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Jessica Cheng April 19, 2011

Putative GSK-3β Phosphorylation Sites Mediate SOX4 Lysosomal Degradation

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An abstract of
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of Emory University in partial fulfillment
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Bachelor of Sciences with Honors

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Abstract

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Sex-determining region Y Box (SOX4) is an intronless gene highly upregulated at the mRNA and protein level in many different cancers including prostate cancer. Recent evidence has linked SOX4 with β-catenin stability and the promotion of cell proliferation through the Wnt pathway. As one of the most commonly misregulated signaling pathways in human malignancies, the Wnt pathway is a perfect target for cancer treatments. In this paper, we mutated three putative GSK-3β phosphorylation sites on SOX4 and examined the effects of these sites on SOX4 abundance and degradation. We treated ARCaPE and LNCaP cell lines with proteasome and endosome inhibitors in an effort to discover the pathway through which SOX4 is degraded. The results suggest SOX4 is degraded through the lysosomal pathway. Understanding SOX4 degradation will provide further insight into the Wnt pathway and may lead to a promising new target for cancer therapeutics.

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Acknowledgements

First and foremost, I would like to thank Dr. Carlos Moreno for giving me the opportunity to work in his lab and on this project. Not only has he provided me with invaluable guidance as a mentor, but he has also encouraged my passion for science and inspired my interest in research. Without his support and patience, I would never have learned as much as I did. I would like to thank Yu-Heng Lai, the graduate student in out lab, who never failed to help me overcome obstacles I encountered throughout my project. Her friendship and kindness helped me through the weekends spent in lab running Westerns, and I truly owe the success of a lot of my research endeavors to her. From the Moreno lab, I would like to thank Soma Sannigrahi, Cornel Phillip, Zhongming Mou, and Lea Remolona for always making lab a cheerful place to work and managing to put a smile on my face every day. Finally, I would like to thank all of my friends and my parents for supporting me and listening to me excitedly ramble about my latest results. I am so grateful to have shared this accomplishment with all of you.

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INTRODUCTION

With an estimated number of 217,730 cases in 2010, prostate cancer is the most common cancer among men in the United States and the second leading cause of cancer death in men. Based on prostate cancer cases diagnosed between 1999 and 2005, the American Cancer Society estimates 92% of all new cases will be diagnosed at local or regional stages. Localized cases of prostate cancer are generally clinically asymptomatic, indicate early-stage disease and have a 5-year relative survival approaching 100%. Prostate cancer in a subpopulation of patients, however, develops into a highly invasive, androgen-independent disease with a 20-30% survival rate (Jemel et al., 2010). This type of advanced prostate cancer is usually initiated by a transient process termed Epithelial-to-Mesenchymal Transition (EMT) and is accompanied by lethal metastasis to the bone. During carcinoma progression, advanced tumor cells downregulate epithelial markers and cells switch from a sessile phenotype to a motile, mesenchymal phenotype (Christiansen et al., 2006; Sethi et al., 2011). Moreover, EMT promotes loss of contact inhibition, enhanced invasiveness, and altered growth control (Christiansen et al., 2006). Increasing evidence suggests EMT and the microenvironment of the cell are crucial components to understanding tumor invasion and metastasis (Sethi et al., 2011).

Several cellular signaling pathways have been discovered to affect cell communication and the development of prostate cancer. These critical pathways include the androgen receptor (AR), Wnt, Hedehog (Hh), and Notch pathways (Moreno, 2010). The Wnt pathway, in particular, is one of the most common aberrantly activated signaling pathways in human cancers and has been linked to many different cancers, including colorectal cancer, leukemia, and melanoma (Willert and Jones, 2006; Figure S1). The involvement of Wnt signaling on embryonic developmental processes such as cell proliferation, differentiation, and epithelial-

mesenchymal interactions makes the Wnt pathway a perfect target for cancer cells (Angers and Moon, 2009). Although the exact mechanisms through which Wnt acts on prostate cancer is still unclear, Wnt signaling assists in bone metastasis and plays a role in the regulation of epithelial branching morphogenesis (Dai et al., 2008; Wang et al., 2008). The 19 highly conserved Wnt genes in humans encode cysteine-rich secreted glycoproteins that remain closely associated with the extracellular matrix. During canonical Wnt signaling, phosphorylation and transcriptional activation of β-catenin is the key to regulating the pathway. In the presence of Wnt, the APC β-catenin "destruction" complex is disrupted, preventing phosphorylation by GSK-3β and degradation of β-catenin (Zeng et al., 2005). The accumulation and stabilization of cytoplasmic β-catenin allows β-catenin to translocate into the nucleus and bind to T cell factor/lymphoid enhancer factor (TCF/LEF) DNA-binding proteins (Sinner et al, 2007). Several downstream targets of the β-catenin/TCF/LEF complex include *c-myc* and *cyclin D* that can both act as oncogenes (Moreno, 2010). In the absence of Wnt signaling, the degradation pathway maintains low cytoplasmic and nuclear β-catenin by ubiquitination and proteasomal degradation (Aberle et al., 1997).

The ubiquitin pathway is responsible for the majority of intracellular proteolysis and is initiated when a 76-residue ubiquitin (Ub) moiety is conjugated to the lysine residues of the target protein (Johnson et al., 1995). Ub is coupled to proteins through a multi-step ATP-dependent process involving ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases termed E3. The ubiquitin pathway can result in either proteins tagged with a single ubiquitin moiety, or poly-ubiquitinated proteins with multiple ubiquitin moieties present. In vivo, the polyubiquitination appears to signal the protein for degradation by the proteasome (Jentsch, 1992). The proteasome, a multisubunit protease complex, has recently

become a popular target for cancer therapeutics. In androgen-sensitive prostate cancer, the removal of androgens induces apoptosis and patients enter remission. Quite often, however, patients develop androgen-refractory prostate cancer that is more aggressive than the primary cancer. Patients with androgen-refractory prostate cancer can be treated with proteasome inhibitors to disrupt regulatory proteins. The cell's inability to degrade 70-90% of cellular proteins induces cellular stress that ultimately leads to cell cycle arrest and/or cell death (Shirley et al., 2006). Along with their ability to prevent degradation of IκB and activation of the NF-κB survival pathway, proteasome inhibitors have been shown to effectively induce apoptosis in prostate cancer cells and sensitize PC-3 prostate cancer cells to ionizing radiation (Shirley et al., 2005; Pajonk et al., 2005). The ability to target androgen-refractory prostate cancer with proteasome inhibitors promises to be a very powerful step towards controlling aggressive prostate cancer cases.

In addition to ubiquitin-mediated degradation by the proteasome, eukaryotic cells also participate in lysosome-mediated protein degradation. This process accounts for the remaining percentage of proteins that are not degraded through the proteasome. The lysosome contains multiple hydrolases and is responsible primarily for the degradation of membrane associated proteins or extracellular proteins acquired through endocytosis (Koh et al., 2005). Degradation of extracellular proteins received through endocytosis is the most well characterized lysosomal degradation pathway, but autophagy has also been increasingly illustrated as a pathway to degrade cytoplasmic components. Autophagy is divided into three classes of pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy (Reviewed by Komatsu et al., 2010). Lysosomal protein degradation involves the development of moderately acidic early endosomes into more acidic late endosomes that contain multiple enzymes (Reviewed by Seglen

& Bohley, 1992). Following the maturation of an early endosome into a late endosome, the endosome fuses with the lysosome. Endosomal fusion with the lysosome has become a therapeutic target for many diseases including cancer. In 2008, targeting lysosomal degradation was shown to induce p53 dependent and caspase independent apoptosis in mouse embryonic fibroblasts. The same study found that inhibiting lysosome-mediated degradation also prevented cancer in mouse models of lymphomagenesis (Maclean et al., 2008). In experiments dealing with the epidermal growth factor receptor (EGFR), activating the autophagy pathway caused cell death in DiFi colorectal adenocarcinoma cells (Li et al., 2010). These results indicate the potency of interfering with normal lysosomal degradation. From a clinical standpoint, the use of an antimalarial lysosomotrophic agent Chloroquine (CQ) has been identified as a possible therapeutic agent against the aggressive astrocytic cell neoplasm glioblastoma (Geng et al., 2009). CQ has also been reported to assist in amplifying the effects of radiation-triggered inhibition of cancer cell proliferation, and is effective in eliminating chemotherapy-resistance cancer cells (Sotelo et al., 2006). Many cancer researchers have studied the effects of inhibiting different pathways involved in lysosomal degradation and all of these studies have confirmed that lysosomal degradation remains an important pathway in the understanding of human malignancies. The pathways involved in protein degradation are continuing to be explored and may eventually lead to the development of new and effective cancer treatments.

In addition to the study of signaling pathways and protein degradation in cancer, expression profiling of prostate cancer has led to possible targets for cancer treatment. Multiple independent studies of prostate cancer have repeatedly detected sex-determining region Y-Box 4 (SOX4) overexpression. SOX4 is an intronless gene that encodes a transcription factor important in development, cell fate, and proliferation (Moreno, 2010). Not only is SOX4 upregulated at the

protein and mRNA level in prostate cancer cell lines, but SOX4 is also linked with many other human malignancies, such as leukemias, melanomas, and glioblastomas (Scharer et al., 2009). SOX4 has been shown to play a role in cell migration and invasion in human cutaneous melanoma, and overexpression of SOX4 regulates p53-mediated apoptosis in hepatocellular carcinoma (Jafarnejad et al., 2010; Hur et al., 2010). In our lab, SOX4 overexpression was shown to trigger anchorage independent growth, a well recognized cancer phenotype (Liu et al., 2006). Data has shown SOX4 to be one of the most common retroviral integration sites and also possessing oncogene activity. As classification of SOX4 as an oncogene becomes more substantive, the interaction of SOX4 with other proteins reveals more about the function of SOX4 in tumorigenesis. Specifically, the interactions of SOX4 with epidermal growth factor receptor (EGFR), tenascin C, Integrins, and rac1 provide evidence linking SOX4 with metastasis (Scharer et al., 2009). Not only is SOX4 active in metastasis, but recent evidence has also shown that SOX4 regulates the tumor suppressor activity of p53. SOX4 is required for the activation of p53 in response to DNA damage by physically interacting with p53 and inhibiting Mdm2-mediated p53 ubiquitination (Pan et al., 2009). Playing roles in both metastasis and DNA damage response, SOX4 appears to have dual functionality as an oncogene and as a tumor suppressor.

SOX4 protein also seems to play an important role in the Wnt pathway. SOX4 is a 47kDa protein that has sequence and structural homology to TCF/LEF family of transcription factors (Sinner et al., 2007). β-catenin converts TCF/LEF from a transcriptional repressor into an activator of target genes and as a consequence regulates important pathways throughout development. In the absence of activated β-catenin, TCF/LEF transcription factors block Wnt target genes by interacting with the Groucho family of transcriptional repressors (Sinner et al.,

2007). The importance of the TCF/LEF transcription factors in mediating Wnt signals and the sequence homology of SOX4 to TCF/LEF leads us to believe SOX4 plays a significant role in the Wnt pathway. Further evidence has demonstrated that SOX4 interacts and cooperates with β-catenin to activate gene expression (Sinner et al., 2005; Scharer et al., 2009). SOX4 promotes Wnt signaling activity and acts as an agonist in promoting proliferation. Together SOX4 transcription factors stabilize β-catenin leading to the implications that SOX proteins regulate developmental and pathological processes through the regulation of Wnt signaling (Sinner et al., 2007). A recent paper has shown that SOX4 stabilizes β-catenin activity through induction of CK2. CK2 was shown to be upregulated based on SOX4 concentration suggesting that SOX4 stimulated the Wnt signaling pathway by inhibiting degradation of the β-catenin via a CK-2 dependent mechanism (Lee et al., 2010).

Nevertheless, very little is known about SOX4 protein homeostasis or how SOX4 protein is targeted for degradation. By specifically exploring SOX4 degradation, we anticipate that we may gain new insights into the function and regulation of SOX4 in the Wnt pathway and prostate cancer progression.

HYPOTHESIS

To examine the role of SOX4 in the Wnt pathway, we have studied the possible association of SOX4 with GSK-3β and the pathway by which SOX4 is degraded.

GSK-3β is a serine-threonine kinase crucial in the Wnt pathway due to its role in regulating the proteolysis of β-catenin (Ali et al., 2001). Analysis of the amino acid sequence of SOX4 revealed sites that strongly correlate with GSK-3β phosphorylation motifs. The activated kinase stucture of GSK-3β obtains an orientation of its two domains that optimally recognizes two Ser residues separated by three residues – SXXXS (Haar et al., 2001). This motif appears in the SOX4 amino acid sequences at the locations of Ser372, Ser 376, and Ser 380. Here, we mutated the three putative phosphorylation sites on SOX4 and saw increased SOX4 protein levels in the mutant compared to the wild-type (WT). We hypothesized that the increased abundance of mutant SOX4 protein was due to increased resistance to degradation by the ubiquitin-dependent proteasome process, in a manner similar to that of β-catenin.

METHODS

Site-Directed Mutagenesis

Analysis of the amino acid sequence of SOX4 indicated three sites that strongly suggest GSK-3β recognition motifs. A former rotating graduate student, In Ki Cho performed site-directed mutagenesis to the pcDNA3.1HASOX4 vector (Figure S2) to mutate Thymine to Guanine at three sites- 2096, 2108, 2120 (Figure S3). Using the Change-IT™ Multiple Mutation Site Directed Mutagenesis Kit (USB) kit in combination with a total of 5μM of Betaine (Sigma) added to the PCR reaction, hybridization of a mutation inducing primer to the wild type plasmid in the PCR cycle was used successfully in inducing a mutation in the newly synthesized plasmid. PCR was used to amplify the mutated plasmid while the original plasmid was digested by a methylation site-specific restriction enzyme DPnI. The plasmid was transformed into XL-10 gold competent cells (Stratagene) which were then plated and selected for growth on LB amp plates.

At amino acid sites 372, 376, and 380, the triple mutant SOX4 clone carried three serine to alanine mutations in putative GSK-3β phosphorylation sites. The mutations were confirmed by sequencing analysis.

Cell Lines

The experiments were performed using the $ARCaP_E$ human prostate cancer cell line (Novicure). $ARCaP_E$ cells exhibit markers indicative of epithelial cells and serve as a model of tumor behavior before EMT. Cells were incubated at 37ºC in Prostate Epithelial Cell Medium (MCaP) growth media (Invitrogen) with 5% Fetal Bovine Serum (FBS), 100u/ml penicillin/streptomycin, and 1% L-glutamine.

In addition to $ARCaP_E$ cells, we used two forms of LNCaP cells: LNCaP and LNCaP-HASOX4 (ATCC). LNCaP cells are an androgen-sensitive human prostate cancer cell line, and the LNCaP-HASOX4 cell line stably expresses HASOX4. LNCaP cells were incubated at 37ºC in T-Medium with 5% FBS, 100 u/ml penicillin/streptomycin, and 1% L-glutamine.

Transient Transfections

$ARCaP_E$ prostate cancer cells were transiently transfected at 80% confluency using Lipofectamine 2000 (Invitrogen) and Reduced Serum Media Opti-MEM (Invitrogen). Cells were transiently transfected with 10µg of pcDNA3.1-HASOX4 triple mutant (Mut), 10µg of the pcDNA3.1-HASOX4 9 (WT), and 10µg of pcDNA3.1 (V) as a negative control. After 48 hours, cells were lysed with IP lysis buffer (137mM NaCl; 20mM Tris pH 8.0; 10% glycerol, 1% Triton X-11). To 1mL of lysis solution we added 10µl of phenylmethylsulfonyl fluoride (PMSF) and 2µl of Aprotinin (Fisher Chemical). Cells were lysed for 20 minutes rocking at 4ºC, and then whole cell lysate was collected.

Western Blots

Lysate samples collected from each treatment were boiled in Laemmli sample buffer (Biorad) for 5 minutes at 100 ºC and then analyzed on 10% or 7.5% SDS-PAGE gel at 100V. The gel was transferred to a nitrocellulose membrane or PVDF membrane at 4ºC, 120V for 90 minutes. The membrane was blocked with 5% Non-fat dairy milk (NFDM) in PBS-T for 40 minutes and then probed with primary antibody overnight at 4ºC in 3% NFDM in PBS-T (Table S1). The blot was also probed with the secondary antibody in 3% NFM at room temperature for

90 minutes. Blots were developed using RapidStep ECL Reagent (CalBiochem) on either Blue Basic Autorad Film 8X10" (GeneMate) or Amersham Hyperfilm^{TM} ECL (GE Healthcare).

Proteasome Inhibition

MG132 reduces degradation of ubiquitin-conjugaed proteins in mammalian cells by interfering with the 26S complex. Varying concentrations and time points of MG132 treatment were tested before determining an optimal concentration and treatment time. MG132 was purchased from Calbiochem and stock solution of 100mM was reconstituted in DMSO. ARCaP$_E$ cells were transiently transfected with the control plasmid pcDNA3.1-HASOX4 and the vector only pcDNA3.1. After 48 hours, the cells were treated with 10µM of MG132 for 5 hours and then cell lysate was collected. Detection of ubiquitination was confirmed by the detection of a laddering effect of poly-ubiquitinated SOX4 in Western Blots.

Endosomal Acidification Inhibition

Chloroquine is a lysosomotropic agent that prevents endosomal acidification by accumulating inside the endosomes and lysosomes. The accumulation disrupts the acidic pH within the endosome and lysosomes leading to inhibition of lysosomal enzymes and prevention of endosome and lysosome fusion. Chloroquine was purchased from InvivoGen and stock solution of 100mM was reconstituted in water. Varying concentrations for a 24 hour time point were tested. LNCaP-HASOX4 cells stably expressing SOX4 were treated with 10µM, 50µM, and 100µM. After 24 hours, the cells were lysed and analyzed on 10% SDS gel.

Immunoprecipitation

Immunopreciptation was performed to detect ubiquitination of immunoprecipitated HASOX4. 50µl of Fast Flow G Agarose beads (Millipore) slurry was used with 4 µl of either 12CA5, anti-HA monoclonal antibody, or Mouse IgG as a control. Whole cell lysates were incubated with the beads rotating for 90 minutes at 4ºC. The samples were washed with IP lysis buffer 2 times and washed with cold 1X Phosphate Buffered Saline (PBS) 3 times. Samples were boiled in 50µl of SDS sample buffer and then loaded on SDS gel.

Confocal Microscopy

To examine the localization of SOX4 in $ARCaP_E$ and LNCaP cells, confocal microscopy was performed. With the help of Mattie Feasel in the Nusrat lab, an antibody to HASOX4 was used to detect SOX4 localization following transient transfections. For immunofluorescent staining, cells were fixed with 3.7% Paraformaldehyde (PFA) and permeabilized with 100% EtOH for 20 minutes at -20ºC. In between fixing, staining, and antibody incubation, the cells were washed with Hank's Balanced Salt Solution with Ca^{2+} and Mg^{2+} (HBSS) pH 7.4. Cells were incubated with the primary anti-HA antibody 16B12 and secondary antibody Mouse HRP in blocking buffer of 3% BSA in $HBSS^{+}$. Nuclei were stained with the DNA stain Topro. Coverslips were mounted on slides using 10µ of p-phenylene and then imaged on a Zeiss 510M confocal microscope.

RESULTS

The first blot detects differences in SOX4 expression between the wild type (WT) plasmid and the mutant (Mut) plasmid made by In Ki Cho. The S. Mut lane was also a mutant made by In Ki Cho, but for the purpose of this paper, we focus only on the triple mutant (Mut). In Figure 1, the band seen around 75kDa is HASOX4. The predicted molecular weight for SOX4 is 47kDa. The blot shows two bands in the WT lane and three bands in the Mut lane. The bands for mutant SOX4 are visibly darker and suggest a higher abundance of SOX4 in the mutant than the wild type. While the cause of the varying number of SOX4 bands is still unknown, we believe the bands represent some type of post-translational modification. The dark middle band in the SOX4 Mutant suggests a possible accumulation of an intermediate post-translationally modified SOX4. This intermediate form of SOX4 may normally rely on GSK-3β phosphorylation for its modification into the 75kDa SOX4.

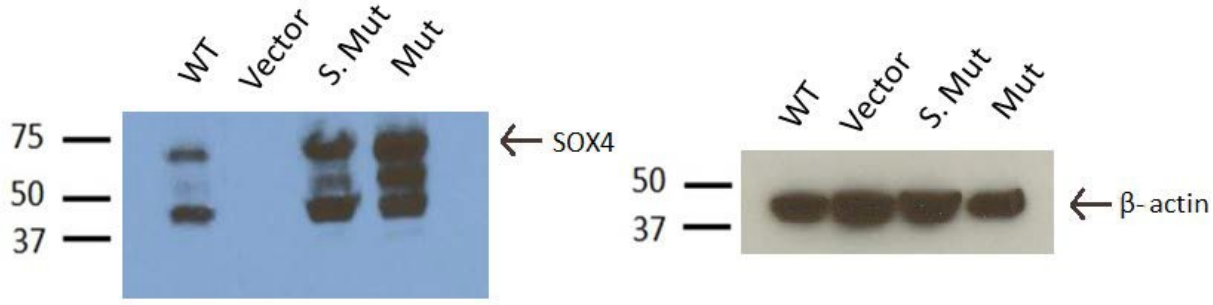

Figure 1: Expression of HASOX4 in the Wild Type Plasmid, Vector Only, and Mutant Plasmid
100mm tissue culture dishes of $ARCaP_E$ cells were transiently transfected with 10µg of the mutant (Mut) plasmid and 10 µg of the wild type (WT) plasmid. Cells were lysed and 60µg of protein was analyzed on a 7.5% SDS gel. The blot was probed with 1:1000 dilution of 16B12 anti-HA antibody and 1:10000 dilution of Mouse HRP secondary antibody.

To optimize the conditions of the MG132 proteasome inhibitor treatment, we tested a 10µM concentration of MG132 at varying time points. Figure 2A shows a five minute exposure of a blot probed for β-catenin – a protein known to be degraded by the ubiquitin-proteasome pathway. The band at 94kDa shows a slightly increased amount of β-catenin after the 24 hour treatment, but the detection of poly-ubiquitinated β-catenin only appears as a faint laddering effect.

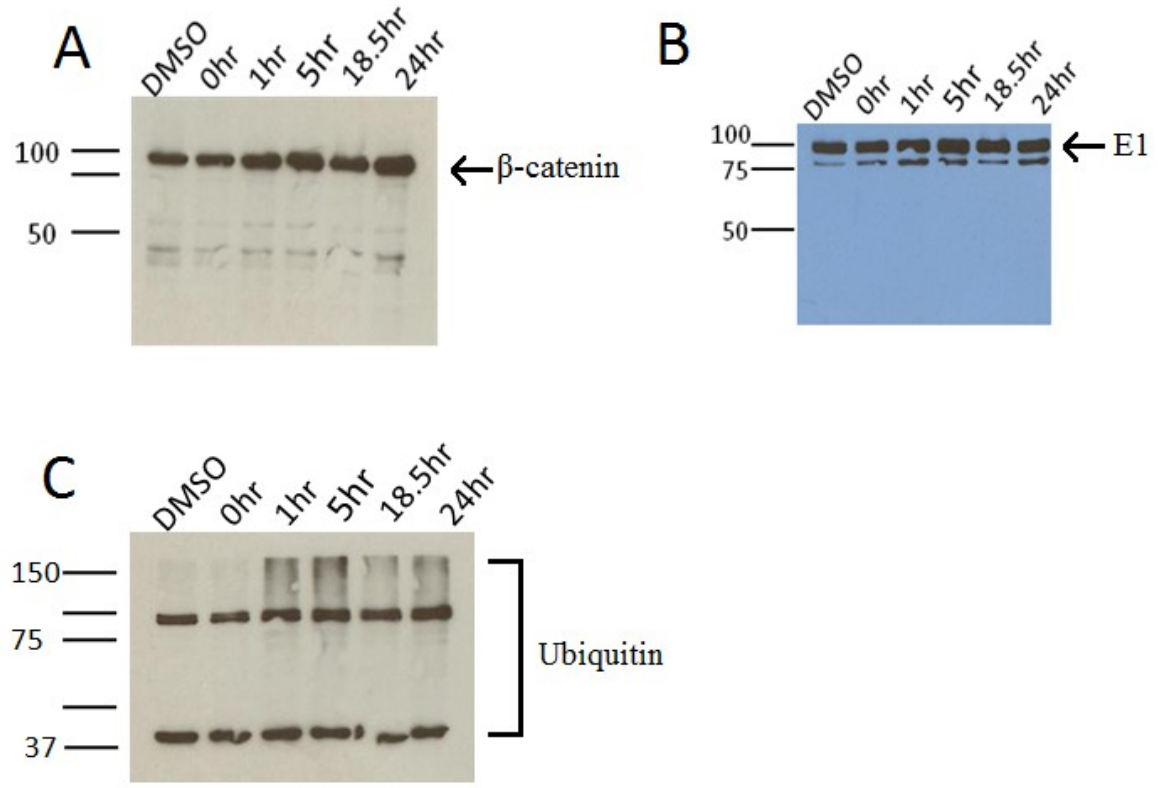

Figure 2: Optimization of MG132 conditions. Membrane probed for the following proteins: (A) β-catenin, (B) Ubiquitin E1 (C) P4D1
6-well plates of $ARCaP_E$ cells were treated with 10µm of MG132 for 0 hr, 1hr, 5hr, 18.5hr, and 24 hr. Cells were lysed with a solution of 50µl of lysis buffer, PMSF, and Aprotinin. Whole cell lysates were loaded into each lane and analyzed on a 7.5% gel.

The same nitrocellulose membrane was probed for Ubiquitin E1 without stripping the membrane (Figure 2B). E1 is detected at 110kDa as the expected doublet and is present

throughout the lanes in relatively equal amounts. E1 catalyzes the first step of ubiquitination, and its detection suggests the presence of the ubiquitin pathway. In Figure 2C, the membrane was probed with P4D1 an antibody to ubiquitin-conjugated proteins. The smears seen after MG132 treatment seem to indicate an accumulation of ubiquitin in the cell. Although the amount of ubiquitin-conjugated proteins does not appear to accumulate with a longer MG132 treatment, the results show weak MG132 effects. We next examined the effect of MG132 on SOX4 protein.

We tested varying concentrations of MG132 and probed for HASOX4 to see the effect of MG132 on SOX4 levels. In Figure 3, the blot was probed with anti-HA antibody to detect HASOX4. MG132 had only a small effect on SOX4. The uneven SOX4 protein

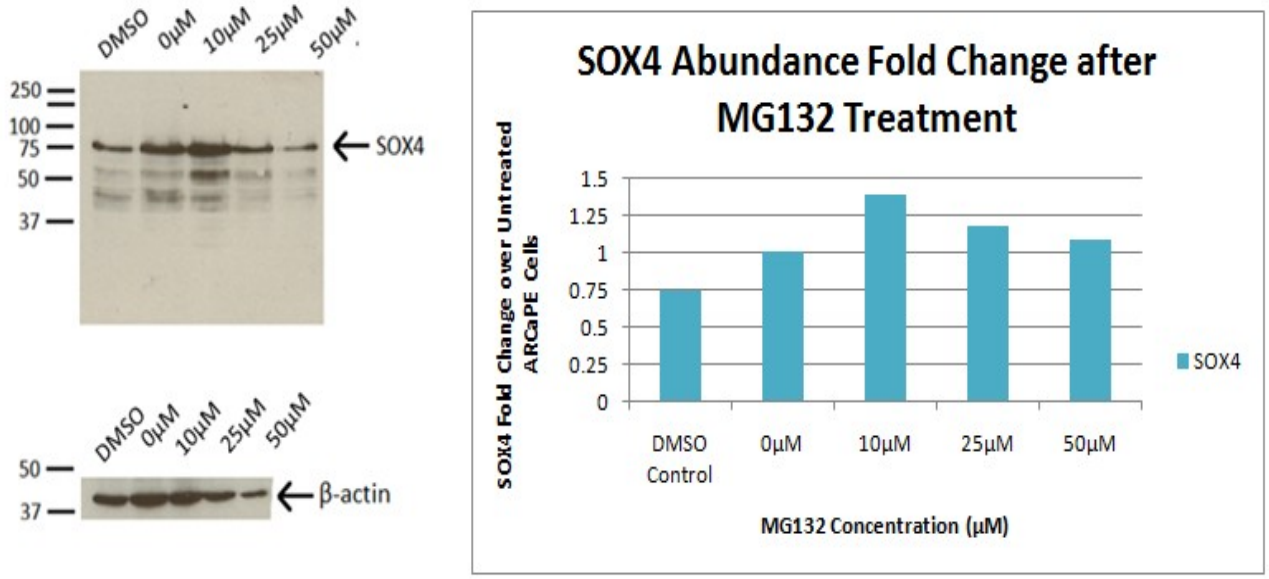

Figure 3: Detection of HASOX4 after treating ARCaP$_E$ cells with varying MG132 concentrations for 5 hours

100mm tissue culture dishes of ARCaP$_E$ cells were transiently transfected with 10μg of pcDNA3.1HASOX4 plasmid. 48 hours after the transfection, cells were treated with a DMSO control, 0μM MG132, 10μM MG132, 25μM MG132, and 50μM MG132 for 5 hours. 50μg of protein was loaded onto a 10% gel. SOX4 fold change was normalized to β-actin loading control.

levels directly correlate with the amount of β-actin in each lane. The β-actin control suggests the darker SOX4 band in the 10μM MG132 lane is partially a direct result of more protein loaded into the well.

Although these results suggest SOX4 is not degraded by the proteasome, we next tested whether we could observe changes in ubiquitination of SOX4 by immunoprecipitation of SOX4 followed by probing for SOX4 and ubiquitin.

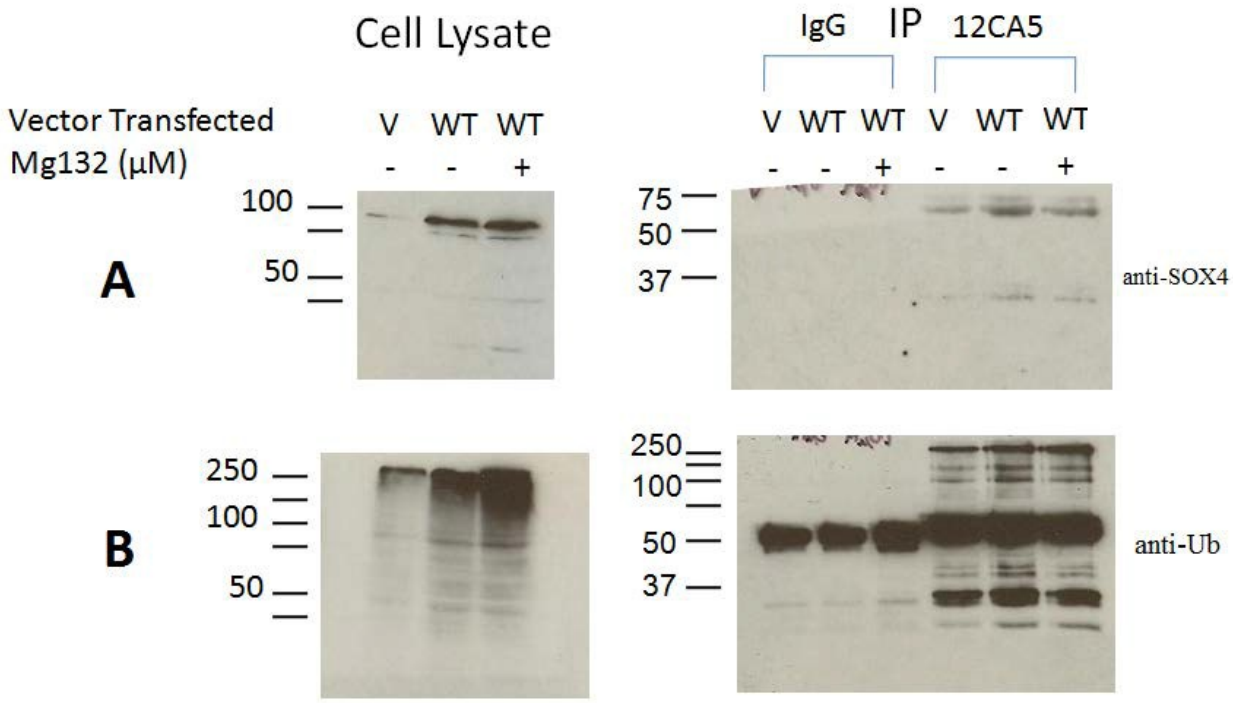

Figure 4: Immunopreciptation of SOX4 and Detection of Ubiquitination. A)anti-SOX4, B) anti-Ub
$ARCaP_E$ cells were transiently transfected with 10μg of vector plasmid or with 10μg of wild type plasmid. The experiment involved vector only (V), wild type (WT) plasmid untreated, and wild type plasmid treated with 10μM of MG132 for 5 hours. 50μl of bead slurry and 4μl of 12CA5 or Mouse IgG were used to immunoprecipitate SOX4.

In Figure 4A, the blots were probed for SOX4 (Lifespan). The bands around 75kDa indicate relatively equal SOX4 expression even after MG132 treatment (Figure 4A). The

presence of more SOX4 in the WT lanes compared to the V lane suggests the transfection was successful. Looking at the IP results in Figure 4A, the absence of bands in the lanes of Mouse IgG also confirms the success of the negative control. Both blots in Figure 4A do not show a laddering effect of poly-ubiquitinated SOX4. To ensure the MG132 treatment worked, the same blots were then probed for ubiquitin to detect any differences in the levels of ubiquitin in the cell. In Figure 4B, the blot of the whole cell lysate provides evidence of a laddering effect, suggesting that the MG132 worked. The cells transfected with WT plasmid and treated with MG132 show a much darker laddering effect compared to the vector and the untreated WT. The laddering demonstrates the accumulation of ubiquitin-conjugated proteins that cannot be degraded by the proteasome. The IP results show similar band patterns between the V and the WT lanes suggesting the IP might have been unsuccessful.

While SOX4 does not appear to be degraded through the ubiquitin-proteasome pathway, another possibility for the abundance of SOX4 (Figure 1) is a change in the localization of SOX4 within the cell. To investigate possible changes in SOX4 localization in $ARCaP_E$ cells we performed confocal microscopy. From the confocal results, SOX4 can be seen mostly concentrated in the cytoplasm of $ARCaP_E$ cells. Figure 5D and 5G show no significant difference in the localization of SOX4 in the WT-HASOX4 and the Mut-HASOX4. The data suggests the abundance of SOX4 seen in Figure 1 is not due to a movement of SOX4 or increased amount of SOX4 in the cytoplasm.

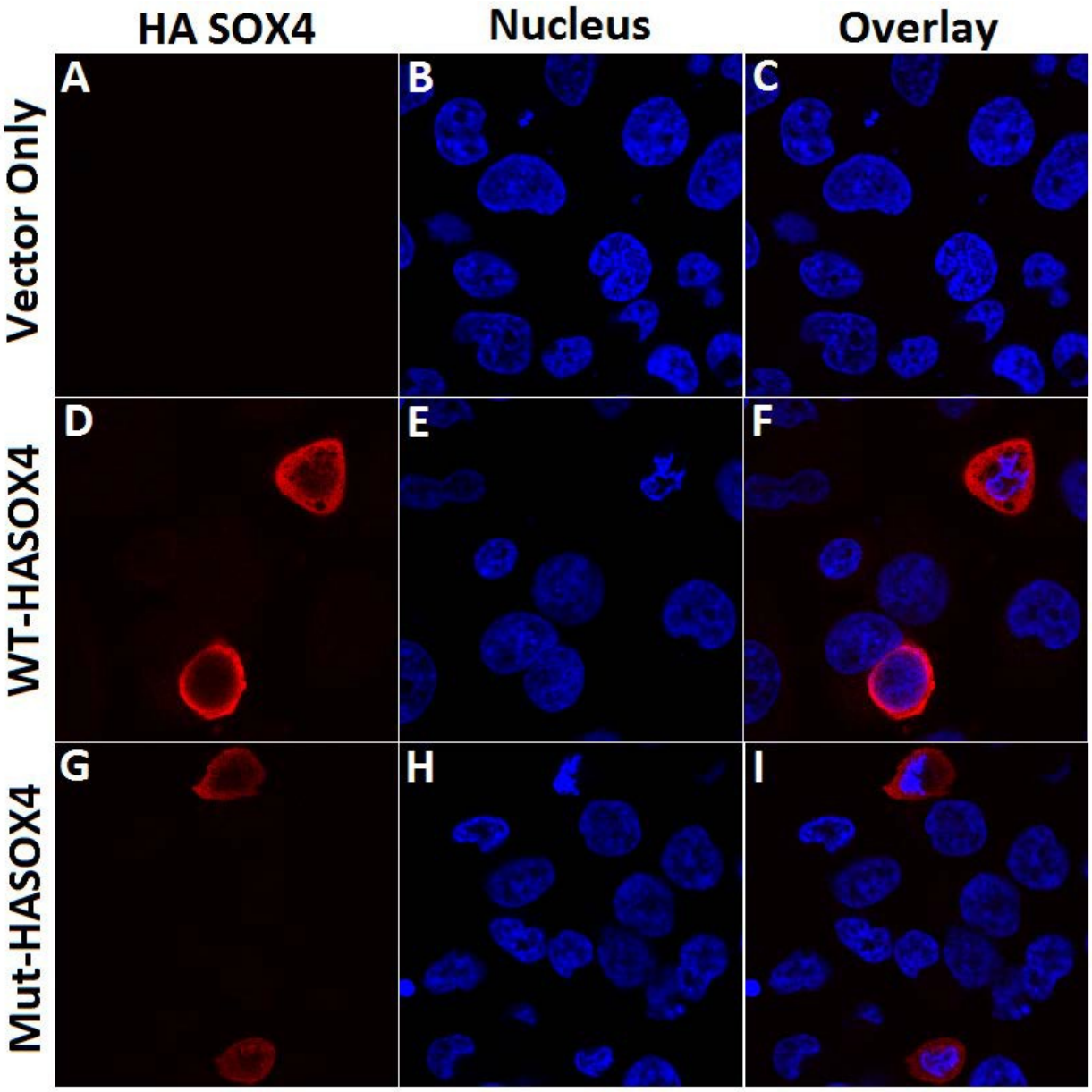

Figure 5: Confocal Microscopy of ARCaP$_E$ Cells Stained for HA-SOX4 and Nuclei – 100X Magnification

6-well plates of ARCaP$_E$ cells were transfected with 2µg of vector-only plasmid, wild type HASOX4 plasmid, and the triple mutant plasmid. Cells were fixed and stained 48 hours after transfection. HASOX4 was detected with 16B12 and the nuclei were stained with Topro.

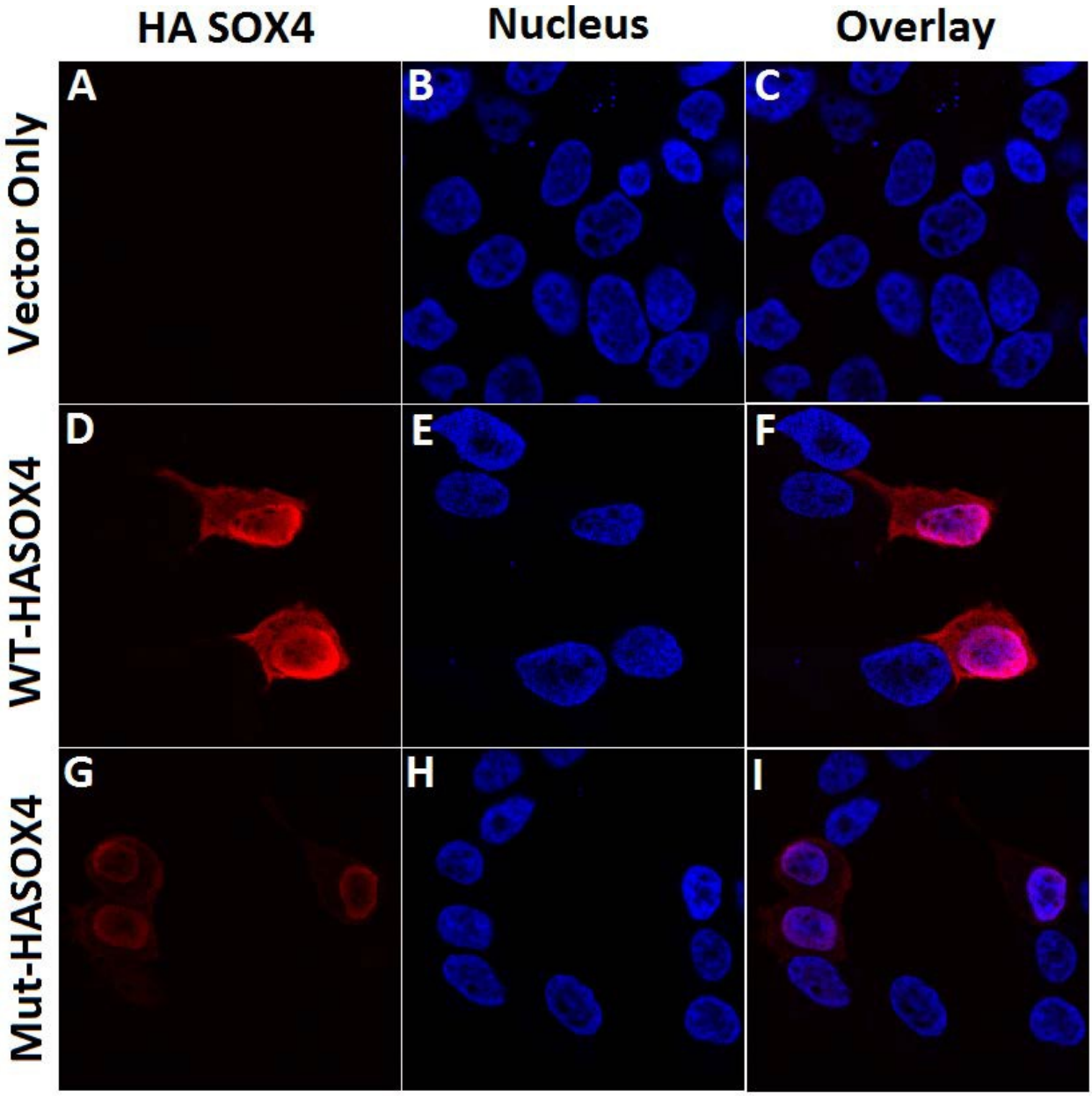

Figure 6: Confocal Microscopy of LNCaP Cells Stained for HA-SOX4 and Nuclei – 100X Magnification

6-well plates of LNCaP cells were transfected with 2µg of vector-only plasmid, wild type HASOX4 plasmid, and the triple mutant plasmid. Cells were fixed and stained 48 hours after transfection. HASOX4 was detected with 16B12 and the nuclei were stained with Topro.

In our lab, SOX4 was previously shown to be localized in the nucleus of LNCaP cells. In order to examine if the localization changes when the mutant is transfected into LNCaP cells, we also performed a confocal microscopy with LNCaP cells. The same amounts of each plasmid were transiently transfected into LNCaP cells. In Figure 6, SOX4 seems predominantly localized in the nucleus in LNCaP cells. Similar to the results found in the $ARCaP_E$ cells, the Mut-HASOX4 and WT-HASOX4 fail to induce any noticeable differences in SOX4 localization (Figure 6D, 6G). The only conclusive result that can be drawn from these two figures is the difference in SOX4 localization in different cell lines. While the $ARCaP_E$ cell line showed SOX4 more cytoplasmic, LNCaP cells show SOX4 more localized in the nucleus. Unlike the results of the western (Figure 1), the confocal results show a lower expression of SOX4 in the Mut-HASOX4 than in the WT-HASOX4.

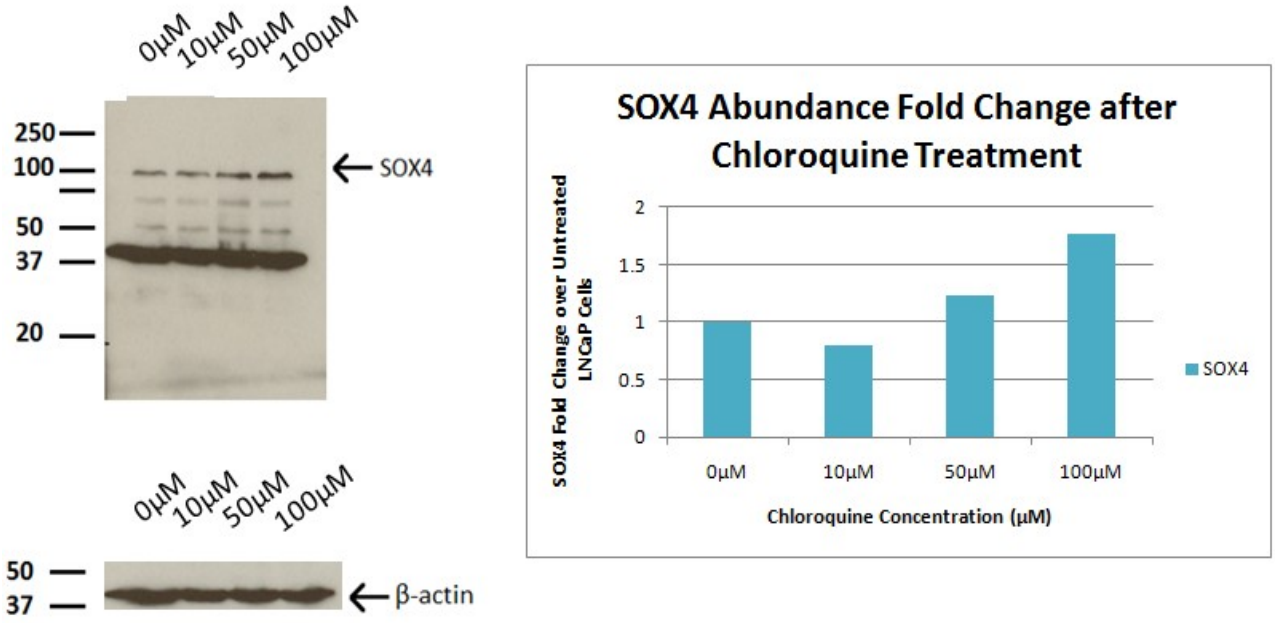

Figure 7: LNCaP- HASXO4 treated with Chloroquine for 24 hours
6-well plates of LNCaP-HASOX4 cells were treated with 0μM, 10μM, 50μM, and 100μM concentrations of Chloroquine for 24 hours. Whole cell lysates were analyzed on a 10% gel. SOX4 fold change was calculated by normalizing SOX4 abundance to theβ-actin loading control.

With the results from Figure 5 and Figure 6 indicating that the abundance of SOX4 is not merely an issue of localization, we returned to the possibility that the three mutated SOX4 sites may still confer resistance to an alternative degradation pathway. Using LNCaP cells that stably express HASOX4, we treated the cells with the endosome acidification inhibitor Chloroquine. In Figure 7, the increasing amount of SOX4 appears to be Chloroquine concentration-dependent. The β-actin bands suggest the samples are relatively equally loaded, but the HASOX4 band after 100μM treatment of chloroquine still appears darker. This blot shows that when the endosome and lysosomes are inhibited, SOX4 accumulates in the cell.

DISCUSSION

Our interest in SOX4 and the Wnt pathway formed the basis for the experiments that were performed in this project. Previous research indicating that SOX4 may function to stabilize β-catenin and increase β-catenin/TCF activity links SOX4 with the Wnt pathway, but the exact role of SOX4 in the Wnt pathway is still unclear (Sinner D et al., 2007). Hoping to discover more about the association of SOX4 with the Wnt pathway, we analyzed the SOX4 protein expression of a mutant and wild type SOX4. We examined the effects of mutating three putative GSK-3β phosphorylation sites and found that these sites play a role in the SOX4 degradation pathway. By inhibiting the proteasome degradation pathway in $ARCaP_E$ cells and the lysosomal pathway in LNCaP cells, we discovered that the inhibition of the lysosomal degradation pathway seems to have a greater effect on SOX4 accumulation than the inhibition of the proteasome-dependent degradation pathway. This leads us to believe the degradation of SOX4 is possibly associated with the lysosomal degradation pathway. A recent paper showing that GSK-3β plays a role in regulating Rab5-mediated endocytosis of AMPA receptors implies the possible role of GSK-3β in mediating lysosomal protein degradation (Wei, 2010). Our new hypothesis suggests that GSK-3β plays a role in targeting SOX4 to the lysosome for degradation. The role of GSK-3β in regulating SOX4 degradation is also supported by our data showing increased expression of SOX4 in the SOX4 mutant. The resistance of mutant SOX4 to phosphorylation by GSK-3β ultimately may lead to SOX4 accumulation in the cell.

Difficulties that we encountered during this project involved the optimization of MG132 conditions and the transfection efficiency. The poly-ubiquitinated laddering effect we looked for in many western blots was not as pronounced as we expected and was not distinct enough to make conclusive judgments. In addition to the weak detection of the ubiquitin laddering effect,

transfections efficiency also seemed low. Transfection of cells with GFP plasmid showed a high transfection efficiency; however, the same transfection conditions did not improve the transfection efficiency of the cells with our vector-only, wild type, and mutant plasmids. A low transfection efficiency results in difficulty in determining whether the effects we saw were just the results of a few over-expressing cells. Improving the transfection efficiency would help improve the western blots and immunoprecipitation. In addition to improved western blots and immunoprecipitations, the localization of SOX4 can also be more definitively examined when more cells are transfected in the confocal experiments. The localization of SOX4 seems to be following a cell-line specific established trend, but seeing this trend in more cells would strengthen this conclusion.

In addition to the data gathered about SOX4 localization, we noticed the mutant appeared less abundant in the confocal experiments. This may be due to epitope masking, since the mutant was more abundant in western blots. Lastly, the immunoprecipitation of SOX4 never seemed to be able to pull down enough SOX4. Although the Mouse IgG negative controls worked, the IP still did not seem to be performing optimally (Figure 4). The similar band patterns between the WT plasmid and the vector-only plasmid (V) suggest the bands seen are just background. Unfortunately, the results of the IP do not conclusively demonstrate that SOX4 protein levels are unaffected by inhibiting the proteasome.

Another interesting result is the slight accumulation of SOX4 after 10μM treatment of MG132. Although the increase of SOX4 is more prominent with the chloroquine inhibition, the results from Figure 3 suggest we cannot fully rule out the SOX4 degradation via the proteasome pathway. Figure 4 suggests there was an accumulation of ubiquitin-conjugated proteins, but a lack in increased SOX4 levels. Further experiments need to be performed to determine the

possibly role of the proteasome on SOX4 degradation. The implications of this project's findings are ultimately important to understanding SOX4 and the development of cancer therapeutics.

The results of the project have given us a fundamental understanding of SOX4 regulation. The possible regulation of SOX4 by GSK-3β phosphorylation and degradation of SOX4 through the lysosome pathway is a novel discovery, and the implications of these findings provide additional insights into GSK-3β and SOX4 inhibitors. With SOX4 overexpression in many different types of human malignancies, the ability to regulate SOX4 abundance could be an excellent target for controlling tumorigenesis. In Hepatocellular carcinoma (HCC), SOX4 has been found to serve as a molecular indicator for HCC prognosis (Zheng et al., 2010). As SOX4 increasingly becomes a method of predicting cancer disease outcomes, the development of SOX4 inhibitors will certainly be beneficial. In addition to potentially influencing SOX4 inhibitors, these results also shed light onto the mechanisms of GSK-3β inhibitors. As more cancer drug developments focus on GSK-3β inhibitors, the understanding of GSK-3β role in the cell will lead to more discoveries. From the results of this project, we have learned how SOX4 is degraded and by applying this fundamental knowledge, we can continue to explore tumorigenesis and find ways to develop drugs against cancer.

FUTURE DIRECTIONS

To further solidify the data collected in this project, we can perform mass spectrometry to confirm whether the three sites are phosphorylated. Additionally, the overexpression of SOX4 was seen in transiently transfected $ARCaP_E$ cells while the detection of increased SOX4 after Chloroquine treatment was seen in LNCaP-HASOX4 cells. We need to make sure the results are consistent even in different cell lines. For example, the overexpression of SOX4 after transfecting the mutant HASOX4 plasmid should be tested in LNCaP cells while the Chloroquine treatment should be performed on $ARCaP_E$ cells. With these additional experiments, we can develop stronger evidence for GSK-3β regulation of SOX4 degradation. Another possible experiment is to inhibit GSK-3β with specific small molecule inhibitors to and see if there is also an accumulation of SOX4.

Finally, we would like to assess the effect of Wnt on SOX4 protein levels. Wnt disrupts the ability of GSK-3β to phosphorylate β-catenin, and if our new hypothesis is correct, we should see increased levels of SOX4 mutant compared to the wild-type after treatment with Wnt. Data showing whether SOX4 is enhanced or inhibited by Wnt would provide further understanding of the role of SOX4 in the Wnt pathway. Ultimately, the goal of this project is to determine the pathway by which SOX4 is degraded and to discover the proteins involved in this process. As the function and role of SOX4 becomes more defined, SOX4 could become an important target in future cancer therapies.

SUPPLEMENTARY FIGURES

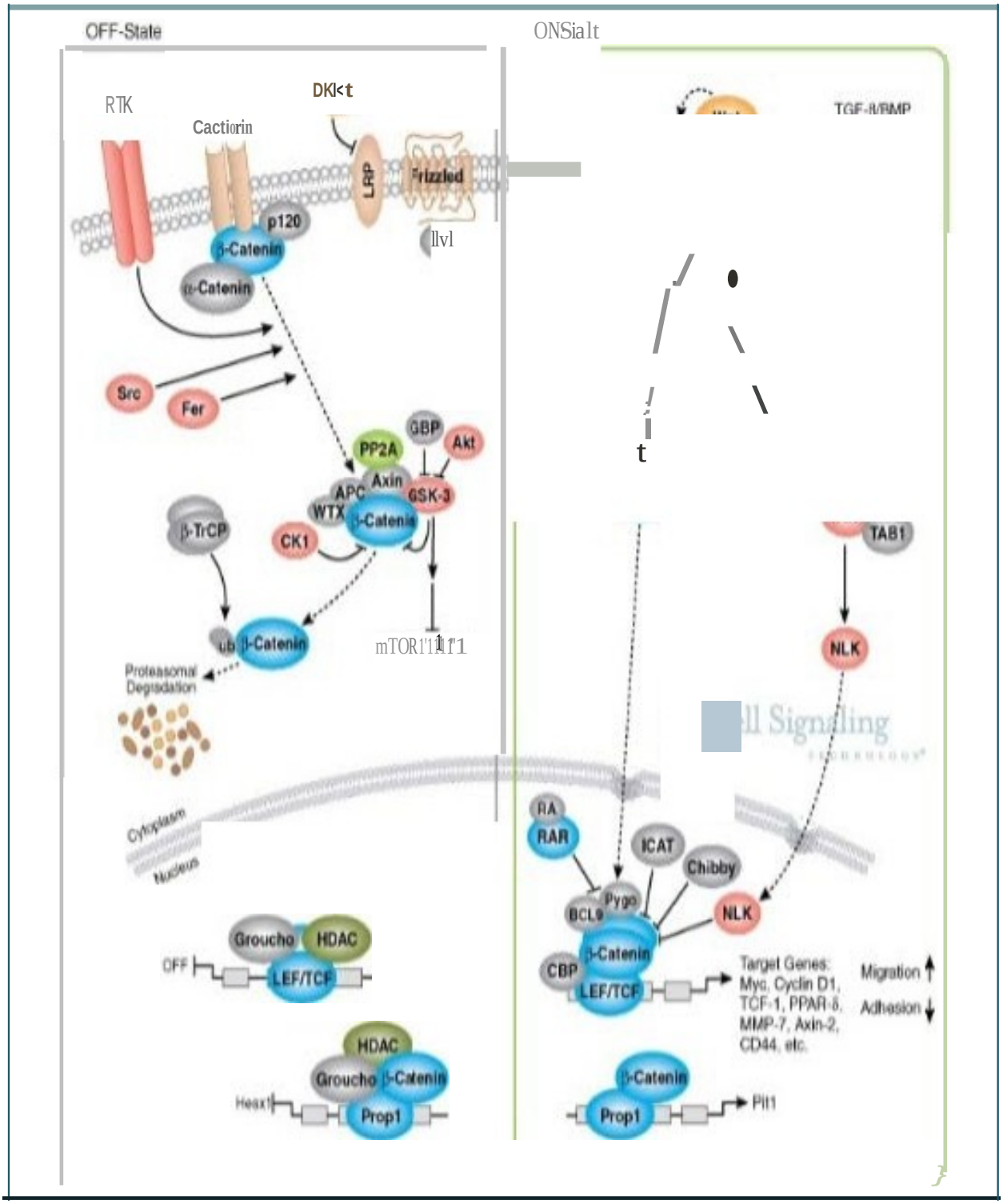

Figure Sl. WntlfJ-camnin Signaling Pathway. Adapted from Cell Si ing Teclmology, Inc (2010) http://www.cellsi.cornlreference/pathway!Wnt_beta_Catenin.html.

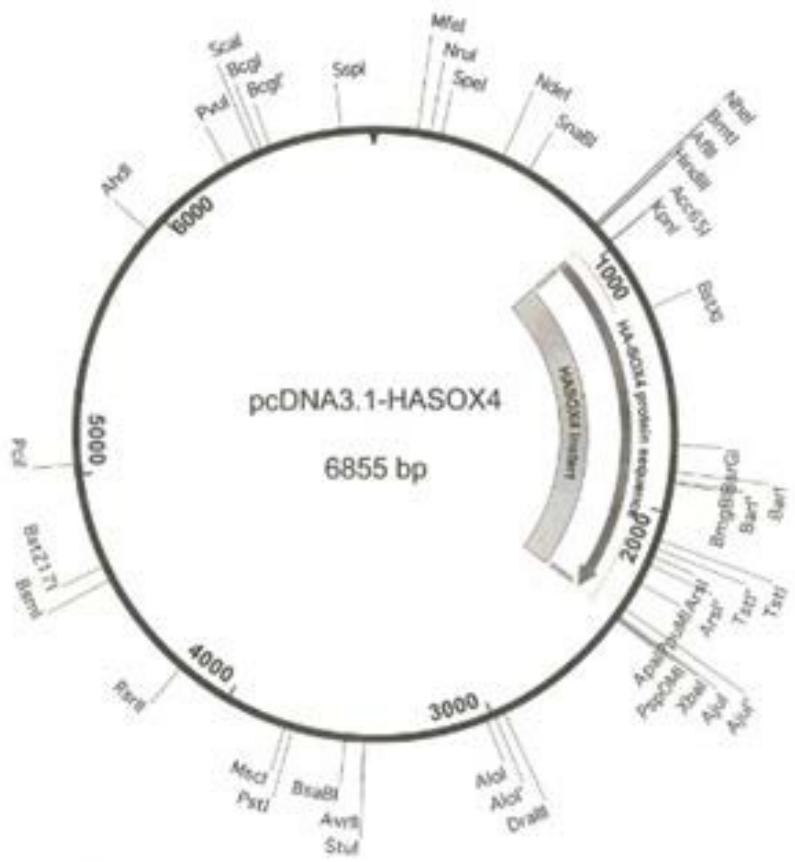

Figure S2: pcDNA3.1-HASOX4 wild type plasmid

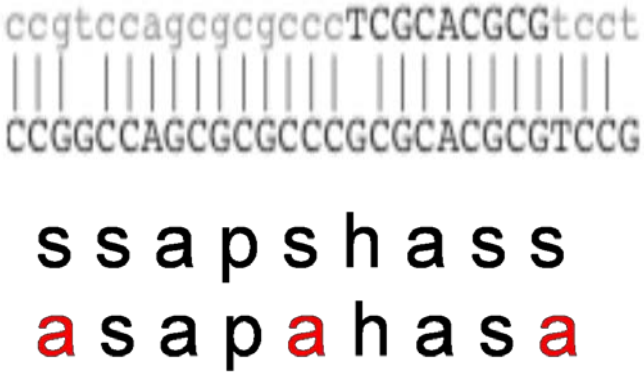

Figure S3: Site-directed Mutagenesis

Primary Antibody	**Dilution**	**Company**
HA.11 Monoclonal Antibody 16B12	1:1000	Covance
β-Actin (8H10D10) Mouse mAb	1:5000	Cell Signaling Technology
P4D1	1:1000	Covance
SOX4	1:1000	Lifespan
B-catenin	1:1000	Santa Cruz
12CA5	1:1000	Ascites

Table S1. Western Blot and Immunoprecipitation Antibodies Used

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