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Table of Contents

Page

Introduction……..4

Body……..5

Key Research Accomplishments…….. 22

Reportable Outcomes……23

Conclusion…… 23

References……23

Appendices…… 23

Abbreviations:
AR: Androgen receptor
ADT
AIPC
ASPC
Hsp:
Hsp40: Heat shock protein 40
CHIP: C-terminal Hsp interacting protein
TRAMP: transgenic adenocarcinoma of the mouse prostate
C: Adeno-X-CHIP
CT: Adeno-X-CHIP and Adeno-Tet-Off
CTD: Adeno-X-CHIP and Adeno-Tet-Off and Doxycycline
MOI: multiplicity of infection
OD: optical density
PI: propidium iodide

INTRODUCTION

Androgen ablation, or androgen deprivation therapy (**ADT**), is the mainstay of treatment for patients with locally advanced or metastatic prostate cancer. This therapy is only temporizing, however. Within 3-4 years, the vast majority of patients develop androgen independent prostate cancer (**AIPC**). Though several new treatments have recently been approved, once a patient develops AIPC, options are less effective, with first line chemotherapy providing only 13-15 month survival.(1) The shift from androgen sensitive prostate cancer (**ASPC**) to AIPC is a seminal event in disease progression and thus it has been extensively studied. At the center of much of this research is the androgen receptor since many believe that the overexpression, mutation and/or constitutive activation of this receptor play a critical role in the progression from ASPC to AIPC.

The chaperone proteins heat shock protein-90 (**Hsp90**) and **Hsp40** are necessary for the correct formation of AR's tertiary structure. Subsequently a second co-chaperone, C-terminal Hsp interacting protein (**CHIP**), was characterized and found to bind HSP 70 and 90. CHIP contains E3 ligase activity which targets proteins for proteosomal ubiquitination and can also directly bind to a highly conserved portion of AR which increases degradation. In cells where CHIP is overexpressed, AR synthesis is decreased and much of the AR produced has a defective tertiary structure which prevents degradation. The addition of proteosomal inhibitors to the cells, does not restore AR levels to normal, indicating that AR degradation/suppression is occurring by non-proteosomal pathway(s) as well.

The role of this study is to better characterize the interaction of CHIP and the androgen receptor and assess its role in disease progression.

BODY

Specific Aim 1: To determine the mechanism through which CHIP controls growth of androgen sensitive prostate cancer cells, both in the presence and absence of androgen and androgen refractory prostate cancer cells

Specific Aim 1A) Examine the Effect of Androgen on the Effects of CHIP:
Compare effect of CHIP on cell growth: LNCaP, AI, MDA, PCa-2b, C4-2, PC3
Compare effects of Chip on cell growth in the setting of varying levels of androgen
Assess functional status of AR in above cells after transduction with CHIP

Western Blot Analysis of the effect of CHIP overexpression on AR expression: The effect of conditional CHIP overexpression on AR expression was explored in both AR expressing androgen sensitive (LNCaP) and insensitive cells (LNCaP-Tsai and C4-2b) and compared to PC3 cells, which are androgen insensitive, AR negative cells. Using an inducible Tet-Off system, CHIP was overexpressed in all four cell lines. When CHIP was overexpressed, it reduced AR expression in all of the AR expressing cells. There was no AR expression seen in PC3 cells (Figure 1).

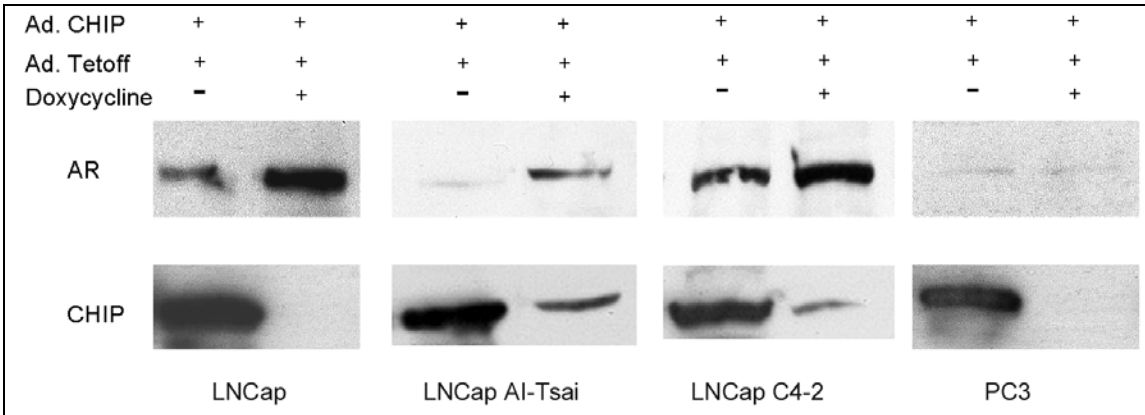

Figure 1: Western blot analysis of CHIP and AR expression in LNCaP, LNCaP-Tsai, C4-2, and PC3 cells. AR expression in AR+ cells which over-express CHIP (Doxycycline -) is reduced.

Table 1: List of Cell Lines used in this study, including AR expression and androgen sensitivity of cell line

Cell Line	Source	AR expression	Androgen Sensitivity
LNCaP	ATCC	Positive	Sensitive
LNCaP^{AI}	Anna Ferrari, MD	Positive	Insensitive
LNCaP-Tsai	Ming Tsai, MD	Positive	Insensitive
C4-2b		Positive	Insensitive
DU-145	ATCC	Negative	Insensitive
PC3	ATCC	Negative	Insensitive
TPC-2	Norman Greenberg, PhD	Positive	Sensitive
RM-1	Timothy Thompson, MD	Positive	Insensitive

Hormone Binding Assay: A hormone binding assay was performed to demonstrate the impact of CHIP overexpression on AR ligand binding. This assay was performed in all of the AR expressing cell lines: LNCaP, LNCaP-Tsai and C4-2b (Figure 2). In all three cell lines, ^{3}H 1881 binding was significantly less in cells overexpressing CHIP than in controls, regardless of whether the cells were grown in the presence of DHT initially (Table 2).

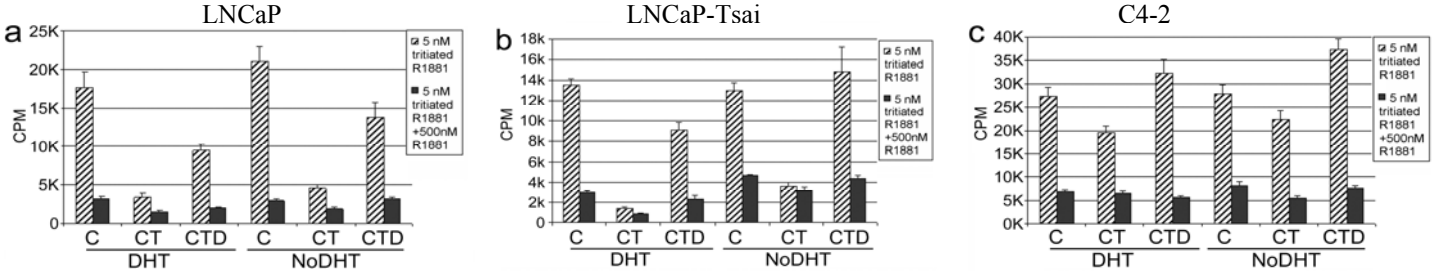

Figure 2: Hormone binding assay in AR+ cells. Hormone binding assay demonstrates that CHIP over-expression reduces AR ligand binding in all 3 AR expressing cells (**a**) LNCaP, (**b**) LNCaP Tsai, (**c**) LNCaP C42b.

Table 2: **p Values for Hormone binding assay:** Hormone binding was significantly less in the cells over-expressing CHIP (C), as opposed to the cells with normal CHIP expression (CTD).

	LNCaP-Tsai		LNCaP		C4-2b	
	C	CTD	C	CTD	C	CTD
CT, DHT+	P=0	P=0	P=0	P=0.001	P=0.006	P=0
CT, DHT-	P=0	P=0	P=0	P=0	P=0.02	P=0

PSA Assay: To assess whether CHIP overexpression alters PSA expression, a PSA assay was performed. PSA, which is an androgen sensitive serine protease, was measured in the media of

LNCaP and LNCaP-Tsai cells after infection with CHIP-pTRE (Tet Response Element) with or without Adeno-Tet-OFF (Figure 3). At 24 hours, PSA levels in LNCaP cells overexpressing CHIP (CT) were comparable to the control cell lines ($p>0.50$), but by 48 hours, LNCaP cells overexpressing CHIP produced less PSA than normal LNCaP cells, with differences nearing significance ($p = 0.073$). A similar pattern was seen in LNCaP-Tsai cells. At 24 hours, PSA production by LNCaP-Tsai cells overexpressing CHIP was similar to cells with normal CHIP expression ($p=0.354$). At 48 hours, however, LNCaP-Tsai cells which overexpressed CHIP produced significantly less PSA than those with normal CHIP expression ($p<0.001$). In both cell lines, PSA levels were reduced both in absolute terms and when normalized to cell counts (as calculated by MTT assay) since overall cell numbers were reduced with CHIP overexpression (data not shown).

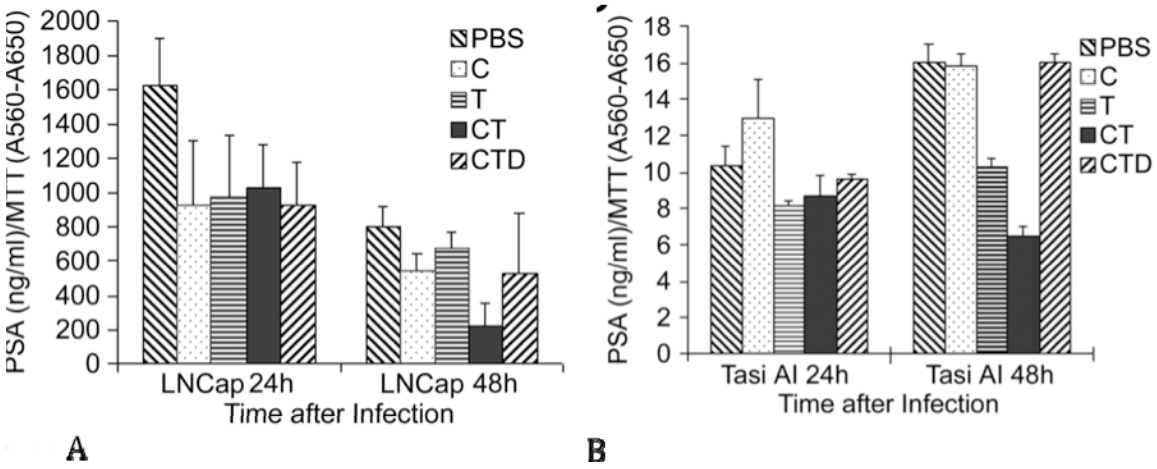

Figure 3: PSA Assay in LNCaP Tsai and LNCaP Cells: PSA was measured in the media of treated LNCaP (A) and LNCaP-Tsai (B) cells, demonstrating reduced levels of PSA following CHIP over-expression. The same held true even when cell numbers were accounted for (data not shown).

MTT Assay: Because CHIP overexpression downregulates AR expression and binding as well as PSA production in AR expressing cancer cell lines, we wanted to determine if cell growth was also affected. Cells were inoculated with CHIP vector at two doses: MOI 20 and MOI 40. An MTT assay revealed that CHIP overexpression decreased the growth and proliferation of all of the AR positive cell lines (C4-2b, LNCaP, LNCaP-Tsai; all $p<0.0005$, Figure 4). As expected, growth of androgen receptor negative PC3 cells, was not affected by CHIP overexpression (MOI = 20, $p = 0.339$; MOI = 40, $p = 0.079$).

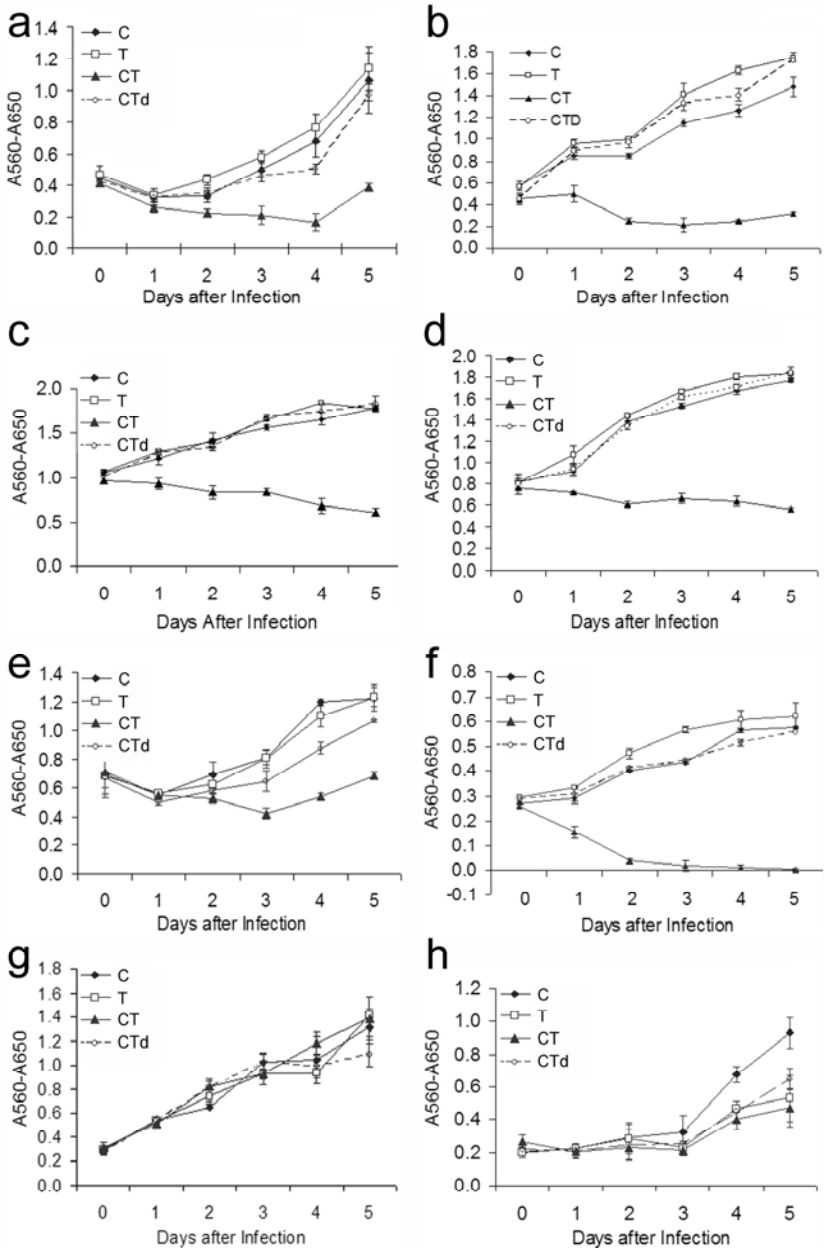

Figure 4: MTT Assay assessing CHIP's effect on prostate cancer cell growth: CHIP over-expression significantly reduced growth of AR expressing cell lines while having only limited effect on non-AR expressing cell lines. (**a, b**) CHIP expression significantly reduced growth of AR expressing Androgen dependent cell line LNCap and MOI dependent; (**c-f**) CHIP expression also significantly reduced growth of AR expressing Androgen independent cell line LNCap Tsai and LNCap C42b cell lines and MOI dependent. (**g, h**) CHIP expression did not have much effect on the cellular growth rate of PC3 cells.
(**a**) LNCap MOI 20, (**b**) LNCap MOI 40, (**c**) LNCaP Tsai MOI 20, (**d**) LNCap Tsai MOI 40
(**e**) C4-2b MOI 20, (**f**) C4-2b MOI 40, (**g**) PC3 MOI 20, (**h**) PC3 MOI 40.
C = Adeno-X-CHIP; T = Adeno-Tet-Off; D = Doxycycline (1μg/ml)

Specific Aim 1B) Examine Effect of Ad.CHIP on Cell cycle of prostate cancer cells

Compare cell cycle status with or without Chip transduction in the presence and absence of androgen

Assess effect of CHIP and androgen withdrawal on cell cycle regulators (p53, p21, p27, p16, rb, cyclins D1/2, cdk2, 4, 5, 6, E/A cdk2, A/B p34cdc2)

Assess changes in Chip and androgen deprivation in the setting of p21 inhibition

Flow Cytometry Analysis of Cell Cycle: To better understand the mechanisms underlying the observed growth suppression, the effect of CHIP expression on cell cycle progression was characterized with propidium iodide flow cytometry at 24, 48 and 72 hours (Figure 5). Regardless of CHIP expression, the vast majority of LNCaP cells (85-92%) appeared to have 2n DNA, cell cycle arrest, as represented by a disproportionately large G1 peak (Figure 5a). Conversely, CHIP over-expression markedly increased the proportion of androgen independent cells (C4-2b and LNCaP-Tsai cells) that were in the sub-G1 (<2n DNA) peak over time, suggesting that CHIP over-expression induces cell death in these cells (Figures 5b and 5c).

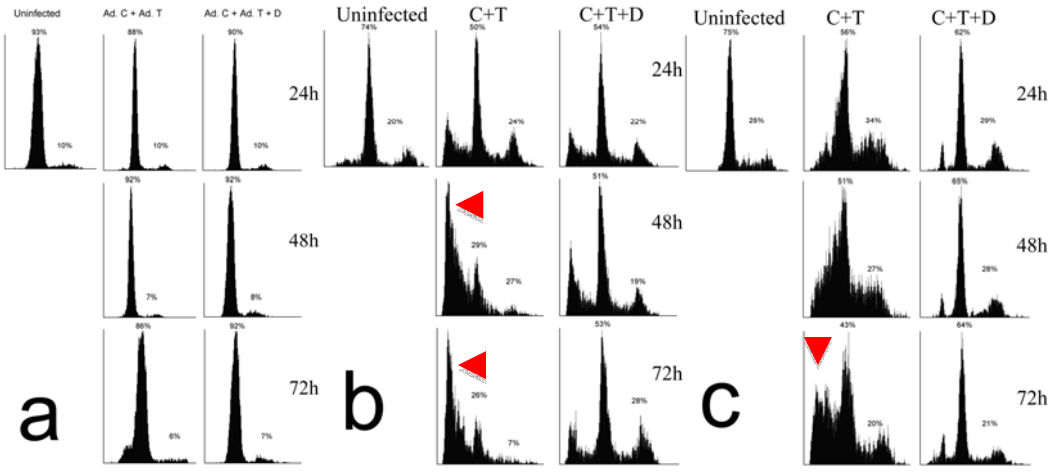

Figure 5: Flow cytometry assessment of the impact of CHIP over-expression on cell cycle. The vast majority of LNCaP cells (a) are found in the G1 peak, which is most likely due to cell cycle arrest. In androgen independent cells (b: LNCaP-Tsai; c: C4-2b) CHIP over-expression resulted in increasing numbers of cells in a sub-G1 (<2n DNA) peak (red arrowheads), indicative of cell death. C = Adeno-X-CHIP; T = Adeno-Tet-Off; D = Doxycycline (1μg/ml)

Annexin V Assay: The method of cell death was determined by Annexin V and propidium iodide staining and FACS at 24, 48 and 72 hours (Figure 6). This assay demonstrated that, with CHIP over-expression, LNCaP-Tsai and C4-2b cells died via a non-apoptotic, or necrotic, mechanism (PI positive, Annexin V negative). Neither cell line demonstrated a significant number of Annexin V positive, PI negative cells, which is characteristic of cells undergoing apoptosis.

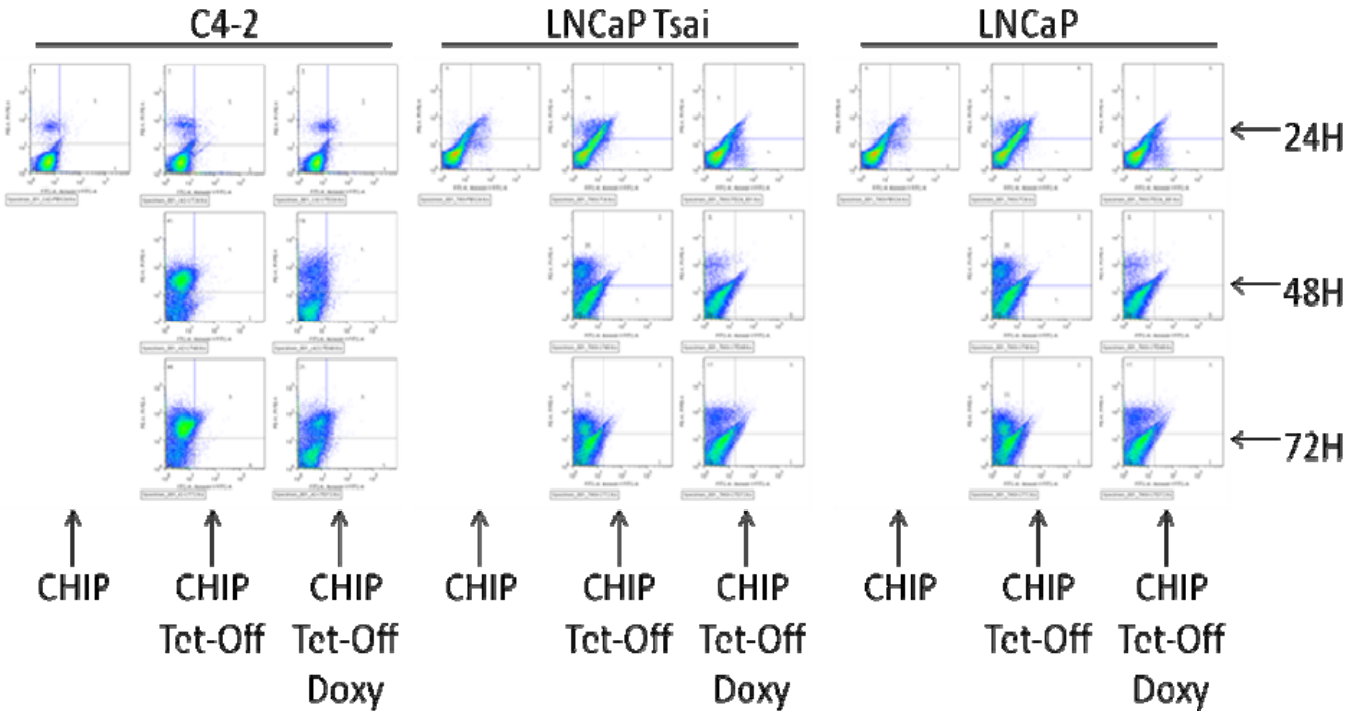

Figure 6: Annexin V analysis of cell cycle. Staining with both propidium iodide (vertical axis) and Annexin V (horizontal axis) in the three cells lines over time shows that CHIP over-expression (CT) results in a marked shift of cells to non-apoptotic cell death (PI+, Annexin V -), particularly in androgen independent cells.

Electron Microscopy: To confirm the findings seen in the Annexin V assay, we examined LNCaP, LNCaP-Tsai and C4-2 cells, all of which were over-expressing CHIP, via electron microscopy (Figure 7). The LNCaP cells examined showed minimal changes though some did exhibit evidence of early apoptosis, such as condensed chromatin (Figure 7A). LNCaP-Tsai cells and C4-2 cells (Figures 7B and 7C, respectively), however, showed much more evidence of cell death. Both showed true lipid droplets as well as lysosomal fat, which are indicators of cell injury and death. The nuclear changes that are characteristic of apoptotic cell death were largely absent, but swelling of the mitochondria, which is an early sign of cell injury, was common in androgen independent cells.

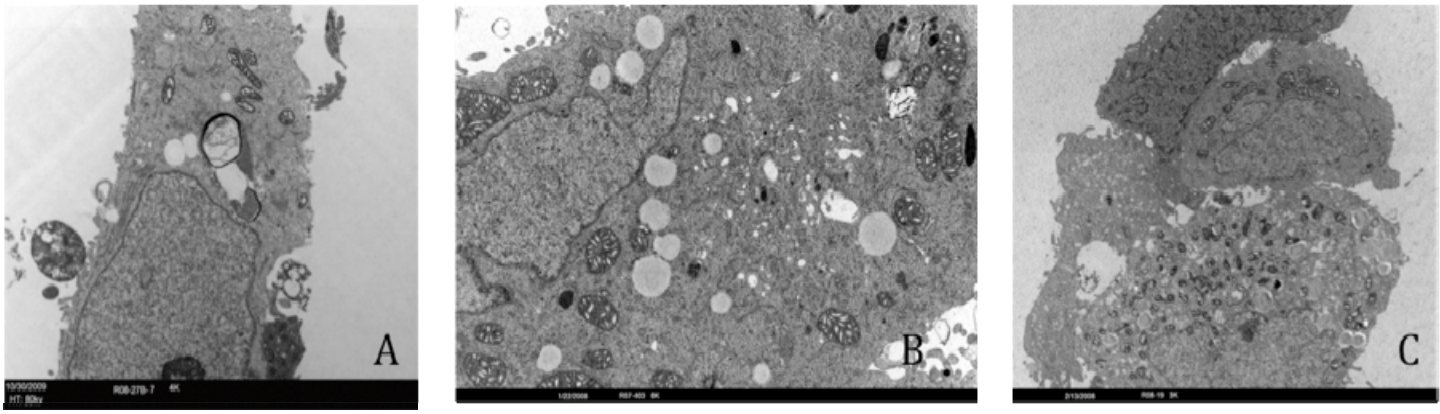

Figure 7: Electron microscopic examination of the effects of CHIP over-expression on prostate cancer cells. A: LNCaP cell (at 36 hours, 4K magnification) showing minimal evidence of apoptosis as demonstrated by lysosomal lipids and condensed chromatin. B: LNCaP Tsai cells (at 48 hours, 6K magnification) showed evidence of cell death including swollen and dilated mitochondria as well as true lipid droplets (light gray) and lysosomal fat (black). C: C4-2 cells (36 hours at 3K magnification) also show lysosomal fat and evidence of the beginning stages of cell death.

Specific Aim 1C) Identify gene products which are up-regulated or down-regulated by CHIP
To examine both hormone sensitive cell lines (LNCaP) and hormone refractory cell lines (LNCaP-Tsai, C4-2b) and identify genes whose expression is up-regulated or down-regulated by CHIP overexpression.

High throughput analysis was used to identify genes that were up or downregulated in androgen insensitive and androgen sensitive cells exposed to CHIP-mediated AR loss to determine the underlying changes in gene expression which predicted cell death or survival. Affymetrix arrays indicated that several gene transcripts were over or underexpressed with excess CHIP expression differentially between androgen sensitive and insensitive cells (Table 3).

Once differentially expressed transcripts were identified, we performed validation quantitative RT-PCR assays in C4-2, LNCaP-Tsai and LNCaP cells transfected with a TET-Off inducible CHIP (Figures 8-15). A summary of their expression can be seen in Table 4. Of the 8 transcripts of interest, only two demonstrated opposite expression levels in hormone sensitive and hormone refractory cells in the setting of increased CHIP expression: SenP1 and Edg4. In cells overexpressing CHIP, both SenP1 and Edg4 had decreased expression in hormone refractory cells and increase expression in hormone sensitive cells. ARC demonstrated increased expression in hormone sensitive cells overexpressing CHIP but no change in expression in either C4-2 or LNCaP-Tsai cells. RhoE was overexpressed in hormone refractory prostate cancer cells which were over expressing CHIP but its expression in LNCaP cells was not impacted by CHIP overexpression.

Table 3: Genes that were differentially expressed in hormone sensitive (LNCaP) and hormone insensitive cells (LNCaP-Tsai). Table 3a lists genes with increased expression in hormone refractory cells but not hormone sensitive cells in the setting of increased CHIP. Table 3b lists genes with decreased expression in the setting of increased CHIP expression in LNCaP-Tsai cells and C4-2b cells but not LNCaP cells.

A. Genes' expression induced by CHIP expression in LNCap-Tsai and LNCap C42b cells but not in LNCap.

GenBank	Gene.Symbol	Tasi LogRatio	C42B LogRatio
AL833762	DKFZp666G057	-5.0855	-4.7688
AI018322	PLAC8L1	-3.0452	-2.8869
BF968097	---	-3.3016	-2.6168
BE463997	ARL9	-4.6413	-3.5082
AB018333	SASH1	-2.8681	-2.4297
AA129774	LOC400793	-3.3986	-2.0182
BG540494	PALM2-AKAP2	-3.8419	-3.4354
BG054844	RND3	-3.9733	-4.7805
AA018968	PIK3R1	-2.1466	-2.7619
AI967987	MUM1L1	-4.2419	-3.1636

B. Genes' expression decreased by CHIP expression in LNCap-Tsai and LNCap C42b cells but not in LNCap.

GenBank	Gene.Symbol	Tasi LogRatio	C42B LogRatio
NM_001543	NDST1	3.06	3.02
Z83838	ARHGAP8 /// LOC553158	2.32	2.75
AI570493	LOC283377	3.28	3.42
AV734843	FLJ22833	2.88	2.39
AK001080	WDR6	2.74	2.46
L11669	TETRAN	2.44	2.08
AI093963	EID-3	4.23	5.10
NM_001517	GTF2H4	2.63	2.03
NM_018056	TMEM39B	2.12	2.27
AW135740	FLJ36812	2.62	2.92
AL530748	GEMIN7	2.62	3.71
AB028127	PIGM	2.80	2.40
AI912351	NOL3 (ARC)	2.55	2.00
NM_024509	LRFN3	2.89	2.04
AA150455	KIAA0276	2.03	4.05
NM_001105	ACVR1	2.46	2.90
BF345728	LOC147727	2.04	2.51
NM_003536	HIST1H3H	2.84	3.57
AW467472	APPL	2.62	2.06
AB037853	KIAA1432	2.44	2.28
AL531790	MGC88387	3.03	2.13
AW002273	FBXL17	2.71	2.82
AI681419	LOC388327	2.26	3.03
BC005810	CLEC11A	2.15	3.51
AI264247	ATP1A1	2.32	2.17
AF131747	KIAA0830	2.87	2.30
BC001428	PLEKHB2	2.14	2.23
NM_005227	EFNA4	3.73	3.44
NM_004085	TIMM8A	2.14	2.31
NM_022492	FLJ12788	2.35	2.34
BE740761	HIST1H4H	2.16	2.73
BE326857	CYP4V2	3.04	2.75
BF977145	C1orf85	4.16	2.25
AK024446	ABCC10	3.09	2.92
BE042976	MGC17330	2.18	3.04
AL122088	LYSMD1	2.40	3.54
X76775	HLA-DMA	2.83	2.67
AI431931	GIMAP2	2.57	2.60
BF939830	LOC254128	3.13	3.35
AW080835	C1orf51	2.76	2.72
NM_006858	TMED1	2.01	2.47
BE467260	DCBLD1	2.44	2.84
AF011466	EDG4	2.38	2.16
NM_019082	DDX56	2.08	3.60

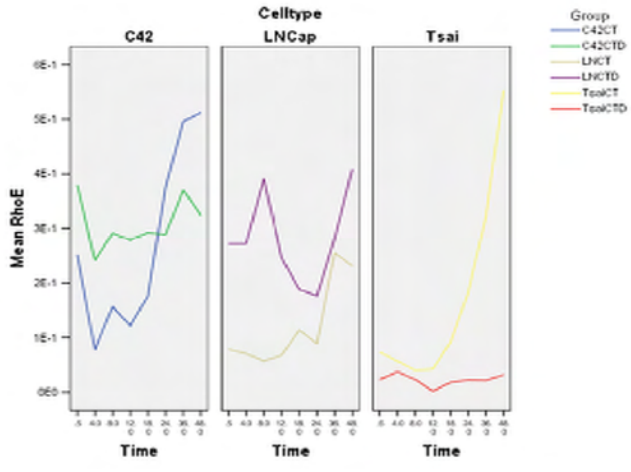

Figure 8: CHIP gene expression induced RND3 (RhoE) significantly expression in LNCap Tsai and LNCap C4-2 but not in LNCap cells.

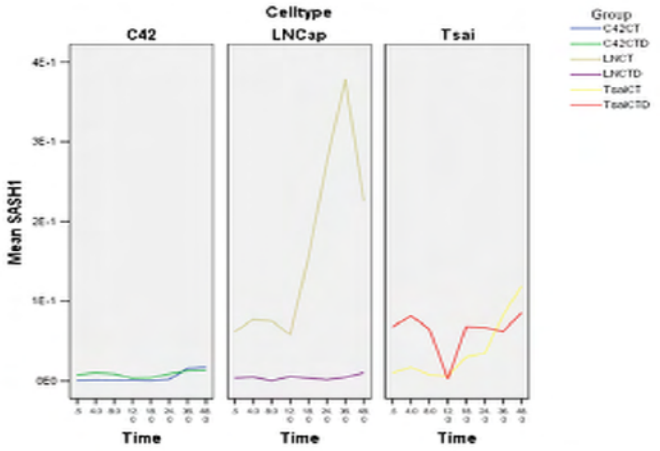

Figure 9:. CHIP gene expression induced SASH1 gene expression in LNCaP cells but not in LNCap Tsai and LNCap C4-2 cells.

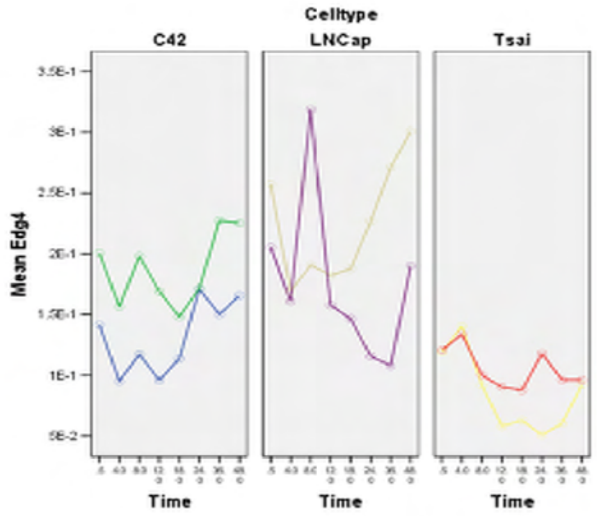

Figure 10: CHIP gene expression lead to significant elevation of **Edg4** expression in LNCap cells, but to reduction of Edg4 expression in LNCap Tsai and LNCap C4-2 cells.

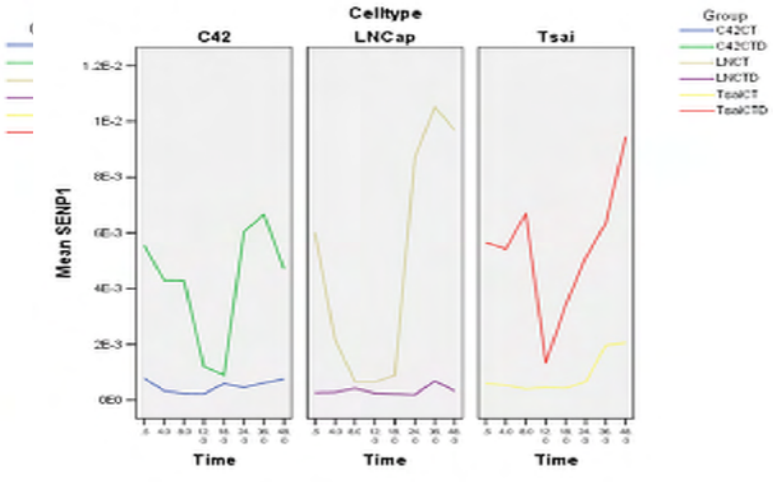

Figure 11: CHIP gene expression lead to significantly elevation of **SENP1** expression in LNCap cells, but to significantly reduction of SENP1 expression in LNCap Tsai and LNCap C4-2 cells.

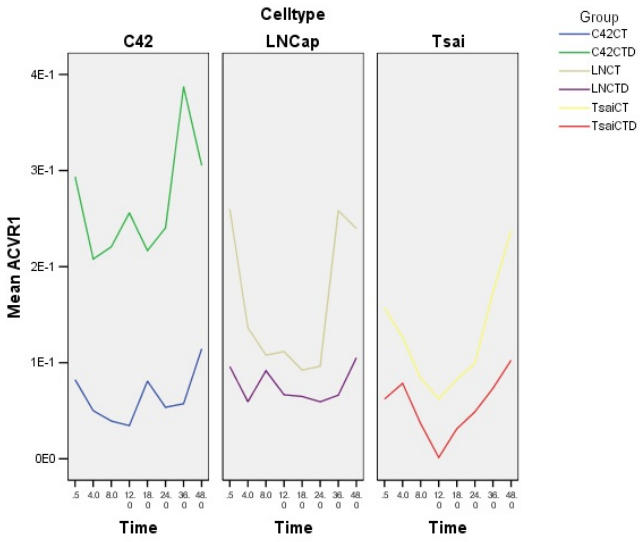

Figure 12: Quantitative RT-PCR of ACVR1 in C4-2, LNCaP and LNCaP-Tsai cells both with and without CHIP over-expression. ACVR1 expression was significantly suppressed by CHIP overexpression in C4-2 cells and moderately upregulated in LNCaP and LNCaP Tsai cells.

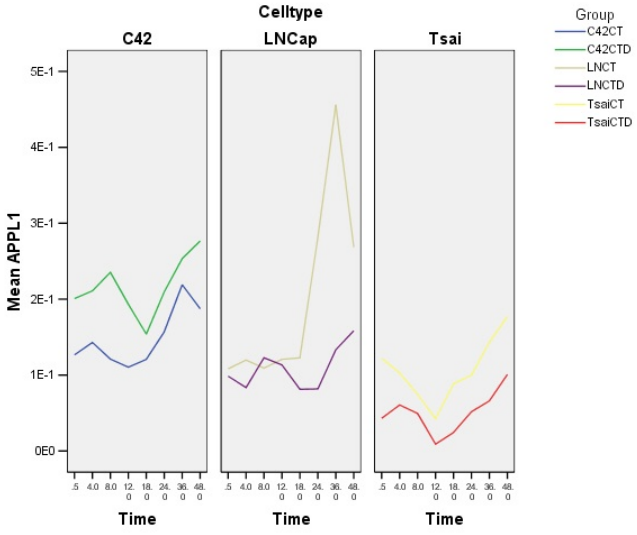

Figure 13 Quantitative RT-PCR of APPL1 in C4-2, LNCaP and LNCaP-Tsai cells both with and without CHIP over-expression. CHIP overexpression increased APPL1 in LNCaP cells but had no impact on its expression in C4-2 or LNCaP-Tsai cells.

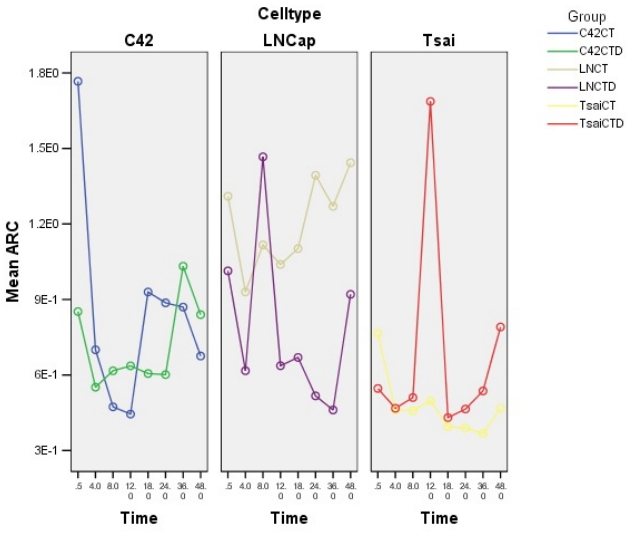

Figure 14: Quantitative RT-PCR of ARC in C4-2, LNCaP and LNCaP-Tsai cells both with and without CHIP over-expression. ARC expression was moderately increased by CHIP overexpression in LNCaP cells only.

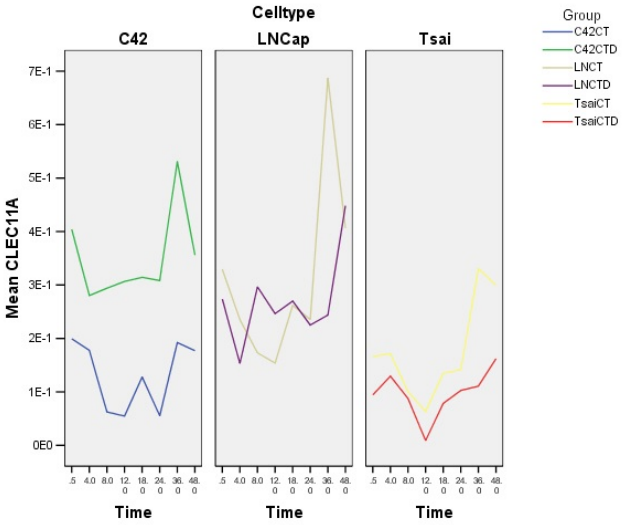

Figure 15 Quantitative RT-PCR of CLEC11A in C4-2, LNCaP and LNCaP-Tsai cells both with and without CHIP over-expression. CLEC11A expression was decreased in CHIP overexpressing C4-2 cells and slightly increased in LNCaP Tsai and LNCaP cells.

Table 4 The impact of CHIP overexpression on selected gene's expression in 3 cell lines subjected to quantitative RT-PCR

Gene Product (*Gene*)	C4-2 Cells	LNCaP Cells	LNCaP Tsai Cells
RhoE (RND3)	Increased	Unchanged	Increased
SASH1	Unchanged	Brief Increase	Unchanged
Edg4	Reduced	Increased	Reduced
SENP1	Reduced	Increased	Reduced
ACVR	Reduced	Moderate Increase	Moderate Increase
APPL1	Unchanged	Brief Increase	Unchanged
ARC (NOL3)	Unchanged	Moderate Increase	Unchanged
CLEC11A	Decreased	Brief Increase	Moderate Increase

Table 5: Western blot antibodies, their manufacturers and dilutions used

Antibody	Manufacturer	Dilution
Anti SENP1	Abgent, San Diego CA	1:100
Anti β-tubulin	Cell Signaling Technology	1:1000
Anti ARC	Cayman Chemical	1:500
Anti RhoE	Millipore	1:400
Anti CDC2	Thermo Fisher Scientific	1:200
Anti Cyclin B	Thermo Fisher Scientific	1:200
Anti-Rock1 (1113) Cleavage Specific Product	Millipore	1:500
Anti-Edg4	Abcam	1:100

We then performed Western blots on the four transcripts of interest: SenP1, Edg4, RhoE and ARC. Multiple attempts at Western blot analysis of Edg4 using commercially available antibodies failed to detect the protein using this method. Thus our efforts focused on the RhoE, ARC and SENP1. Cyclin B1, Rock1 and CDC2 were also included in our Western Blot as these are all downstream from RhoE. Results of Western blot analysis of these proteins are shown in Figures 16-19. Overexpression of CHIP did not significantly increase or decrease protein production of ARC or RhoE in LNCaP, LNCaP-Tsai, or C4-2 cells (Figures 16-18). Accordingly, there were no discernible protein production changes in RhoE's three downstream targets that were tested: Cyclin B1, Rock1, and CDC2.

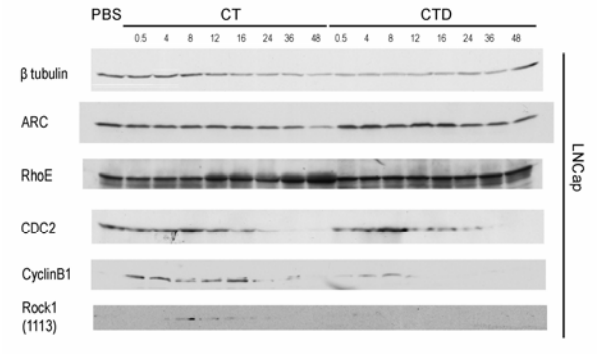

Figure 16: Western blot analysis of ARC, RhoE, CDC2, Cylin B1, and Rock1 in LNCaP cells. (CT = CHIP Tet-Off; CTD = CHIP Tet-Off Doxycycline). CHIP overexpression did not significantly increase or decrease protein production of ARC or RhoE in LNCaP cells overexpressing CHIP.

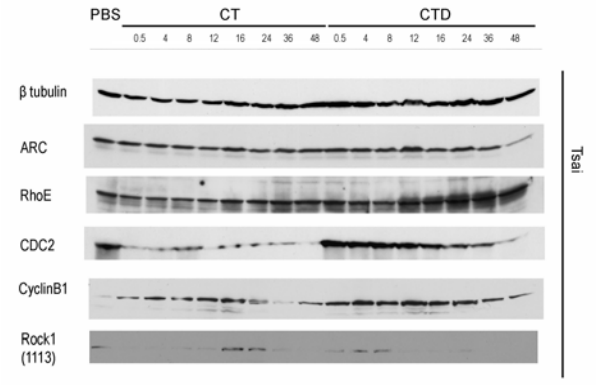

Figure 17: Western blot analysis of ARC, RhoE, CDC2, Cylin B1, and Rock1 in LNCaP-Tsai (Tsai) cells. (CT = CHIP Tet-Off; CTD = CHIP Tet-Off Doxycycline). CHIP overexpression did not significantly increase or decrease protein production of ARC or RhoE in LNCaP-Tsai cells overexpressing CHIP.

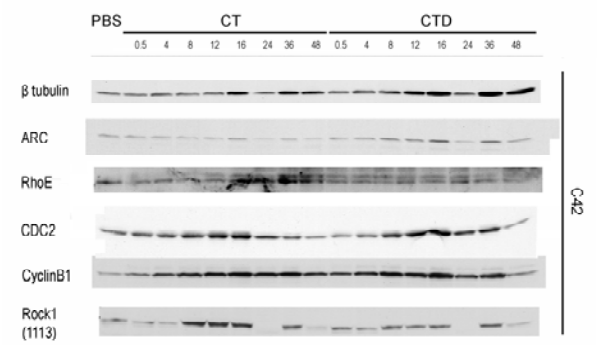

Figure 18: Western blot analysis of ARC, RhoE, CDC2, Cylin B1, and Rock1 in C4-2 cells. (CT = CHIP Tet-Off; CTD = CHIP Tet-Off Doxycycline). CHIP overexpression did not significantly increase or decrease protein production of ARC or RhoE in C42 cells overexpressing CHIP.

Western blot analysis of SENP1 (Figure 19), however, did show results which were consistent with initial quantitative RT-PCR tests. In hormone sensitive LNCaP cells, CHIP overexpression upregulated SENP1 protein production while in androgen independent LNCaP-Tsai and C4-2 cells, CHIP overexpression decreased SENP1 protein production.

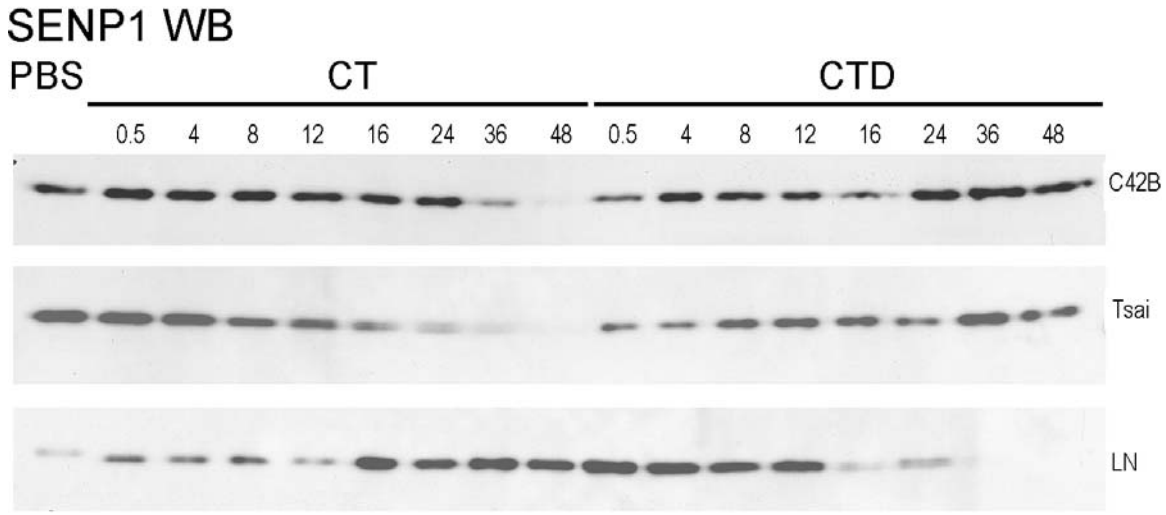

Figure 19: Western Blot analysis of SENP1 in C4-2B, LNCaP Tsai (Tsai) and LNCaP (LN) at different time points (0.5 hours to 48 hours) after transfection with a Tet-Off CHIP inducible system. (CT = CHIP Tet-off; CTD = CHIP Tet-off Doxycycline). CHIP over-expression upregulates SENP1 in LNCAP cells 6 hours after transfection and downregulates SENP1 in C4-2B and LNCaP-Tsai cells 16-36 hours after transfection.

SENP1 is an enzyme involved in the SUMOylation pathway. This pathway was first described in 1996-1997, when a new ubiquitin-like protein was characterized. This protein, which is now called SUMO for small ubiquitin-like modifier, is also called sentrin-1, GMP1, PIC1, SMT3 and UBL1 in these initial reports (*1*). There are three proteins in the SUMO family: SUMO1, SUMO2, and SUMO3. SUMO2 and SUMO3 have 93.6% homology to eachother while SUMO1 has 52.4% homology. SUMO proteins are highly conserved. Homologues of these proteins have been found in plants and animals. Similar to ubiquitin, SUMO's require activating (E1), conjugating (E2) and ligating (E3) enzymes for sumoylation. Unlike ubiquitination, however, sumoylation does not target proteins for degradation. Instead, sumoylation may stabilize proteins or alter their localization, function, or degree of function. Interestingly, SUMO proteins can act concomitantly or compete with ubiquitin. Since its characterization, a number of proteins have been identified as sumoylation substrates, including androgen receptor and p53 (*1,2*). Androgen receptor (AR) is sumoylated at lysine residues 386 and 520, and mutation of these residues increases transcription of AR's downstream targets. This implies that sumoylation of these residues acts to downregulate AR activity.

There are also four co-regulators of AR that have recently been identified as sumoylation substrates. Two of these coregulatory agents increase AR activity (SRC1 and GRIP1) while two suppress AR activity (HDAC1 and HDAC4).

Reversal of sumoylation is carried out by a family of proteases known as SENP's (figure 9). In a recent study by Cheng and colleagues, SENP1 produced a ligand-dependent, 23-fold increase in AR's transcriptional activity in LNCaP cells (*1*). This effect could not be produced by any of the other members of the SENP family. When the sumoylation sites on AR were mutated, SENP1 still had the same effect on transcription, which suggests that SENP1's impact on AR is not via direct sumoylation of the receptor.

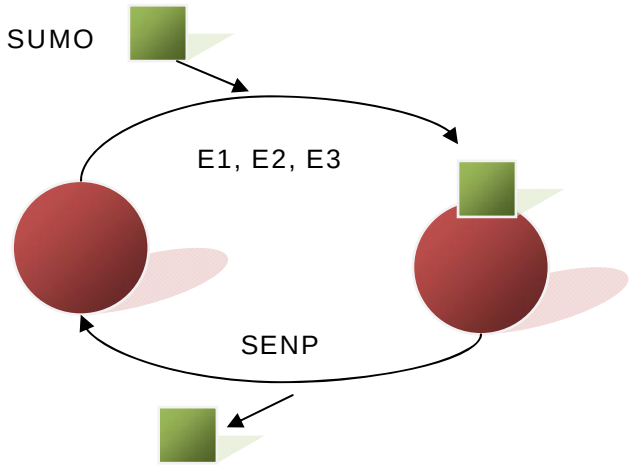

Figure 20: Addition of SUMO proteins via the process of sumoylation, is catalyzed by E1, E2 and E3 sumoylation enzymes. Sumoylation can have one of many effects on the substrate: targeted proteins may be stabilized or their localization, function or degree of function may be altered. SUMO proteins are removed by SENP enzymes.

Our lab sought to further characterize the interaction between androgen, SENP1 and the androgen receptor. Androgen was withdrawn LNCaP cells by changing the cells' media from RPMI 1640 containing 10% FBS to RPMI 1640 containing 10% Charcoal/Dextran treated Fetal Bovine Serum. A Western blot was performed and demonstrated that androgen withdrawal increases SENP1 expression in LNCaP cells (Figure 21), while in hormone insensitive cell lines, the low levels of SenP1 appear to be reduced even further.

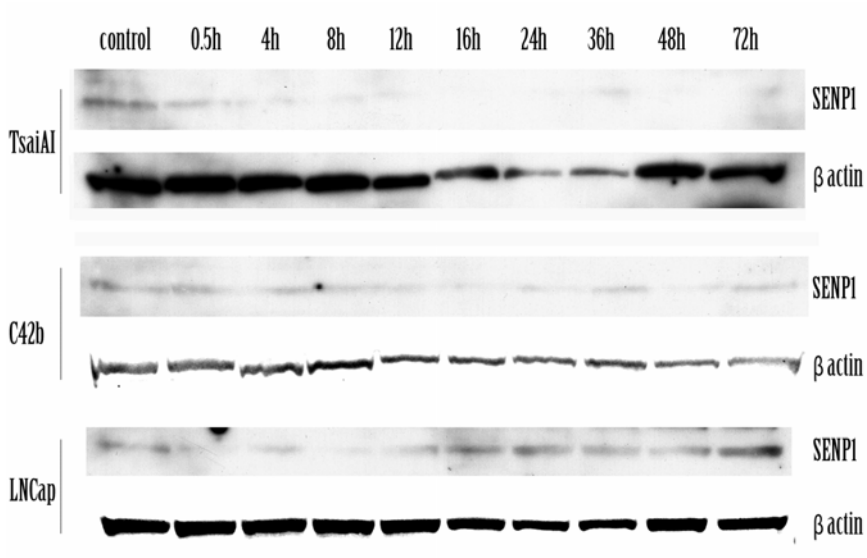

Figure 21: Western Blot analysis of the impact of androgen withdrawal on SenP1 expression in LNCaP, C4-2b, and LNCaP-Tsai (TsaiAI) cell lines. Over the course of 72 hours, the low levels of SenP1 expression in hormone refractory cell lines (C4-2b or LNCaP-Tsai) appear to decrease even further, but there is an increase in SenP1 expression in LNCaP cells.

Based on this, we hypothesized that SENP1 may play a critical role in determining the different outcomes observed with CHIP-mediated AR loss: its expression in LNCaP cells results in growth arrest (but not cell death) while its lack of expression in androgen insensitive cells results in death via autophagy (summary seen in Table 6). In an attempt to understand whether SenP1 or CHIP was responsible for these observations, a SenP1 knockout was made out of each of these cell lines both with and without CHIP overexpression.

Summary of CHIP Overexpression's Impact on Cell Lines			
Cell Line (Hormone sensitivity)	LNCaP Cells (Hormone sensitive)	C4-2 Cells (Hormone refractory)	LNCaP-Tsai Cells (Hormone refractory)
SenP1 Expression	INCREASED	DECREASED	DECREASED
Cell Viability	Growth Arrest	Death via Autophagy	Death via Autophagy

Table 6: Summary of the effect of CHIP overexpression on the SenP1 expression and viability of prostate cancer cell lines, LNCaP, C4-2 and LNCaP-Tsai

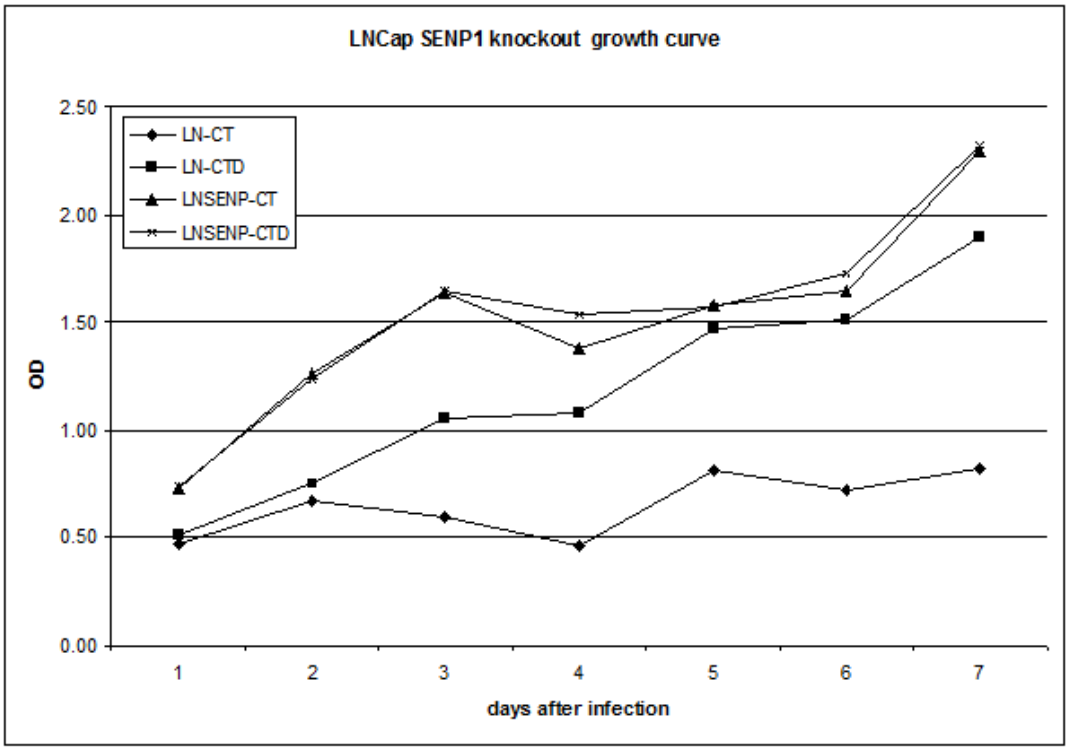

Figure 22: Cell growth was compared in LNCaP cells with intact SenP1 (LN) or LNCaP cells that were SenP1 knockouts (LNSENP). CHIP overexpression (CT) normally increases SenP1 expression in LNCaP cells, but would not have that affect in SenP1 knockout cells overexpressing CHIP (LNSENP-CT). A cell growth curve indicates that LNCaP cells simply overexpressing CHIP (with concomitant upregulation of SenP1) largely remained dormant and did not proliferate. Normal LNCaP cells (LN-CTD) exhibited proliferation. SenP1 knock out cells with both normal CHIP expression or CHIP overexpression, however, both grew faster than baseline, suggesting that the SenP1 may inhibit cell proliferation.

Consistent with previous findings, LNCaP cells overexpressing CHIP (LN-CT) appeared to undergo growth arrest while normal LNCaP cells (LN-CTD) proliferated normally (Figure 22). When SenP1 was knocked out, however, LNCaP cells overexpressing CHIP (LNSENP-CT) grew at rates greater than normal LNCaP cells and similar to cells with normal CHIP expression and no SenP1 (LNSENP-CTD). Similar SenP1 knockouts were made out of C4-2 and LNCaP-Tsai cells, however, these cells did not proliferate or die (data not shown). **When taken together, these results suggest that SenP1 plays nearly opposite roles in hormone sensitive and hormone refractory prostate cancer cells. Regardless of the level of CHIP expression, SenP1 is capable of limiting cell growth in hormone sensitive LNCaP cells but stimulates growth in hormone refractory cell lines.**

Specific Aim 2A: To characterize both the direct and indirect interactions which take place between CHIP and AR.

Several different mechanisms of interaction between CHIP and the AR have been proposed. Studies have demonstrated that while CHIP may regulate AR levels through proteosomal degradation, there is also a component of AR destruction that is non-proteosomal. Using co-immunoprecipitation assays in both LNCaP and C4-2b cells, it is evident that there is direct interaction of CHIP and AR (Figure 23). It can not be discerned from this assay, however, if CHIP overexpression has any impact on the degree or type of interaction.

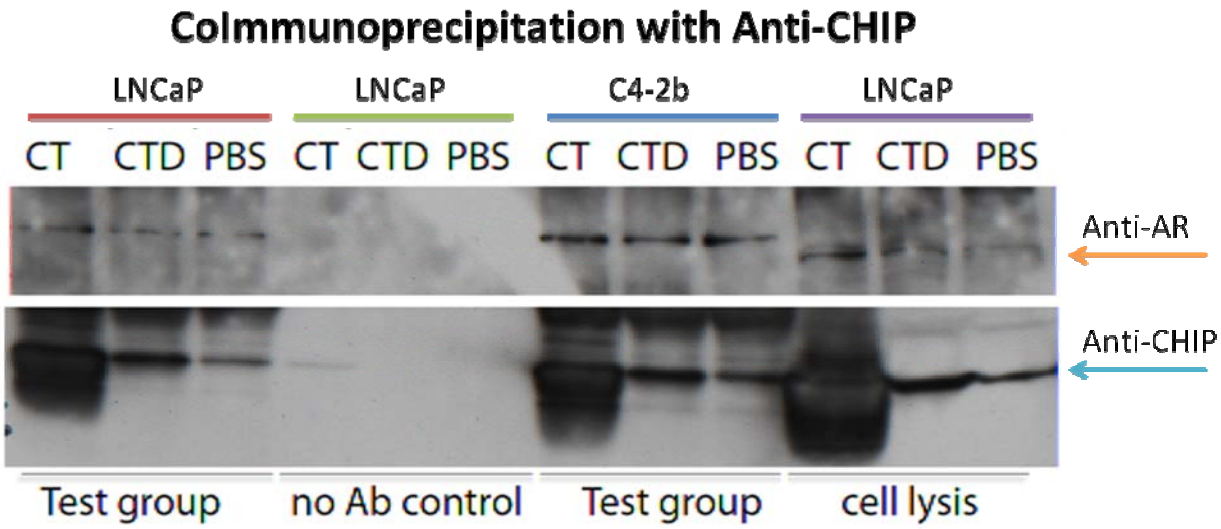

Figure 23: Coimmunoprecipitation of CHIP and AR can be seen in both LNCaP and C4-2b cells that are overexpressing CHIP (CT) and cells that are not (CTD).

Given the differences in cell proliferation, arrest and death, we also studied CHIP's effect on Akt. The Akt and the PI3 pathway are critical in cell survival and inhibiting apoptosis. A Western blot staining for both Akt and Serine 473 phosphorylated Akt was performed (Figure 24). In LNCaP cells, CHIP overexpression appeared to decrease levels of both Akt and phosphorylated Akt. When these studies were repeated in C4-2b and LNCaP-Tsai cells, there was no definitive difference in Akt expression in normal cells and those that overexpressed CHIP (data not shown).

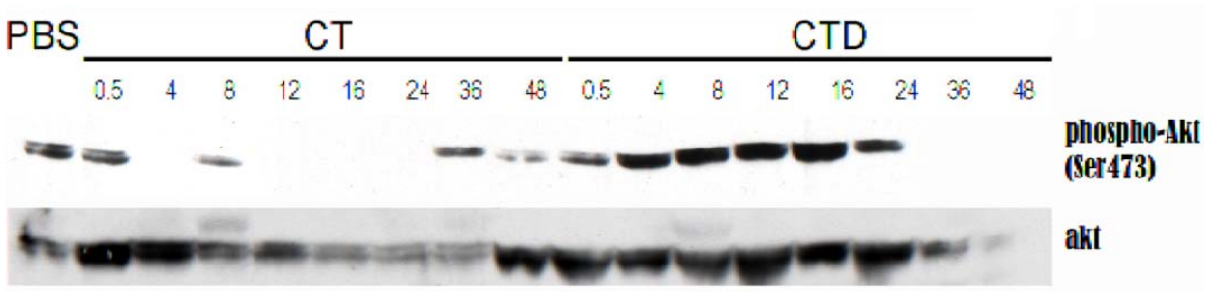

Figure 24: Western blot analysis in LNCaP cells of Akt and Akt phosphorylated at Serine 473 was performed. This study demonstrates that LNCaP cells overexpressing CHIP exhibit lower levels of the proto-oncogene Akt as well as phosphorylated-Akt. Cells expressing normal amounts of CHIP appear to express both Akt and phosphorylated Akt, though these levels appear to attenuate with time.

Specific Aim 2B) Human tissue samples will be examined and tested for mutations in the AR CHIP binding site.

This specific aim was not completed in the time remaining.

Specific Aim 2C) Examine in vitro whether CHIP overexpression will result in the evolution of androgen independent clones over time and whether mutations of the AR will evolve.

This specific aim was not completed in the time remaining.

KEY RESEARCH ACCOMPLISHMENTS

Bulleted list of key research accomplishments from this research

- When CHIP was overexpressed, it reduced AR expression in all of the AR expressing cells. There was no AR expression seen in PC3 cells.
- In all three cell lines, hormone binding was significantly less in cells overexpressing CHIP than in controls, regardless of whether the cells were grown in the presence of DHT initially.
- In both hormone sensitive and insensitive cell lines, CHIP over-expression decreased PSA production.
- An MTT assay revealed that CHIP overexpression decreased the growth and proliferation of all of the AR positive cell lines
- Flow cytometric analysis demonstrated that regardless of CHIP expression, the vast majority of LNCaP cells (85-92%) were in cell cycle arrest. Conversely, CHIP over-

expression markedly increased the proportion of androgen independent cells (C4-2b and LNCaP-Tsai cells) that were in the sub-G1 peak over time, suggesting that CHIP over-expression induced cell death.

- Annexin V analysis revealed that LNCaP-Tsai and C4-2b cells that overexpressed CHIP died via a non-apoptotic, or necrotic, mechanism.
- High throughput analysis revealed that hormone refractory cells overexpressing CHIP express decreased amounts of SenP1 while in hormone sensitive cells, SenP1 expression was increased. This was validated by semi-quantitative RT-PCR as well as Western blot. With androgen withdrawal, the decreased expression in hormone refractory cells and the increased expression in hormone sensitive cells was exacerbated.
- Growth studies in SenP1 knockout cells suggest that SenP1 plays nearly opposite roles in hormone sensitive and hormone refractory prostate cancer cells. Regardless of the level of CHIP expression, SenP1 is capable of limiting cell growth in hormone sensitive LNCaP cells but stimulates growth in hormone refractory cell lines.
- CHIP and AR interact through many mechanisms, but there is direct binding involved.
- In LNCaP cells, CHIP overexpression appears to decrease Akt expression levels.

REPORTABLE OUTCOMES

We believe that all of these results are reportable and will be submitting a manuscript with our findings.

CONCLUSION

CHIP appears to play a critical role in both AR expression and functional behavior of prostate cancer cell lines, likely through a number of mechanisms. The role it plays in hormone sensitive and hormone refractory cells, however, appears to be different and thus it may play a role in the evolution of the disease from a hormone sensitive to hormone refractory phenotype. SenP1, a protease involved in sumoylation, appears to be regulated by CHIP expression, but plays nearly opposite roles in hormone refractory cells. Further studies are needed to elucidate the pathway between CHIP and SenP1 and the downstream targets of SenP1 which dictate its different effects in hormone sensitive and hormone refractory cells. Ultimately, studies in live animals and human tissues will needed to determine if these findings hold true *in vivo*.

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APPENDICES

None