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The increase of signal transducer and activator of transcription (STAT6) has been correlated with increased prostate tumor size as well as Gleason score. This molecule's exact role in prostate cancer is still unknown. This research focused on the relationships of STAT6 in prostate specific antigen (PSA) expression as well as its novel interaction with annexin A2. These data show that STAT6 is involved in an alternate PSA expression pathway. It is also concluded that the interaction of STAT6 and annexin A2 increased the activated STAT6 (p-STAT) but not total STAT6. Chromatin immunoprecipitation also confirmed the novel protein-protein interaction between STAT6 and annexin A2 is nuclear.

STAT6 AND ITS RELATIONSHIPS WITH PSA AND ANNEXIN A2 IN HUMAN PROSTATE CANCER

Cherice P. Roth, B. S.

APPROVED:

Major Professor

Committee Member

Committee Member

University Member

Chair, Department of Molecular Biology & Immunology

Dean, Graduate School of Biomedical Sciences

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Cherice P. Roth, B.S.

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Mentor: Jamboor K. Vishwanatha, Ph.D.

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LIST OF ABBREVIATIONS

STAT	Signal transducer and activator of transcription
IL-4/IL-4Ra	Interleukin-4 / Interleukin-4 receptor a
PSA	Prostate specific antigen
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase – polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
PI3-K	Phosphatidylinositol 3'-kinase
Akt (PKB)	Protein kinase B
Jak	Janus Kinase
SH2	Src homology 2 domain
T_H2	T-Helper cell type 2
IgE	Immunoglobulin Epsilon
ANX2	Annexin A2
ANX3	LNCaP cells that express GFP-Annexin A2
PIN	prostate intraepithelial neoplasia

siRNA	Short interfering RNA
ShRNA	Short hairpin RNA
ChIP	Chromatin immunoprecipitation
ARE	Androgen response element
SRE	Steroid response element
CREB	cAMP response element-binding
AP-1	Activator protein-1

CHAPTER 1

INTRODUCTION

Prostate Cancer

The American Cancer society estimates that close to 200,000 men will be diagnosed with prostate cancer in 2008[52]. There are two different methods of diagnosing prostate cancer, the first and most used is a blood test that measures the level of prostate specific antigen[52]. Although an easy test, it is an imperfect method of testing because of the tendency of African American males to have high levels of PSA. An abnormal blood test for leads PSA leads to a rectal examine[11] – which is an uncomfortable and unwelcome test to say the least. In African American males, this high level of PSA leads to a rectal examine, which usually leads to the conclusion of a "false positive" for prostate cancer[53]. Continual false positives in men lowers the chances of them continually being tested. While this may not seem like a crisis, the fact that 55% of African American men will get prostate cancer makes the refusal of continual testing a crisis[54]. There are many aspects of prostate cancer that are poorly understood but one of the major gaps is in trying to understand the signaling differences between the non-metastatic androgen dependent prostate cancers and the metastatic androgen independent prostate cancers. This difference is vital to understand because before the metastasis and androgen independence is established prostate cancer is quite treatable by several methods ranging from anti-androgen therapies

to radial prostatectomies and are rather effective[52]. However, once the cancer has metastasized the treatments have to be tailored towards the whole body and are generally less effective[52]. This work focuses on three proteins that have the potential to uncover part of the mystery behind how these two types of prostate cancer respond to similar stimuli differently. The experiments discussed here after will show that prostate specific antigen, annexin A2, and signal transducer and activator of transcription 6 all have distinct characteristics and interactions based on the androgen dependent status of prostate cancer. Briefly, this work established 1) that STAT6 is involved in PSA expression and 2) that the novel interaction between STAT6 and annexin A2 occurs in the nucleus (Figure 1); but in order to understand the gravity of the findings there are several characteristics of these proteins that need to be understood.

STAT6 Characteristics and Involvement in Prostate Cancer Progression

Many cytokines, hormones and growth factors use STAT signaling to regulate various biological responses. These responses include cell proliferation, differentiation, development, and survival. In a wide array of human cancers, constitutive activation of STAT signaling has been observed[1]. Classically, STAT6 is activated through tyrosine phosphorylation (Y641) by interleukin-4 (IL-4) signaling[2]. IL-4 binds to its receptor IL-4Ra, which phosphorylates Janus Kinases 1 & 3 (Jak1 and Jak3). Jak1 & Jak3 phosphorylate STAT6 initiating its SH2 domain homodimerization. This homodimerization allows for the translocation of STAT6 to the nucleus for gene transcription. Besides cytokines, other proteins

can activate STATs. For example, activation of STAT5 by type IV collagen has been reported in MCF7 human breast cancer cells[3]. Another report showed activation of both Jak2 and STAT6 in response to angiotensin II in cardiomyocytes[4].

STAT6 activity in prostate tumors was shown to be significantly higher than matched normal tissues adjacent to tumors[5]. This is also true for the tissues of normal prostate donors[6]. Identification of STAT6 as one of the robust marker genes for prostate cancer was observed by integrating microarray datasets from three different prostate cancer studies[7]. STAT6 signaling is a major contributing factor towards an immunological T_H2 biased tumor microenvironment[9]. T_H2 polarization by STAT6 mediated signaling has a major role in immunosurveillance by dampening anti-tumor immunity (generally anti-tumor immunity is cytotoxic and driven by T_H1 cells), which favors tumor metastases[8-10]. Understanding the primary role of the STAT6 pathway in prostate cancer may lead to great insight into the ability of prostate cancer to progress.

PI3'-Kinase Signaling is the Known Mechanism for Prostate Specific Antigen Secretion

Currently, there are two ways of diagnosing prostate cancer in the early stages: the first way is a digital rectal examination (DRE) which consists of a physical examination of the patients' rectum, the second being a blood test for the prostate specific antigen (PSA) levels[11].

In androgen dependent prostate cancer, IL-4 binds to its receptor, IL-4Ra, this leads to the phosphorylation of several receptor associated kinases including Jak 1, Jak 3, and IRS-1/2[12]. The IRS1/2 proteins interact with SH2-containing signaling proteins including the p85 regulatory subunit of phosphatidylinositol 3'-kinase[13]. This activates the PI3K/Akt pathway leading to the androgen receptor translocation to the nucleus and finally allows for the transcription of PSA via the genes androgen response element (ARE)[14]. Although IL-4 enhances PSA secretion and activates STAT6, no clear relationship between STAT6 and PSA is known.

Response Elements Located on the PSA Enhancer Region

The enhancer region of the PSA gene is located at -5824 (Accession Number U37672)[15]. The PSA enhancer region contains a SRE/ARE, an AP-1, two other ARE sites, and a CREB binding sequence[15]. Although it requires further confirmation, *in silico* analysis discovered that a STAT6 binding sequence (5' TTC-$N_{3,4}$-GAA 3'[16, 17]) is present in the PSA enhancer region (Figure 2) and is located a site -5241. The presence of a STAT6 DNA binding site on the PSA enhancer region and the ability of PSA to be secreted in response to IL-4 lead to the hypothesis that STAT6 is involved in IL-4 mediated PSA secretion. With IL-4 acting as the initiator molecule for both the PI3K pathway and the STAT6 pathway the possibility of a relationship between STAT6 and PSA cannot be ignored and this relationship was explored for this study.

Annexin Family of Proteins

Annexins are a family of calcium and phospholipid-binding proteins implicated in a number of cellular functions[19, 25, 26, 31]. This family is structurally characterized with a variable N-terminal domain and a conserved C-terminal domain. The variable N-terminal domains are thought to be responsible for the diverse molecular functions of annexins. The C-terminal domain is formed by four or more repeats of approximately 70 amino acids. This C-terminal domain contains the calcium binding sites and is responsible for phospholipid binding[18, 19]. Annexin A2 is an important member of this calcium binding and membrane-associated family of proteins. Annexin A2 is abundantly expressed in most cancers[20-31]. Elevated levels of annexin A2 in cancers supports its important roles in cell proliferation, extracellular matrix organization, migration, fibrinolysis and neoangiogenesis[25, 32-34]. Vishwanatha et. al. has reported a role of annexin A2 monomer in cell proliferation and DNA synthesis[28,32,33]. They have also shown that nuclear annexin A2 is phosphorylated in a cell cycle-dependent manner[35] and that aberrant accumulation of nuclear annexin A2 retards proliferation of LNCaP cells[33]. Annexin A2, as a suppressor of prostate cancer cell migration has also been reported[36].

Annexin A2 knock-out mice show changes in regulation of microvascular fibrin homeostasis and macrovascular clearance of thrombi, and show markedly impaired postnatal, angiogenesis due to the failure of plasmin localization and

activation of matrix metalloproteinases (MMPs), MMP-9 and MMP-13[34]. Directed migration was also reduced in annexin A2 null endothelial cells[34].

Annexin A2 expression is lost in prostate cancers and in a majority of prostate intraepithelial neoplasia (PIN). This loss of annexin A2 appears to be specific for prostate cancer[29-31]. Among the human prostate cancer cell lines, LNCaP cells are null for annexin A2 expression. The other androgen-dependent cell lines MDA PCa 2A and MDA PCa 2B either lack annexin A2 or show greatly reduced annexin A2 protein levels. In contrast however, the androgen-independent PC-3 and DU-145 cells express abundant annexin A2[30]. Over expression of annexin A2 is observed in other human cancers, including lung, pancreatic and breast cancers and brain tumors[37].

Annexin A1, another member of the annexin family suppresses proliferation of human esophageal tumor cells and loss of Annexin A1 leads to uncontrolled proliferation of epithelial cells[38]. Annexin A7 expression is significantly reduced in both metastatic and locally recurrent hormone refractory prostate cancers. Annexin A7 also suppresses prostate cell proliferation *in vitro*[39]. Interestingly, metastatic prostate cancer cells regain annexin A2 expression[30]. Loss of annexin A2 expression in androgen dependent prostate cancer and its subsequent reappearance in the hormone refractory stage suggests an important function of annexin A2 as a stimulator of prostate cell proliferation. Annexin A2 may

function as both an inhibitor of primary prostate cancer and a stimulator of metastatic prostate cancer.

The lack of clear mechanistic studies on annexin A2 regulation in prostate cancer progression led to the search for novel interactors. The interaction of STAT6 with annexin A2 in yeast two hybrid screening system (Figure 3) observed in our laboratory formed a basis to further characterize this novel interaction using over expression and down regulation experiments carried out in prostate cancer cells during present studies.

This work **hypothesized** that STAT6 is **involved in the IL-4 mediated PSA expression and that STAT6 activity is regulated by the annexin A2 interaction as prostate cancer progresses to an androgen-independent state.**

CHAPTER II

MATERIALS AND METHODS

Cell culture: LNCaP and DU145 cells were cultured in RPMI 1640 containing 7% fetal bovine serum (FBS) (*Gibco-BRL, Rockville, MD*), 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37° in a 5% CO_2 cell culture incubator. LNCaP cells with stable annexin A2 expression, polymerase chain reaction (PCR) amplified full length annexin A2 cDNA was cloned as a BamHI-NheI fragment into the pEGFP-C1 vector (Clontech, CA, USA). Selection was achieved by using the aminoglycoside, G418 (Sigma, MO, USA) at a concentration of 0.5mg/ml. One of the stable clones expressing annexin A2 as a GFP fused protein was named as ANX-3 cell line. Expression of GFP fused annexin A2 and GFP proteins in these stable cell lines were confirmed by western blot and immunocytochemical analyses. The ANX-3 cells were maintained in complete RPMI 1640 medium containing 7% fetal bovine serum (FBS) (*GIBCO, Invitrogen, Carlsbad, CA, USA*), 100 I.U./ml penicillin and 100ug/ml of streptomycin.

SiRNA Transfection- The STAT6 siRNA were purchased from *Dharmacon* (*Lafayette, CO*). To obtain effective silencing of STAT6 expression, the STAT6 SMARTpool siRNA reagent were used, which is a combination of four SMART selection-designed STAT6 siRNAs in a single pool. Transfections were done

according to the manufacturer's instructions using Dharmafect 1 as the transfection reagent (*Dharmacon, Lafayette, CO*). The day before transfection the cells were seeded at a density of 80,000-100,000 cells into 6 well plates in RPMI 1640 media containing 5% FBS without antibiotics. The following day, transfections of cells with siRNA were performed. STAT6 siRNA were used at a fixed effective concentration (100nM). The cells were incubated overnight in the presence of a pool of STAT6 SiRNA in RPMI media without antibiotics. The plates were incubated at 5% CO2 and 37°C for different time intervals. Two methods of control were used in the siRNA experiments. The controls consisted of cells in medium without siRNA but containing the transfection reagent to rule out any possible cytotoxic effect from the transfection reagent. Lastly, non-targeting SiRNA (*Dharmacon*) was used to be certain that the addition of non-specific RNA to the system would not mimic the STAT6 SiRNA results. After the incubation period in the presence of the SiRNA,, the cells were placed in full media for 72 hours. The cells were then washed once with PBS and Trizol was added in order to isolate RNA. The total RNA was then amplified using Reverse Transcription –Polymerase Chain Reaction (One-Step superscript -*Invitrogen*) using custom primers specific for STAT3, STAT5, and STAT6 (Table 1)(*IDT DNA*) . RT-PCR of b-actin was used as the loading control.

Nanoparticle down regulation of Annexin A2 – DU-145 cells were treated in normal media with either 500μg nanoparticles loaded with SH annexin A2 or

500μg blank nanoparticles. The nanoparticles were formulated by Dr. Arthur Braden[51].

Chromatin Immunoprecipitation – Approximately 10^6 cells from a T-25 flask were used for the Chromatin immunoprecipitation (ChIP). The DNA was cross-linked at 37°C by adding 1% paraformaldehyde to the media for 10 minutes. The cells were washed with ice cold PBS and lysed in the presence of a protease inhibitor (PI) cocktail. After collecting the cells by scraping the flask, they were then centrifuged for 4 minutes at 2000rpms. The cells were then lysed (in the presence of PI cocktail) in a buffer containing 1%SDS, 10mM EDTA, and 50mM Tris (pH 8.1). The lysate was sonicated on ice until the mixture no longer appeared viscous (Figure 4). The lysate was diluted in the ChIP dilution buffer (.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris at pH 8.1, and 16.7mM NaCl) and centrifuged for 10 minutes at 4°C and 13,000 rpm. After the centrifugation 5ug of either the annexin A2 or control IgG then left to rotate at 4°C overnight. This incubation was followed by the addition of Protein A/G beads with rotation at 4°C for 1 hour. The mix was centrifuged at 700rpm and the bead-protein-DNA complex endured a number of washes. One wash each in a low salt wash (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris at pH 8.1, and 150mM NaCl), a high salt wash (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris at pH 8.1, and 200mM NaCl), and a LiCl wash (0.25M LiCl, 1% NP-40, 1% deoxycholic acid, 1mM EDTA, and 10mM Tris at pH 8.1). The bead complex was washed twice with a TE buffer (10mM Tris at pH 8.1). Following

the washes the DNA could be eluted from the beads, this was done by vortexing the beads with an elution buffer. The beads were then diluted in SDS sample buffer for western blot analysis and the DNA portion was set aside for cross-linking reversal. The cross-linking was reversed by adding 20µl of 5M NaCl, and incubating the mixture at 37°C. The DNA was removed from the suspension via phenol::chloroform extraction.

Phenol::Chloroform Extraction – The phenol::chloroform extraction was initiated by adding an equal volume of phenol::chloroform to the mixture. This was vortexed for 30 seconds and spun down at room temperature for 5 minutes. The aqueous phase that remained contained the DNA with trace amount of phenol. The excess phenol was removed by adding an equal volume of chloroform and centrifuging a second time for 3 minutes. The aqueous phase was moved to a new tube, 2.5 volumes of ice cold 100% ethanol was added to the mix which was incubated at -20°C for 30 minutes. After the incubation, the precipitant was collected by centrifugation at max speed for 10 minutes in 4°C. The pellet was washed in 70% ethanol and allowed to air dry. The DNA was re-suspended in TE made with nuclease free water and frozen for polymerase chain reaction (PCR) amplification.

Polymerase Chain Reaction – The PCR methodology was performed according to the *Promega* protocol for the One Step PCR kit. The IgE promoter primers

used can also be seen in Table 1 and yielded a product of 174 base pairs. Equal amounts of DNA were added to the PCR mixture. DNA concentrations were measured using the Pharmacia GeneQuant RNA/DNA calculator. The PCR program was 50°C for 30 minutes, 94°C for 2 minutes, 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 1 minute for 40 cycles with a final 15 minutes extension at 72°C. Fifteen microliters of each PCR product was taken for electrophoresis in 1.5% agarose gel and the gel was stained with ethidium bromide (10μg/ml). The gel was visualized with UV light and photographed under gel documentation chamber.

Enzyme Linked Immunosorbent Assay – Approximately 80,000-100,000 LNCaP cells were plated and grown in regular growth conditions for 2 days. On day 3, the medium was changed into serum-free medium. Twenty-four hours later, the cells are treated with either 50ng of IL-4 (diluted in water), 20μM LY294002 (diluted in DMSO), or both. This concentration was selected based on the IL-4 response in LNCaP cells dictated by published data[14]. The 20μM concentration of LY294002 is the concentration recommended by the manufacturer. The control cells are not treated but the culture contains 1% DMSO. The cells were then incubated another 24 hours. Twenty microliters of media, from each treatment well was removed and loaded in the PSA ELISA kit (*DSLabs*) in triplicates to determine PSA concentrations. In the assay standards, controls and samples were incubated in 96 wells following the manufacturer's

instructions. Each original experimental sample was assayed in triplicate. PSA values (ng/mL) were determined using log-log curve fit to standard curve.

Computer Analysis – Statistical analysis for all studies were performed using SPSS. Microsoft Excel 2007 was used in order to generate graphs. The densitometry and fluorescence intensity measurements were both measured using the ImageJ software. *In silico* identification of the STAT6, binding site on the PSA enhancer region was completed using Microsoft Word 2007. Schematic diagrams were created using BioDraw.

CHAPTER III

RESULTS

PI3K inhibition does not abolish PSA secretion

PSA secretion is controlled by the PI3 kinase pathway via NF-κB. In order to explore the possibility of a secondary pathway involved in PSA expression initial experiments were conducted to test whether or not PSA secretion is inhibited by the known inhibitor of PI 3 kinase pathway. When LNCaP cells were treated with 20μM LY294002 (LY), a PI3K inhibitor, PSA secretion was not abolished. The treatment of LY alone and IL-4 with LY significantly decreased ($p=0.05$) the amount of PSA secreted (Figure 5).

There are no studies that explore the PSA secretion differences between androgen depended and independent LNCaP Cells. In order to find out if androgen independent LNCaP cells secrete PSA at higher levels than androgen dependent LNCaP cells both cell types were treated with IL-4 alone, LY alone or both IL-4 and LY together. There were significant increases in PSA secretion in LNCaP C42 cells in all treatment categories (Figure 5). The control population of androgen independent LNCaP cells secreted significantly more PSA. The increase in PSA secretion was the opposite response seen in the low passage, androgen dependent LNCaP cells. PSA secretion in LNCaP cells was not

abolished upon the addition LY that opened the possibility of STAT6 being involved in PSA gene transcription.

STAT6 SiRNA down regulation effects on PSA

PSA secretion was not eliminated in the presence of the PI3K inhibitor but to establish whether STAT6 was involved in the gene transcription of PSA changes in PSA mRNA were evaluated upon STAT6 down regulation. Significant ($p \leq 0.001$) siRNA down regulation of STAT6 showed significant increase ($p=0.002$) in PSA in the low passage LNCaP cells (Figure 6). This increase was not seen in androgen independent LNCaP cells. The similarities in other STAT sequences lead to the control PCR to evaluate whether STAT6 siRNA was also down regulating STAT3 and STAT5 (Figure 7). There were no significant changes to the other STATs by the STAT6 siRNA.

STAT6 and its Novel Interaction with Annexin A2

Chromatin Immunoprecipitation of Annexin A2 Yields STAT6 Specific Promoter

Immunoprecipitation and immunocytochemistry both support the novel interaction of STAT6 and annexin A2 (Roth, CP, submitted). However, the novel interaction of STAT6 and annexin A2 has yet to be assigned a mechanism. In order to find out whether this interaction takes place in the nucleus of prostate cancer cells a chromatin immunoprecipitation (ChIP) was performed. The annexin A2 antibody was used for the ChIP. The protein fraction yielded STAT6 protein by

western blot analysis. The PCR analysis yielded the gene product for the IgE promoter in DU-145 cells (Figure 8). The primary population of STAT6 in the nucleus is phosphorylated which lead to the question of whether annexin A2 affects p-STAT6.

Over-expression of Annexin A2 Increases p-STAT6

The ChIP yielded the IgE promoter region, which is a target of transcription by p-STAT6. The previous findings lead the question of whether annexin A2 expression affects levels of p-STAT6. The consequences of annexin A2 over expression in LNCaP cells were analyzed using immunocytochemistry. ANX3 cells and normal LNCaP cells were exposed to antibodies for total STAT6 and p-STAT6. After the appropriate secondary antibodies were added the resulting fluorescence was analyzed for intensity using the ImageJ software. All of the images were taken at the same settings so that the fluorescence intensity could be accurately compared. ImageJ software measured a significant ($p = 6.93E-06$) increase in phosphorylated STAT6 depicted in Figure 9. Although there was a slight increase in total STAT6, this change was not significant ($p=0.22$). The over expression of annexin A2 increased p-STAT6 so it was also important to establish the consequences of annexin A2 down regulation in relation to STAT6 expression.

SiRNA down regulation of Annexin A2 decreases STAT6

In order to establish the consequences of annexin A2 down regulation in relation to STAT6 expression, DU-145 cells were treated with annexin A2 specific Sh-

loaded nanoparticles in order to down regulate annexin A2. A ~60% decrease of annexin A2 mRNA led to a ~17% decrease in STAT6 mRNA ($p \leq .003$). The down regulation of annexin A2 did not alter mRNA for STAT3 or STAT5 (Figure 10). On the protein level, a significant decrease in annexin A2 protein also led to a significant ($p=0.005$) decrease in STAT6 protein by western blot analysis.

CHAPTER IV

DISCUSSION

STAT6 and its Role in PSA Expression

PI3K inhibition does not abolish PSA secretion

The ability of the PI3K pathway to stimulate PSA secretion in the presence of IL-4 has been established in LNCaP cells[14]. A major issue with this work is that Lee et. al. failed to describe the androgen status (usually determined by the passage number)[14]. Studies have shown that as the passage number of LNCaP cells increase the population moves toward androgen independence[40]. The first portion of this work aimed to first determine whether STAT6 was involved in the PSA expression, and second whether there is a difference in the secretion profile of PSA in relation to the androgen dependence of LNCaP cells. According to Lee et. al. The treatment of LNCaP cells with IL-4 increased PSA secretion while treatment with IL-4 with the PI3K inhibitor LY decreased this response significantly but did not return the secretion to basal levels[14, 41]. This secretion profile however mirrored the results in Figure 4 in low passage LNCaP cells. A completely different response was seen in the androgen independent LNCaP C42 cells. The absence of androgens decreases PSA in the androgen dependent LNCaP cells, but LNCaP C42 cells high very high constitutive levels of PSA even

though they maintain AR function[42]. This is demonstrated in Figure 5 as well however, the constitutive activation increase in the presence of IL-4 and increased a second time with LY alone. This supports that LNCaP cells have an alternate pathway for PSA secretion. These data suggests that this pathway is stimulated by IL-4 but is not negatively affected by the inhibition of the PI3K pathway. This leaves STAT6 as an excellent candidate. In order to deduce the possible role of STAT6 in PSA secretion it was vital to observe the system without the interaction of STAT6 using siRNA down regulation.

STAT6 SiRNA down regulation effects on PSA

STAT6 down regulation allowed for the pinpointing of its role in PSA secretion in LNCaP cells. RT-PCR of PSA mRNA showed a significant increase with the down regulation of STAT6. This is explained by the complex interaction of NF-kB, a known transcription factor for PSA with STAT6[41]. Although activated STAT6 binds NF-kB, the relationship is multifaceted[43]. There is a clear decrease in the efficacy of NF-kB when STAT6 is able to bind to the same gene[43]. The decrease in STAT6 via siRNA may have allowed NF-kB to bind better to its target DNA sequence on the PSA gene. This explains the increase in PSA mRNA, in low passage LNCaP cells, when STAT6 is absent (Figure 6). However, the increase in PSA mRNA is not significant in the LNCaP C42 cells, possibly due to the constitutive high expression of PSA in these cells, perhaps at its maximum level. These data strongly suggest that STAT6 may be involved in LNCaP PSA expression and warrants further investigation to elucidate the entire process.

STAT6 and its Novel Interaction with Annexin A2

Chromatin Immunoprecipitation of Annexin A2 Yields STAT6 Specific Promoter

Researchers in the Vishwanatha laboratory discovered the novel physical interaction of annexin A2 and STAT6 using both yeast two-hybrid screening and immunoprecipitation (Roth, C. P., submitted 2008). The purpose of this study was to ascertain the cellular location of this novel interaction by first establishing whether this interaction occurs when STAT6 is bound to DNA. This was accomplished using the chromatin immunoprecipitation shown in Figure 8. The IgE promoter precipitated by the annexin A2 antibody supports that this interaction might participate in STAT6 DNA binding. This finding was also supported by an electromobility shift assay (Roth, C. P., submitted 2008). Together, these data strongly suggest that the interaction of annexin A2 and STAT6 is present in the nucleus. This is not surprising because STAT6 interacts with many proteins in the nucleus. Two such co-regulatory molecules are CREB binding protein (CBP) and p300. CBP/p300 complex provides a bridging factor for STAT6 to the basal transcription machinery comprising RNA Polymerase II, RNA helicase and transcription factor IIB (TFIIB)[44, 45]. Another co-activator, p100, was reported to bridge the physical interaction between CBP/p300 and STAT6 and was found to recruit histone acetyltransferase (HAT) activity to STAT6 in vivo[46]. The chromatin immunoprecipitation yielding the IgE promoter adds annexin A2 to this co-activation hypothesis. The data in this report has not been able to ascertain whether it is the monomer, dimer, or heterotetramer, of

annexin A2, that is interacting with STAT6 – which would be an important continuation of this work.

A wide variety of molecules that are regulated by STAT6 mediated transcriptional activation range from cytokines such as IL-4, adhesion molecules such as P-selectin, enzymes such as 15-Lipoxygenase, receptors like MHC-II and opiod receptor to signaling molecules such as Bcl_{XL}[47]. As a result of regulating such a wide variety of genes STAT6 can regulate a diverse range of physiological functions from cell proliferation, differentiation and apoptosis[1]. Annexin A2 may be a part of these functions.

Expression of Annexin A2 affects STAT6 Phosphorylation State

Further characterization of the mechanism for this interaction was done to identify the consequences of annexin A2 over expression on STAT6. Over expression of annexin A2 in LNCaP, cells did not affect the total amount of STAT6 significantly; however, it did increase the amount of p-STAT6 (Figure 9). This again supports that this interaction maybe taking place in the nucleus because p-STAT6 is essentially nuclear. The role of this interaction is still unclear. Additional studies are needed to establish whether annexin A2 is protecting p-STAT6 from proteolysis. It is not well understood, but p-STAT6 is specifically regulated by calpain degradration[49]. *In silico* analysis of the STAT6 protein sequence yielded a possible cleavage site preceded by a possible signal site (Figure 11)[50]. The initial cleavage of a protein can trigger full degradation by the proteosomal pathway[49]. These sites are located in the same position that the annexin A2 interaction is located (Figure 3). It is possible that this novel

interaction may be physically hindering the cleavage of p-STAT6, but further study is needed to support this theory.

After testing the effect of annexin A2 over expression in prostate cancer cells the effect of down regulation of annexin A2 was tested. In DU-145 cells there was a significant down regulation of STAT6 with the down regulation of annexin A2 (Figure 10). This suggests a stability relationship between the two proteins, which is common in proteins that have a physical interaction. It is important to understand how annexin A2 cooperates with STAT6 translocation to the nucleus and whether annexin A2 co-regulates gene expression mediated by STAT6. This interaction has important implications in prostate cancer progression considering the evidence that STAT6 is a robust prostate cancer marker gene and is a survival factor[1, 6, 7] and annexin A2 plays role in angiogenesis and migration[34, 36, 48].

In conclusion, this work aimed to 1) establish whether STAT6 was involved in PSA expression and 2) to determine a possible mechanism of the novel annexin A2 interaction (Figure 1). These data presented in this report supports the hypothesis that STAT6 is a co-transcription factor of PSA. Clinically, examining the prostate cancer cells for STAT6 and NF-kB instead of looking at PSA may give a better idea as to the survival tendencies of the cells which, would lead to a stronger diagnostic tool and hopefully rule out false positives. Further study in this aspect is needed however. These data have also shown strong evidence that the annexin A2 interaction takes place in the nucleus and suggests that this interaction prevents degradation of p-STAT6 (Figure 12). The location of the DNA binding domain and the possible cleavage sites on STAT6 may indicate that annexinA2 is

blocking both the gene transcription capabilities of p-STAT6 as well as the marked increase in the molecule. Future investigations should be directed towards understanding of the role of this interaction in mediating gene expression that may regulate prostate cancer development and progression particularly as it relates to the constitutive activation of STAT6 in prostate cancer.

CHAPTER V

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CHAPTER VI

FIGURE LEGENDS AND FIGURES

Figure 1: Thesis Schematic. This work aimed to 1) establish whether STAT6 was involved in PSA gene transcription and 2) to determine a possible mechanism of the novel annexin A2 interaction.

Figure 2: A potential STAT6 DNA binding is located on the PSA enhancer region. The enhancer region of the PSA gene is located at -5824 (Accession Number U37672)[15]. The PSA enhancer region contains a SRE/ARE, an AP-1, two other ARE sites, and a CREB binding sequence.[15] Analysis of the PSA enhancer region yielded a possible STAT6 DNA binding sequence located at sites -5241 to -5232 (Denoted in red). The STAT6 binding sequence at this site is 5' TTC AGG TGA A 3'.

Figure 3: STAT6 interacts with annexin A2 in a yeast two hybrid screening. **A.** Positive clones were selected on high-stringency medium (synthetic dropout medium) with the selection markers SD/-Ade/-His/-Leu/-Trp/X-α-gal. True interactors only can activate the expression of b-galactosidase leading to the development of blue color. The intensity of blue color also signifies the strength of

any interaction. The right panel shows the positive and negative controls. p53-T antigen interaction was used as a positive control. Annexin A2–p53 interaction served as a negative control and showed no growth of colonies resulting in the blue color. **B.** Sequence analysis results from plasmid DNA of the STAT6 positive clones corresponds to 85 amino acids from the STAT6 protein as indicated by red color. **C.** Schematic representation of the STAT6 interacting domain (red bar) with annexin A2 that lies between coiled coil and DNA binding domains of the STAT6 protein. (This work was carried out by Dr. Sushoban Das)

Figure 4: Flow chart of Chromatin Immunoprecipitation method. A Chromatin Immunoprecipitation starts with the cross linking of the DNA to protein using paraformaldehyde. Next, the DNA/protein complexes are then sheared using sonication. The antibody of interest is incubated overnight in the lysate and then protein A/G beads are added. These beads are precipitated by centrifugation. The cross linking is reversed using a high salt buffer and heat. The DNA is eluted from the mixture and used for polymerase chain reactions (PCR) and the protein fraction is used for western blot analysis.

Figure 5: LNCaP C42 Cells secrete more PSA than low passage LNCaP cells. Low passage LNCaP cells and LNCaP C42 cells were treated with IL-4, LY294002 alone, both IL-4 and LY294002, and DMSO only for the control. PSA levels were determined by ELISA (n=4). There was a significant difference ($p \leq 0.05$) in PSA levels between low passage LNCaP cells and LNCaP C42 cells.

The * denotes a significant difference between cell types at the same treatment level and the ^ denotes a significant difference between the treatments compared to control within the same cell type.

Figure 6: SiRNA down regulation of STAT6 in low passage LNCaP cells increases PSA mRNA. The treatment of LNCaP cells (low passage and C42) with 100nM of STAT6 siRNA resulted in a significant increase ($p=0.002$) in PSA mRNA in low passage LNCaP cells. There was not a significant change in PSA mRNA in LNCaP C42 cells. The density of the RT-PCR band was taken using Image J software and normalized to the amount of b-actin.

Figure 7: Silencing of STAT6 in LNCaP cells (low passage & C42) does not affect STAT3 or STAT5 mRNA. Both LNCaP low passage (Panel A) and C42 (Panel B) cells were treated as above with 100nM STAT6 mRNA with no change seen in STAT3 or STAT5. Densitometric analyses of RT-PCR bands were carried out by Image J software and the values presented were normalized to the density of the b-actin product.

Figure 8: Chromatin immunoprecipitation using the Annexin A2 antibody yields the promoter for IgE. The ChIP showed both the presence of STAT6 in western blot analysis (Panel A) as well as the presence of the IgE promoter, a

known STAT6 DNA target via PCR (Panel B). The X3 for each panel denotes triple the amount of protein and RNA loaded.

Figure 9: Over expression of Annexin A2 increases p-STAT6 without increasing total STAT6. The cell lines shown are ANX-3 and LNCaP. Cells were grown on cover slips in 6-well plates to 70-80% confluency and immunostaining was performed with Alexa flour conjugated secondary antibodies as described in materials and methods. Cells were incubated with the indicated anti-human phosphorylated STAT6 primary monoclonal antibody at 0.2 mg/ml. **A.** There is a significant increase ($p= 6.93e-06$ denoted by *) in the amount of p-STAT6 in ANX-3 cells when compared to the annexin A2 negative LNCaP cells. **B.** The slight increase of total STAT6 in ANX-3 cells is not significant ($p=0.22$).

Figure 10: Short-hairpin down regulation of annexin A2 in DU-145 decreases STAT6. **A.** A fifty percent short-hairpin (SH) down regulation of annexin A2 showed a significant ($p<0.003$) decrease in STAT6 mRNA. No changes were seen in STAT3. DU-145 cells express no STAT5. Lane 1 - the addition of blank nanoparticles and Lane 2 – treatment with SH loaded nanoparticles. **B.** Western blot analysis after SH down regulation of annexin A2 mirrored the significant decrease of STAT6 in DU-145 cells ($p=0.005$), on a protein level. The lanes of the western blot are M (marker), 1 – DU-145 treated with blank nanoparticles and 2 – SH treated DU-145 cells.

Figure 11: *In silico* analysis of the STAT6 protein sequence detects a possible cleavage site in the location of the annexin A2 interaction. The SignalP online server has detected possible cleavage site (C-score denoted by a red peak) as well as a possible signal sequence (S-score denoted by a green peak) just preceding the cleavage site. The blue peak denotes the Y-score, which is a derivative of the C-score combined with S-score. A signal peak preceding a cleavage peak denotes a better cleavage site. This peak complex is enlarged in the bottom corner. The cleavage site close to the 800 amino acid site represents a known cleaved version of STAT6.

Figure 12: Working model based on results. These data suggest that STAT6 is a transcription factor for the PSA gene. STAT6 shares this responsibility with NF-kB but both transcription factors actively binding the gene display STAT6/NF-kB co-suppression. The novel interaction of annexin A2 and STAT6 appears to interfere with the degradation of p-STAT6.

Figure 1: Thesis Schematic

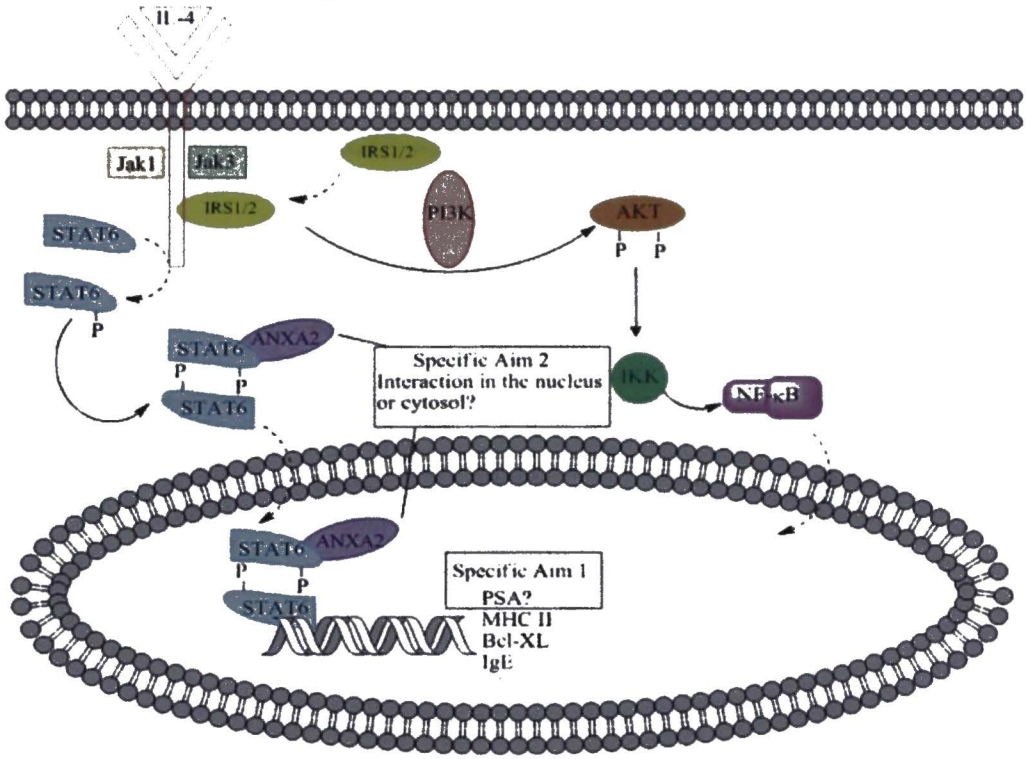


Figure 2: PSA Enhancer Map

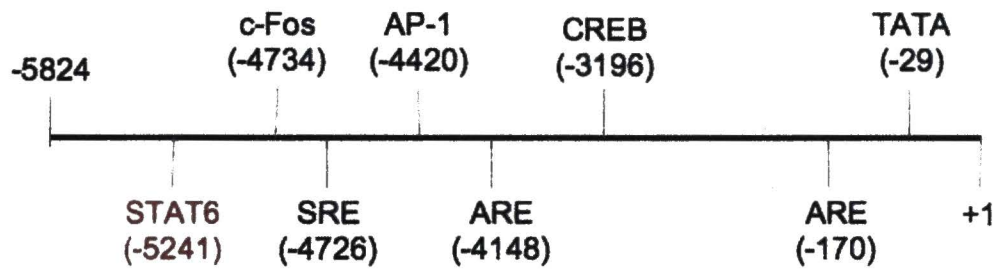
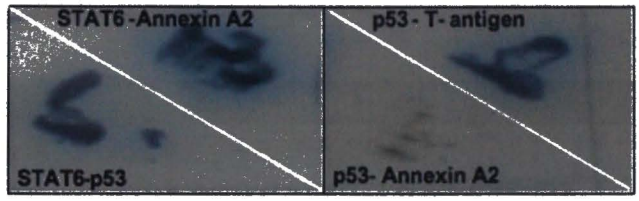


Figure 3: Annexin A2 interacts with STAT6 in a yeast 2-hybrid analysis

A



B

1 mslwglvskm ppekvqrlyv dfpqhlrhll gdwlesqpwe flvgsdafcc nlasall
61 vqhlqasvge qgegstilqh istlesiyqr dplkivatfr qilqgekkav meqfrhlpmp
121 fhwkqeelkf ktgirriqhr vgeihlirea lqkgaeagqv slhslietpa ngtgpseala
181 mllqettgel eaakalvlkr iqiwkrqqql agngapfees laplqerces lvdlysqlqq
241 evgaaggele pktrasltgr ldevlrtlvt scflvekqpp qvlktqtkfq agvrflglr
301 flgapakppl vradm vtekq arelsvpqgp gagaestgei inntvplens ipgnccsalf
361 knllkkikr cerkgtesvt eekcavlfsa sftlgpgklpi qlqalslpl vvivhgnqdn
421 nakatilwdn afsemdrvpf vvaervpwek mcetlnlkfm aevgtnrgll pehflflaqk
481 ifndnslsme afqhrsvsws qfnkeillgr gftfwqwfdg vlditkrclr sywsdrliig
541 fiskqyvtsl llnepdgtfl lrfsdselgg itlahvirgq dgspqleniq pfsakdlsir
601 slgdrirdla qlknlypkkp kdeafrshyk peqmgkdgrg yvpatikmtv erdqplptpe
661 lqmptmvpsy dlgmapdssm smqlgpdmvp qvypphshsi ppyqglspee svnvlsafqe
721 phlqmppslg qmslpfdqph pqgllpcqpq ehavsspdpl lcsdvtmved sclsqpvtaf
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841 iranpsw

C

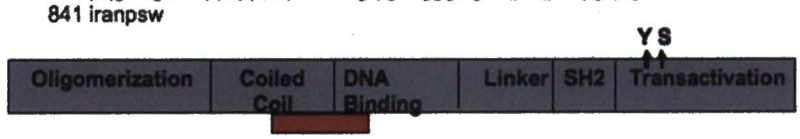


Table 1: Custom Primer Sequences

Gene Name	Primer Sequence (5' > 3')
STAT6 Forward	CTG CCA AAG ACC TGT CCA TT
STAT6 Reverse	GGT AGG CAT CTG GAG CTC TG
STAT5 Forward	CAG GTG AAG GCG ACC ATC AT
STAT5 Reverse	TGC TGT TGT AGT CCT CGA GG
STAT3 Forward	ACC CAA CAG CCG CCG TAG
STAT3 Reverse	CAG ACT GGT TGT TTC CAT TCA GAT
IgE Forward	TGG GCC TGA GAG AGA AGA GA
IgE Reverse	AGC TCT GCC TCA GTG CTT TC
β–Actin Forward	GAG GCT CTC TTC CAG CCT TCC TTC CT
β–Actin Reverse	CCT GCT TGC TGA TCC ACA TCT GCT GG

Figure 4: Flow Chart of Chromatin Immunoprecipitation

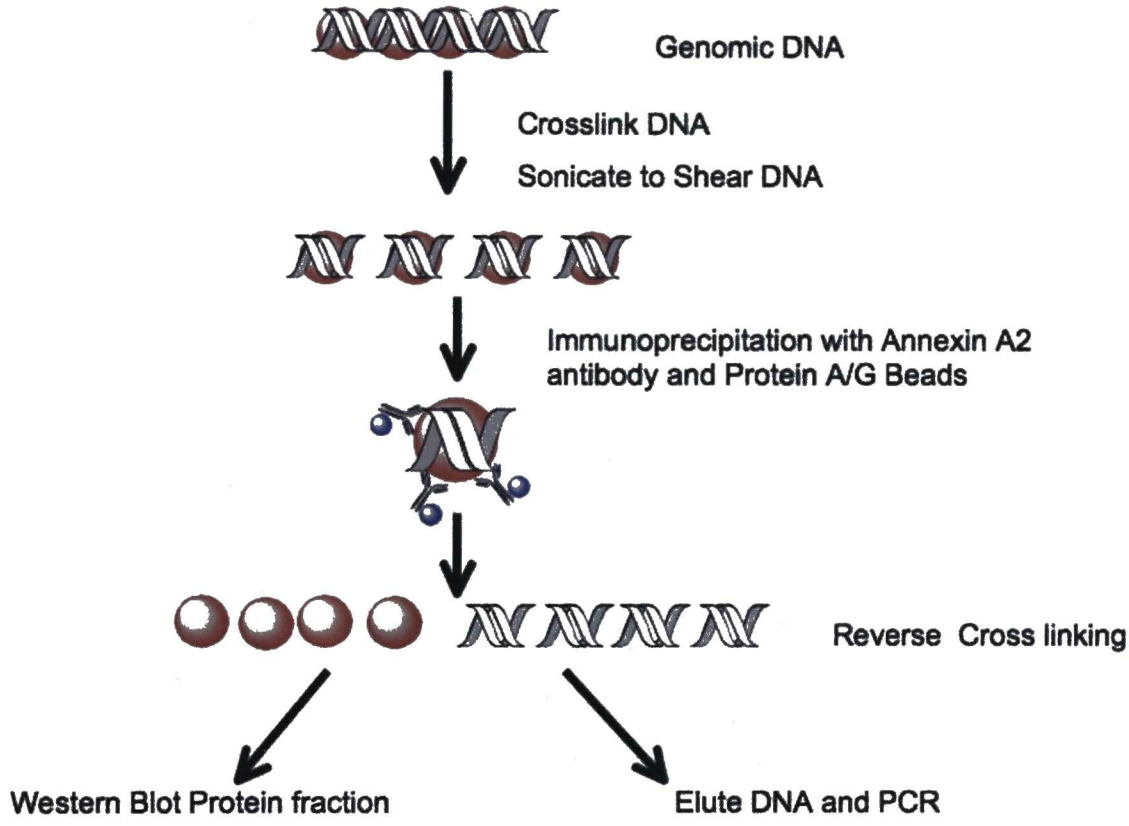


Figure 5: PSA ELISA of increasing passages of LNCaP cells (low passage and LNCaP C42 cells)

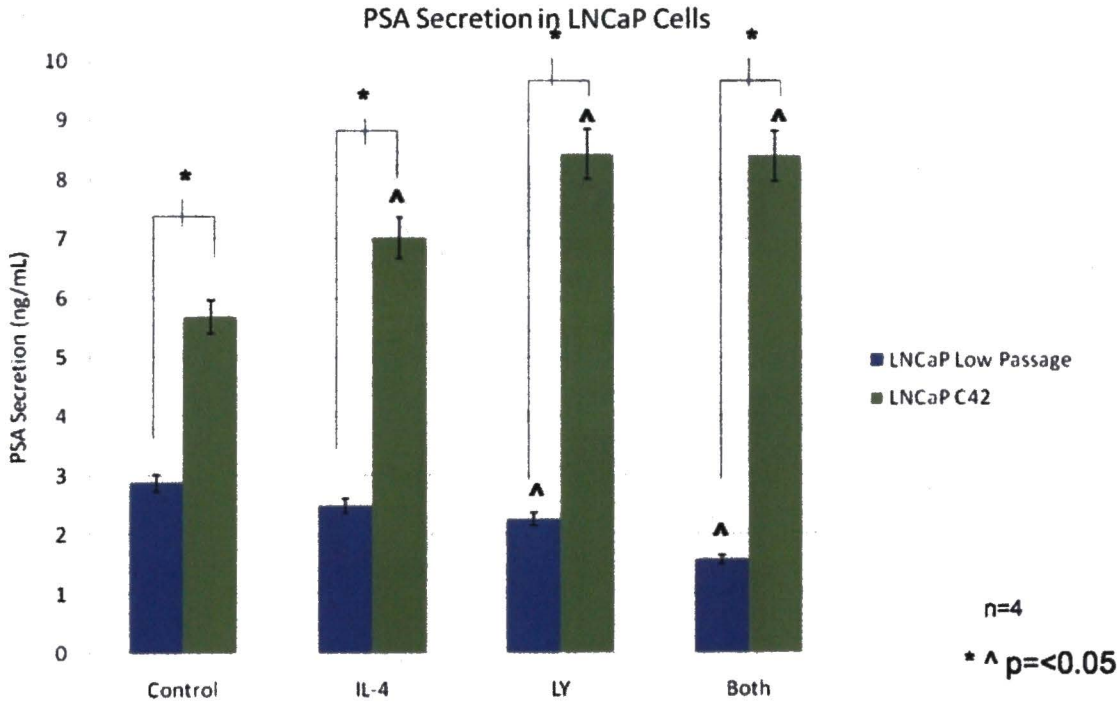
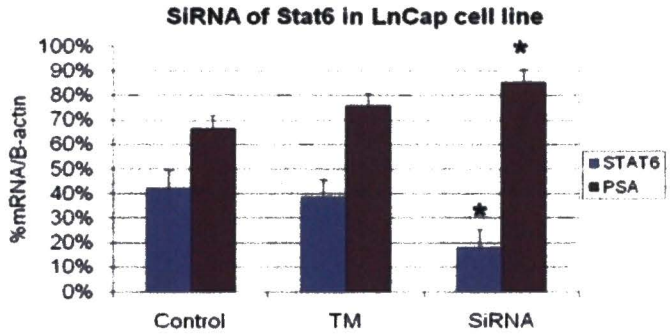


Figure 6: SiRNA Down regulation of STAT6 in LNCaP cells

A



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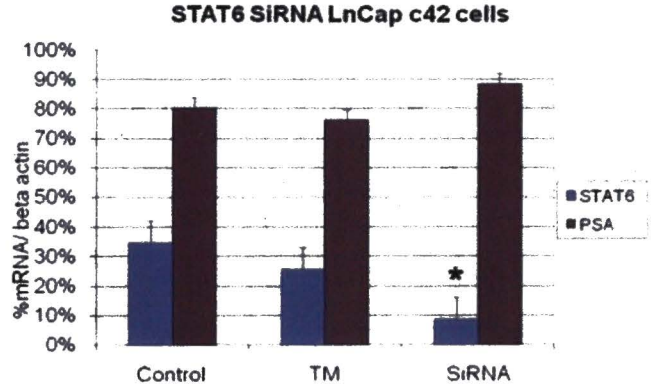
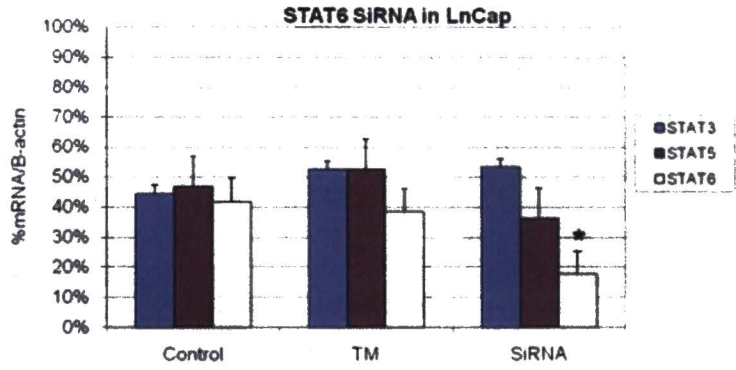


* p= <.001

Figure 7: STAT6 SiRNA does not effect STAT3 or STAT5

A



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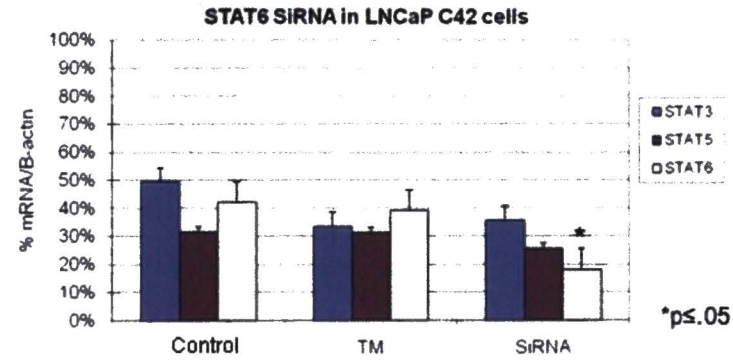


Figure 8: Confirming the novel interaction of STAT6 and annexin A2 using chromatin immunoprecipitation

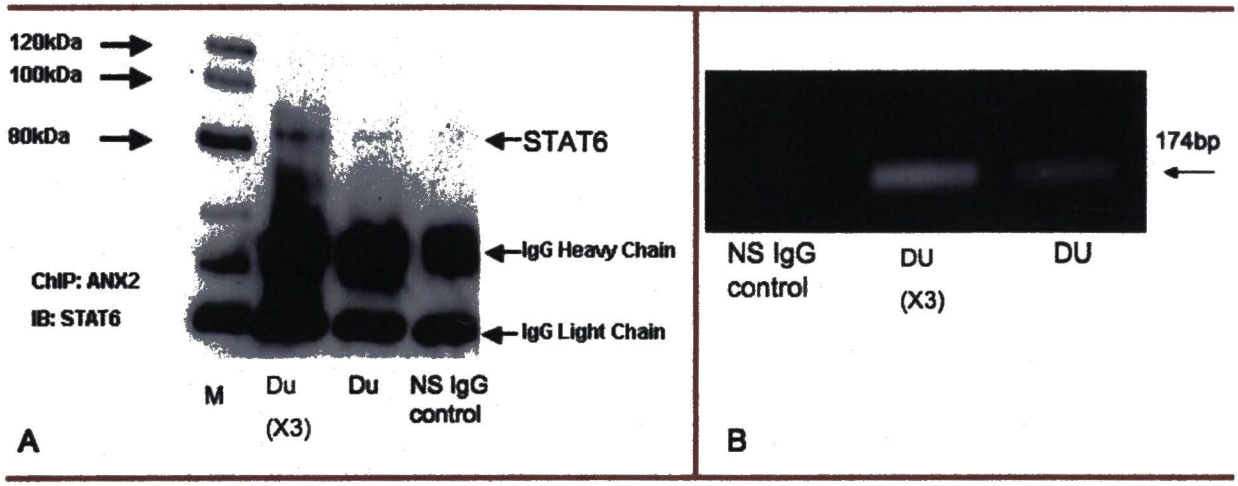


Figure 9: Identifying the effect of annexin A2 over-expression on STAT6 expression and activation using immunocytochemistry

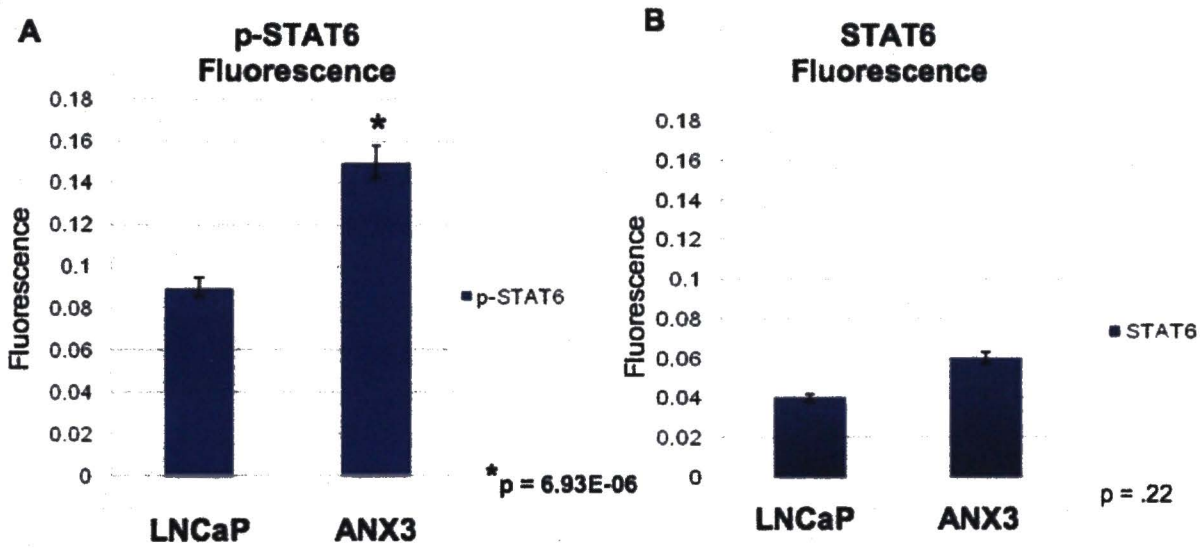
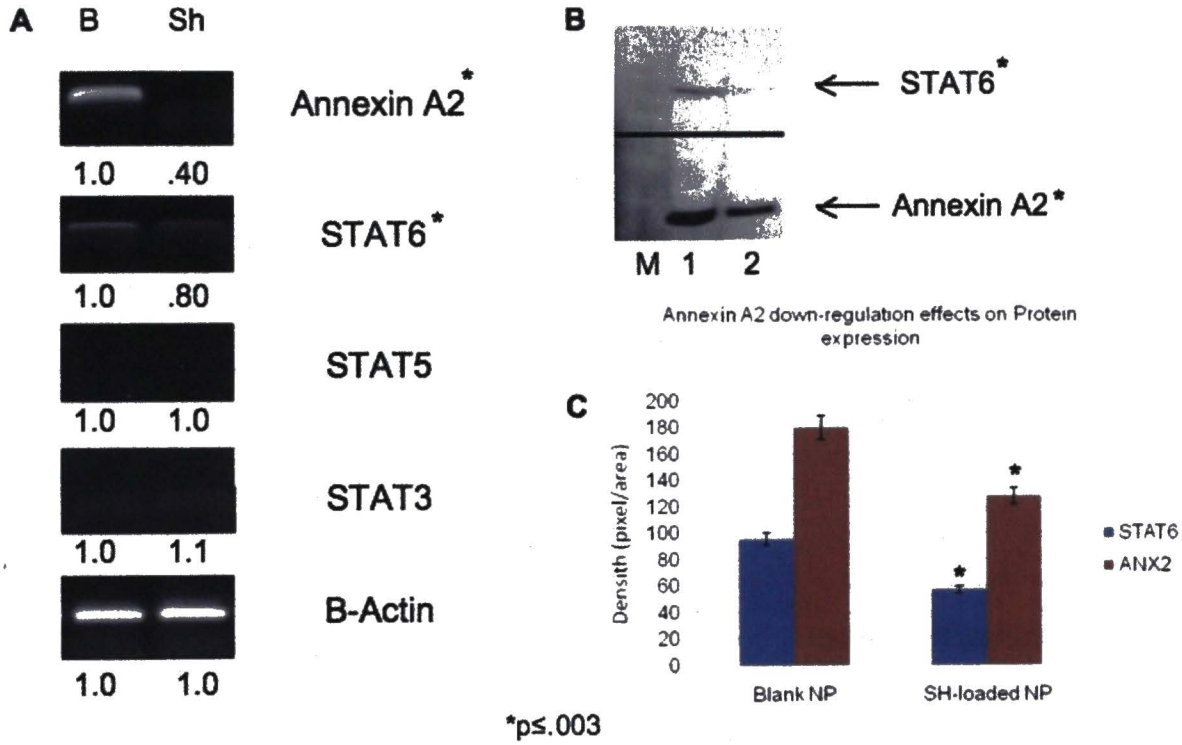
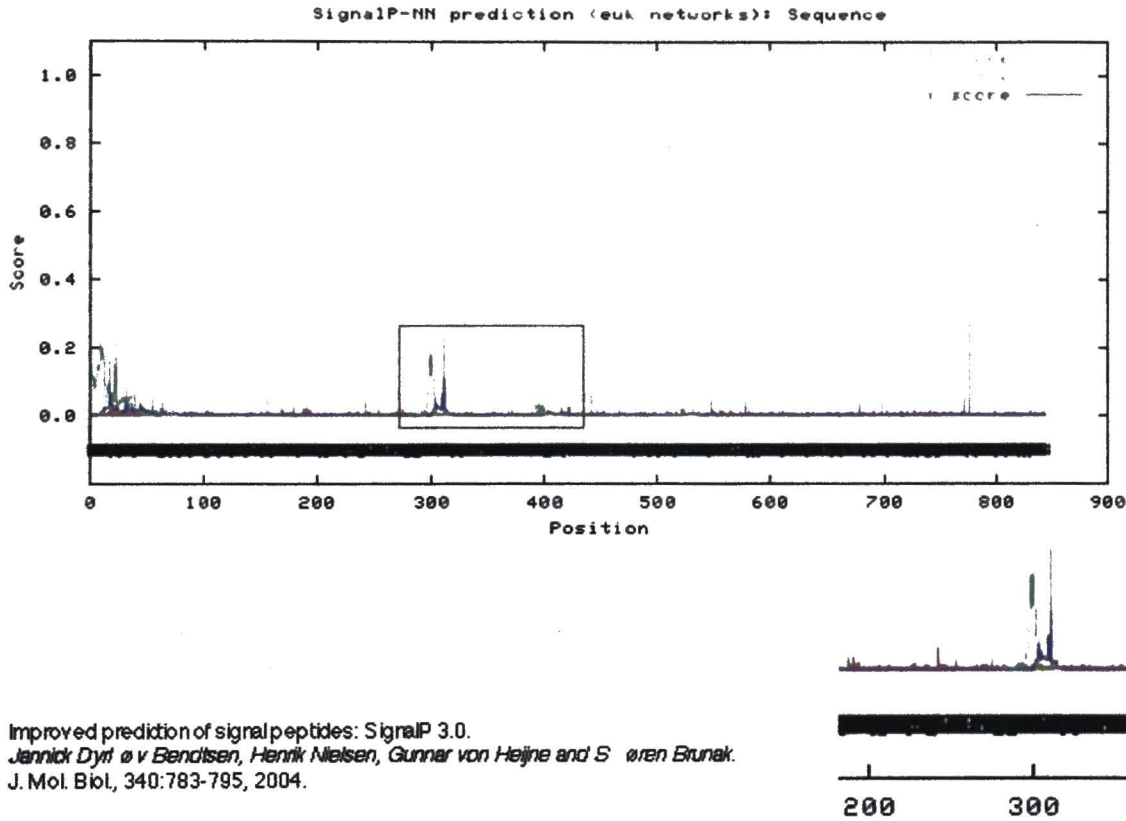


Figure 10: Identifying the effect of annexin A2 down regulation on STAT6 expression



*p≤.003

Figure 11: *In silico* analysis of the STAT6 protein sequence



Improved prediction of signal peptides: SignalP 3.0.
Jannick Dyrl ø v Bendtsen, Henrik Nielsen, Gunnar von Heijne and S øren Brunak.
J. Mol. Biol., 340:783-795, 2004.

Figure 12: Working Model Based on Results