43). In this proposal we plan to use TRAMP mice to help elucidate the role of E6-AP in prostate cancer development. Since p53 is abrogated in TRAMP prostate and E6-AP degrades p53, TRAMP mice will be an exciting model system to study E6-AP function.

6) 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 (44, 45). 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 (see preliminary data). Furthermore, in this proposal we plan to cross these mice models with TRAMP mice, which offers a unique opportunity to study the role of E6-AP in prostate cancer.

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

In our original proposal we had hypothesized that E6-AP, a coactivator of AR, is functionally significant in the development of prostate cancer. Hence, we had earlier proposed the following specific aims:

Aim 1. Generation of E6-AP null TRAMP mice and examine the consequence of loss of E6-AP on prostate cancer development and progression in TRAMP mice.

Aim 2. Generation of E6-AP transgenic TRAMP mice and examine the consequence of overexpression of E6-AP on prostate cancer development and progression in TRAMP mice.

However due to technical difficulties encountered to accomplish the earlier proposed aims, we were forced to redirect the aims to focus on the role of E6-AP on the development of normal prostate gland and tumorigenesis and also to study the role of E6-AP on PI3K-Akt signaling in prostate cancer cell lines. I will be summarizing the technical difficulties encountered and will state our new aim, in addition to our earlier aims.

Technical difficulties encountered:

In order to generate E6-AP null TRAMP mice, TRAMP mice have to be crossed with E6-AP null mice. TRAMP mice are available from Jackson laboratories (Bar Harbour, Maine). Since there was a huge demand for TRAMP mice, we experienced difficulties in procuring TRAMP mice to commence breeding for our project. This placed several months lag on our project. After we procured the mice, we had set up breeding to have adequate number of TRAMP mice to cross with E6-AP null mice. We experienced difficulties in breeding as the parents gave extremely low litter size and these turned out to be transgene negative and eventually the parents ceased to breed. This led us to place a fresh order of TRAMP mice. These mice are breeding well and

right now I am in the process of crossing these mice with E6-AP null mice. Hence, due to the difficulties in procuring TRAMP mice and due to their failure to breed properly, the research on Aim1 of this project will be accomplished in few months. As soon as I realized these difficulties, I wanted to pursue my research in a similar direction, with a distinct set of aims as mentioned above. Even though I have been a new aim, I will be still accomplishing my earlier aims in the second year of my funding period.

Hence, in addition to our earlier aims, I am proposing a new aim which is similar to the one earlier proposed, but distinct with respect to models and approaches.

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

Aim 2: Generation of E6-AP null TRAMP mice and examine the consequence of loss of E6-AP on prostate cancer development and progression in TRAMP mice.

Aim 3: Generation of E6-AP transgenic TRAMP mice and examine the consequence of overexpression of E6-AP on prostate cancer development and progression in TRAMP mice.

Over the past year, I have accomplished most of the goals of Aim1. 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 tumorigenesis.

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 tumorigenesis

Characterization of E6-AP overexpressing transgenic mice:

We had previously 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. Prostate 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 Phospho-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 level of p-Gsk β , which is a target of activated Akt. We found that p-Gsk β was also elevated in E6-AP transgenic prostate glands.

Over expression of E6-AP increases prostate gland size and leads to precancerous lesions:

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 rise 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=7). These tissues were processed, embedded in paraffin and 5 μ 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 hyperplastic 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.

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

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. 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 μ g/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.

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(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. ³⁵S-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 immunoblotting. 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.

Key Research Accomplishments

1. E6-AP is involved in the growth of the prostate gland as over expression of E6-AP increases prostate gland size.
2. Over expression of E6-AP in the prostate gland leads to the development of PIN like lesions
3. Generation of cell lines which stably overexpress E6-AP in LNCaP cells
4. E6-AP regulates PI3K-Akt pathway in the prostate gland and as well as LNCaP cells
5. E6-AP regulation of PI3K-Akt pathway may be due to regulation of RhoA levels by ubiquitin mediated degradation

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.

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. 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

Figure 1

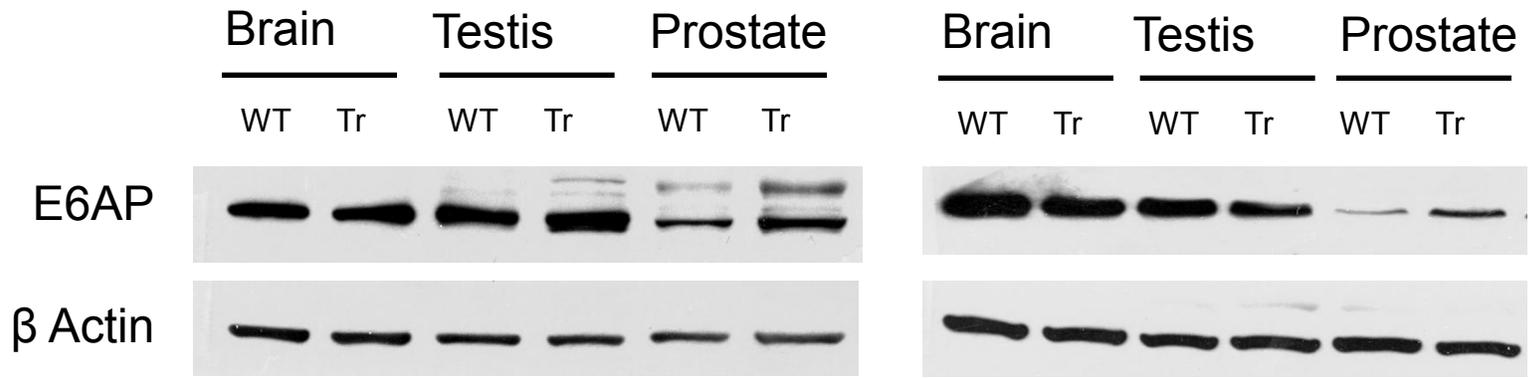


Figure 1: Representative western blots from two lines of E6-AP over expressing transgenic mice showing the expression levels of E6-AP in different organs. E6-AP is specifically over expressed in the prostate glands of transgenic mice. (WT- Wild-type, Tr- Transgenic)

Figure 2

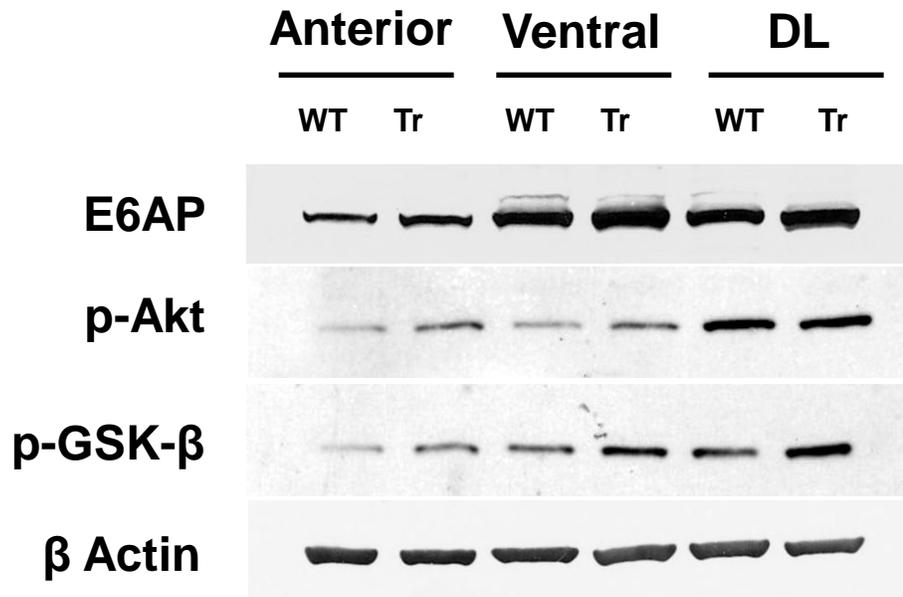


Figure 2: Western blots showing the expression of E6-AP, p-Akt and p-GSK- β in different lobes of the wild-type and transgenic prostate glands. (WT- Wild-type, Tr- Transgenic)

Figure 3

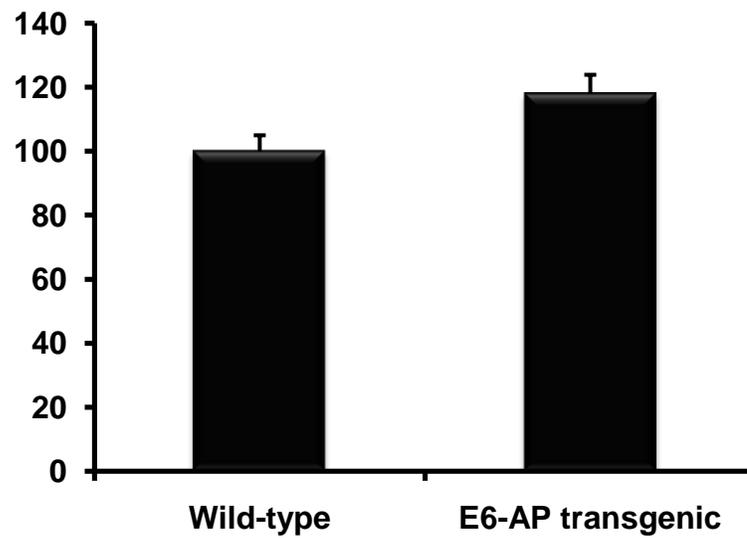


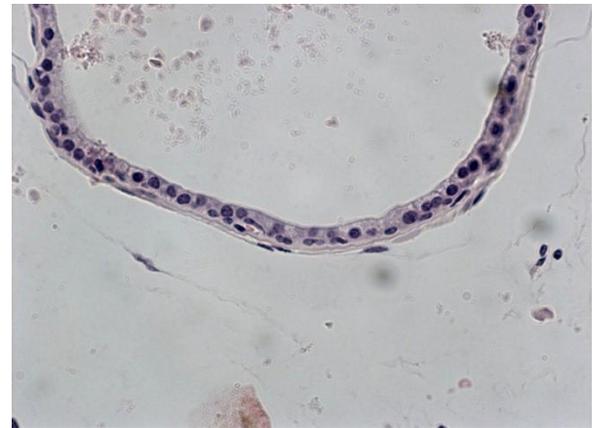
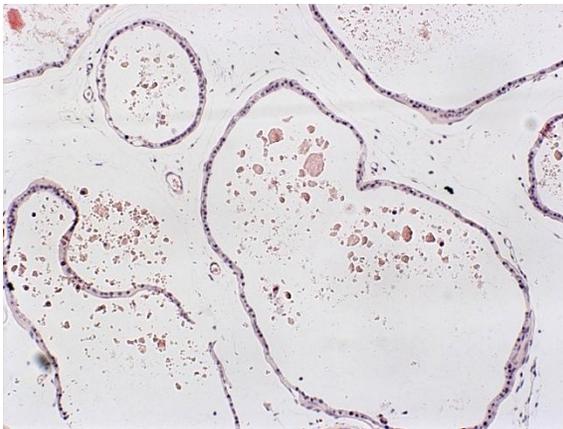
Figure 3: Wet weights of prostate glands from wild-type and E6-AP over expressing transgenic mice. The wet weights are normalized with body weights. n>20

Figure 4

10 X

40 X

WT



TG

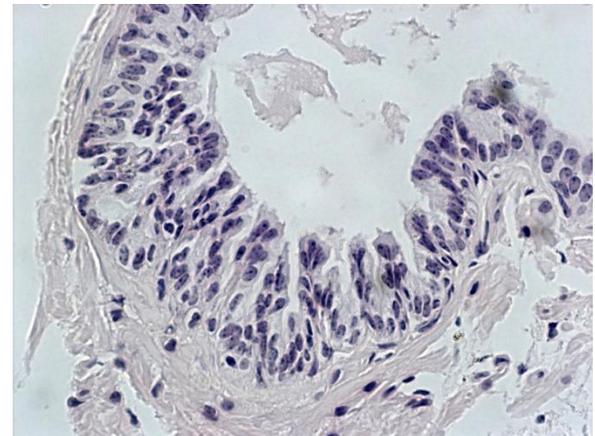
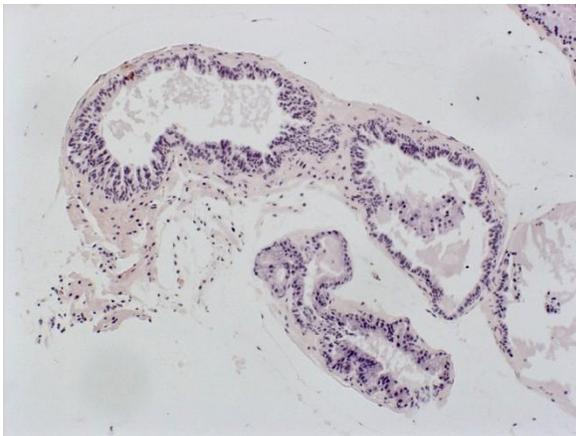


Figure 4: Representative photomicrographs of H & E staining of wild-type (WT) and transgenic mice (TG) showing PIN like lesions only in the transgenic mice.

Figure 5

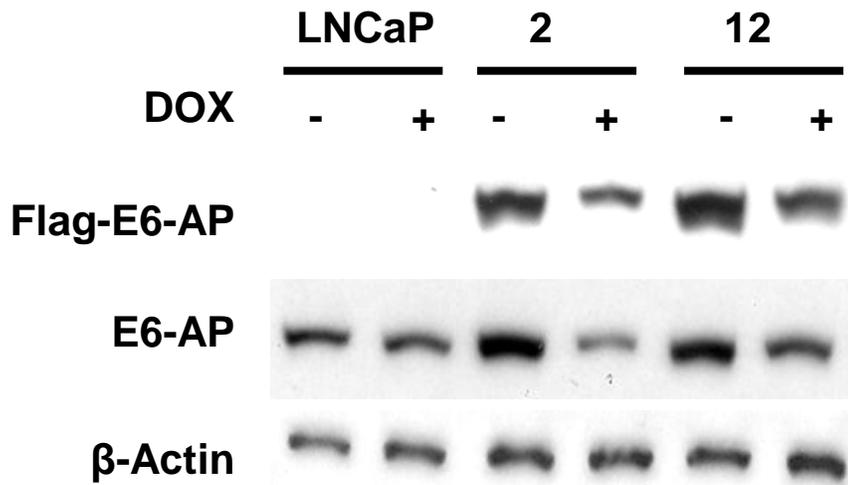


Figure 5: Representative western blots from two lines of E6-AP over expressing stable LNCaP cells lines (2 and 12) showing the expression levels of Flag-E6-AP and total E6-AP in response to Doxycycline (DOX). E6-AP is specifically over expressed under the absence of (DOX)

Figure 6

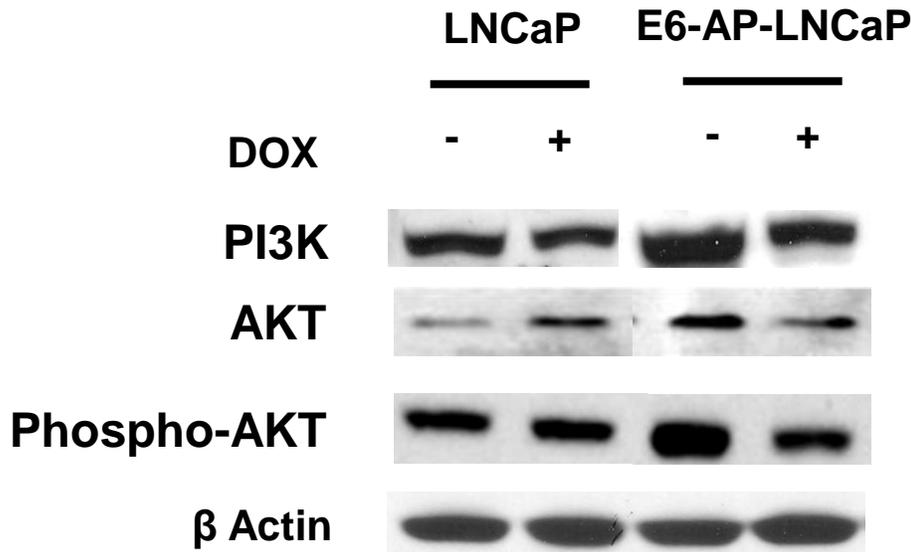


Figure 6: Figure 2: Overexpression of E6-AP induces PI3K-Akt signaling pathway. Parental LNCaP cells and E6-AP-LNCaP cells were treated with or without Dox for 48 hrs. Under inducible conditions (-Dox), the expression of E6-AP is increased in E6-AP-LNCaP cells compared to parental LNCaP cells. Increased 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

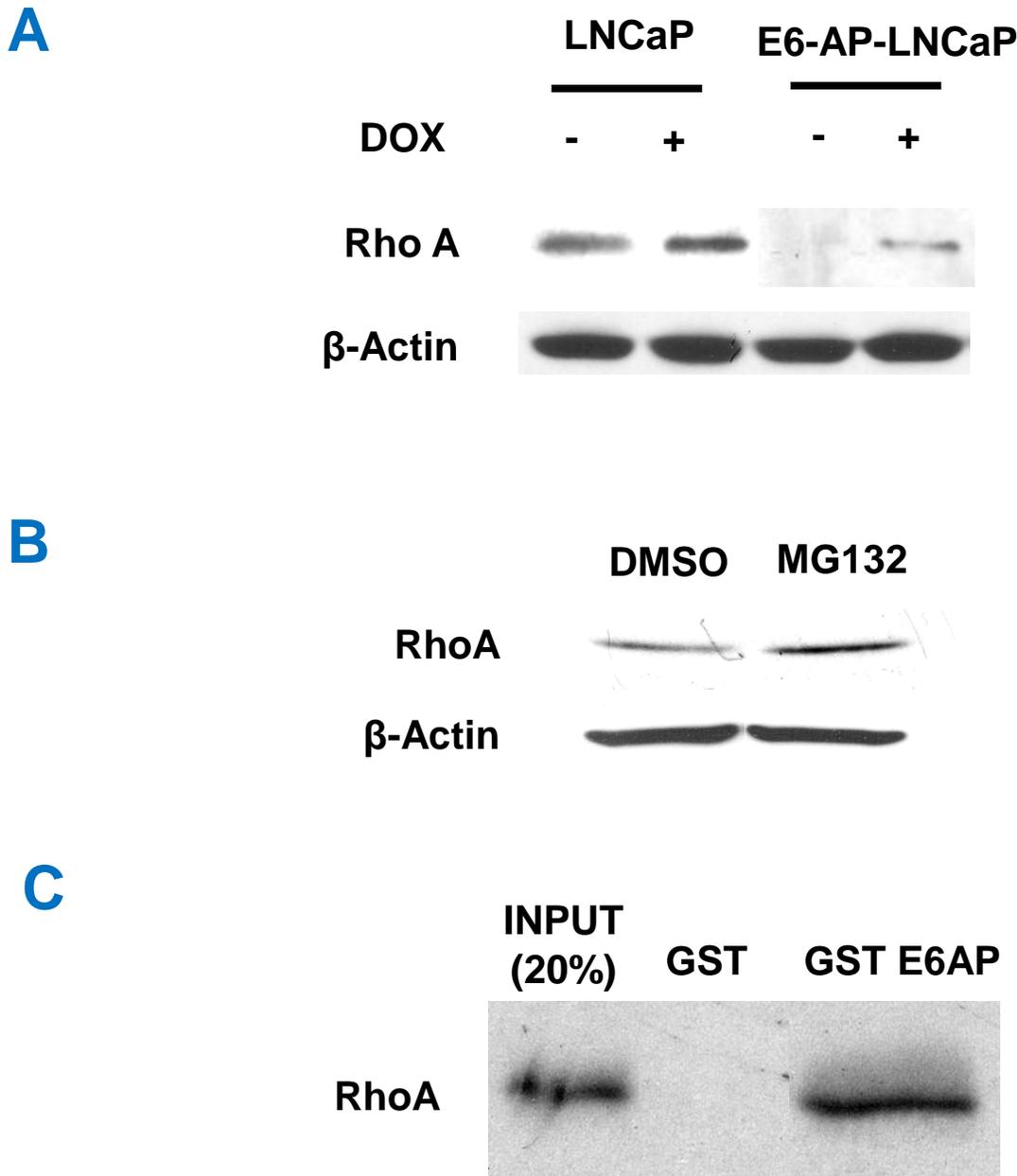


Figure 7: Involvement of RhoA in E6-AP-mediated regulation of Akt activity. (A) Western blots showing that the levels of RhoA are decreased under E6-AP overexpressing conditions (-Dox) in E6-AP-LNCaP stable cells compared to that of parental LNCaP cells. (B) To determine if RhoA is target of the ubiquitin-proteasome 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

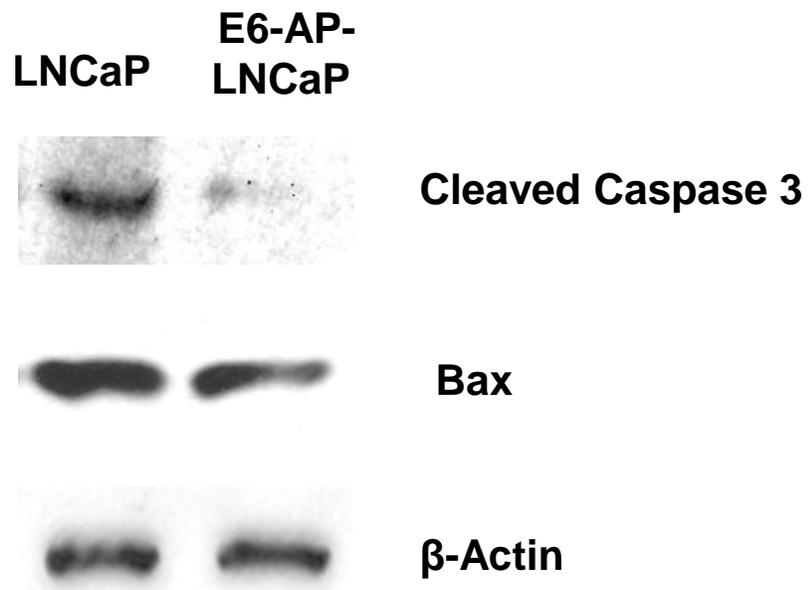


Figure 8: Overexpression of exogenous E6-AP in prostate cells protects cells against apoptosis. Parental LNCaP cells and E6-AP-LNCaP cells were grown in normal serum for twenty four hours and then treated with 100 μ M etoposide for another 6hrs. The cell lysates were analyzed by Western blots using anti-caspase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

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

Abstract Number: 4152

Presentation Title: **Steroid hormone receptor coactivator, E6-associated 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.