The Mechanism of PTOV1 Regulation by 14-3-3, HUWEI1 and SGK2

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The Mechanism of PTOV1 Regulation by 14-3-3, HUWEI1 and SGK2

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

The Mechanism of PTOV1 Regulation by 14-3-3, HUWE1 and SGK2

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Prostate tumor overexpressed 1 (PTOV1) is highly expressed in several forms of cancer. High expression of PTOV1 is associated with tumor aggressiveness in several tumor types, including ovarian and breast cancer. Currently, PTOV1 is known to act both as a translational and transcriptional regulator aiding in the expression of prosurvival genes. Although PTOV1 is known to pass in and out of the nucleus in a cell cycle-dependent manner, the regulation of PTOV1 activity is not well understood and here we identify 14-3-3 as a PTOV1 interactor and show that high levels of 14-3-3 expression, like PTOV1, correlate with prostate cancer progression. Further, we identify SGK2-mediated phosphorylation at S36 of PTOV1 that is required for 14-3-3 binding. Disruption of the PTOV1-14-3-3 interaction results in an accumulation of PTOV1 in the nucleus and a proteasome-dependent reduction in PTOV1 protein levels, which requires ubiquitination at K114 of PTOV1.

We also observed HUWE1 as a PTOV1-interacting partner responsible for the degradation of PTOV1 through the proteasome. We show that loss of 14-3-3 binding leads to an increase in PTOV1-HUWE1 binding, suggesting that 14-3-3 stabilizes PTOV1 protein by sequestering PTOV1 in the cytosol and inhibiting its interaction with HUWE1. Finally, our data suggest that stabilization of the 14-3-3-bound form of PTOV1 promotes PTOV1-mediated expression of cJun. Together, these data support a model that explains how 14-3-3 and HUWE1 regulate the PTOV1 stability, localization, and function within the cell.

Keywords: PTOV1, SGK2, 14-3-3 protein, prostate cancer, HUWE1
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LIST OF ABBREVIATIONS USED

PTOV1 = Prostate tumor overexpress 1
BRCA1 = Breast cancer type 1
HPC1 = Hereditary prostate cancer 1
EGF = Epidermal growth factor
FISH = Fluorescence in situ hybridization
BCL2 = B-cell lymphoma 2
KLK2 = kallikrein-related peptidase 2
NLS = nuclear localization signals
HGPIN = high-grade prostate epithelial neoplasia
AAH = Atypical adenomatous hyperplasia
PC = Prostate Cancer
LSCC = In laryngeal squamous cell carcinoma
HPV = human papillomavirus
MED25 = Mediator of RNA polymerase II transcription subunit 25
RA = Retinoic acids
RAR = retinoic acid receptor
SGK2 = Serum Glucocorticoids kinase 2
LC-MS/MS = liquid chromatography tandem mass spectrometry
CHAPTER 1: INTRODUCTION TO PROSTATE TUMOR EXPRESS 1 (PTOV1)

1.1 Summary
Prostate-Tumor-Overexpressed-1 (PTOV1) is an oncogenic adaptor protein that was first discovered in prostate cancer and is used as a molecular marker to track tumor progression and aggressiveness. Previous studies have shown that PTOV1 is overexpressed in prostate cancer patients and highly expressed in a variety of other cancers as well, including ovarian, breast, nasopharyngeal, squamous laryngeal, hepatocellular and urothelial cancers. PTOV1 interacts with a variety of proteins in the nucleus and cytoplasm to regulate changes in gene expression at the transcriptional and translational levels that promote tumor growth. In addition, the movement of PTOV1 between the nucleus and cytoplasm is accompanied by changes in PTOV1 protein levels. However, the mechanisms that control PTOV1 cellular localization and protein stability are not well understood. This review provides an update on the current findings of the upstream regulation of PTOV1 protein, which will be important in understanding the mechanism that controls PTOV1 localization and stability.

1.2 Introduction

1.2.1 Background of prostate cancer
Cancer is a disease in which tissue homeostasis is disrupted by mutations that activate oncogenes, thereby causing cells to proliferate uncontrollably. However, the mechanisms that control many oncogenes are not completely understood. Prostate cancer is the second most common cancer in males and is caused by genetic changes in prostate cells that drive cancer growth\(^1\). Previous studies have shown that androgen receptor pathways or related signaling pathways are responsible for the conversion of normal prostate cancer cells to malignant cells. Other reasons for this conversion could be mutations in tumor suppressor genes, oncogenes, and other genes such as BRCA1 and HPC1\(^2,3\). When there is one malignant transformation, cells from that malignant population can spread to other
areas of the prostate, giving rise to multiple cancer lesions. Prostate intraepithelial neoplasia is the earliest recognizable lesion containing cells with malignant features in prostate cancer. There are several molecular markers such as the cyclin-dependent kinase inhibitor p27, or the EGF receptor that mark prostate cancer cells. These molecular markers change in intraepithelial neoplasia, including loss of the expression of cyclin-dependent kinases and overexpression of the EGF receptor\textsuperscript{4,5} which may be a reason for the malignant conversion of prostate cells.

1.2.2 Relationship between prostate cancer and PTOV1

To identify the exact cause of this malignant transformation of prostate cancer cells, both genetic and epigenetic approaches have been taken into consideration. These approaches have been used to identify changes in molecular markers in the early stages of prostate cancer\textsuperscript{6,7}. Previous studies identified PTOV1 as an overexpressed protein in prostate cancer patients\textsuperscript{8}. The PTOV1 gene encodes a protein called Prostate Tumor Overexpressed 1, which is a conserved adaptor protein that is overexpressed in early neoplastic lesions and prostate intraepithelial neoplasia. However, due to the lack of known motifs of this protein, the function is not yet clearly understood\textsuperscript{4,8}.

1.2.3 The structure of the PTOV1 gene and protein

1.2.3.1 The gene Structure

Fluorescence in situ hybridization (FISH) identified chromosome 19q13.3 as the location for the PTOV1 gene. Chromosome 19q13.3 contains a variety of prostate cancer-related genes and androgen-regulated genes, such as the apoptotic regulator BCL2 associated X, kallikrein-related peptidase 2 (KLK2), etc\textsuperscript{8–10}. PTOV1 gene size is approximately 9.51Kb, including 12 exons. Differential splicing of PTOV1 results in at least two transcripts. The number of bases for each transcript is 1,875 bps and 1,539 bps, which translate into proteins of 416 residues and 374 residues, respectively\textsuperscript{11}. A
related gene called PTOV2, also has homology to subunit 25 of the Mediator complex which is an essential component of the eukaryotic RNA polymerase II complex regulating eukaryotic transcription.

### 1.2.3.2 The protein Structure

Prostate-Tumor-Overexpressed-1 (PTOV1) is a 46kDa adaptor protein that is conserved in vertebrates and arthropods, but not conserved in fungi. PTOV1 protein is known to interact with proteins in the cytoplasm and nucleus and regulate gene expression at both the transcriptional and translational levels. PTOV1 protein contains 416 amino acids and has two domains known as domain A (146 amino acids) and B (143 amino acids). The sequences of these two domains are related to each other and are located as tandem repeats. These domains express 66% identity and 79% similarity to each other. The protein has two putative nuclear localization signals (NLS), one in the A domain and the second in the B domain. There are two proteins known as PTOV1 and PTOV2, both of which have tandem repeats that are conserved in both types of protein. These two proteins can be found in humans, rodents, and flies. Previous studies from immunohistochemical analysis reveal that PTOV1 is overexpressed in both early and late-stage prostate cancer cells.
1.2.4: PTOV1 protein expression and its role in tumor cells and normal cells.

PTOV1 protein is overexpressed in prostate cancer cells and implicated in different pathological conditions, however, its expression can be detected in normal cells and tissues such as the heart, human brain, skeletal muscles, liver ovary, kidney, salivary glands as well. The function of PTOV1 is not clear in the above-mentioned sites. PTOV1 may have a role in atherogenesis, since PTOV1 expression in human aorta is androgen sensitive. However, PTOV1 expression in normal tissues such as the human brain, liver, etc. shows a very weak staining. The expression intensities of PTOV1 in
different tissues vary depending on the tissue type. Previous studies however show intense staining in some prostate luminal cells in the cytoplasm and nucleus\textsuperscript{8,14,15}.

PTOV1 protein expression levels in preneoplastic lesions of high-grade prostate epithelial neoplasia (HGPIN) associated with prostate cancers are high compared to normal prostate epithelial cells. This finding implicates that the expression of PTOV1 in HGPIN has an association with the cancer presence and can thus be used as a detection marker for prostate cancer, due to its elevated levels in cancerous cells. This overexpression in HGPIN is helpful in identifying premalignant lesions associated with cancer and could be helpful in cancer diagnosis. The other important factor is that PTOV1 overexpression levels in HGPIN close to prostate cancer areas are comparably higher than HGPIN located away from the cancer lesions. This fact also suggests that PTOV1 overexpression levels are associated with the localization of HGPIN around prostate cancers, thus supporting the concept of field cancerization\textsuperscript{16,17}. Atypical adenomatous hyperplasia (AAH) is a proliferative lesion that is similar to low-grade carcinoma. The expression of PTOV1 in AAH is also found to be associated with prostate cancer. Prostate adenocarcinoma has different stages such as T1, T2, T3, and T4. A previous study showed that 71\% of T2 and T3 stages of prostate adenocarcinoma overexpressed PTOV1. In addition, 59 \% of samples showed overexpression in the cytoplasm and the others showed overexpression in both the nucleus and cytoplasm. This overexpression was known to be strong\textsuperscript{8,18}. Another important finding is that the PTOV1 overexpression levels are significantly increased in metastatic tumors that have spread from the part of the body where it started, the primary site, to other parts of the body. Conversely, non-metastatic tumors show low expression levels of PTOV1 compared to metastatic tumors. Moreover, recent studies show that this PTOV1 overexpression is associated with the Ki-67 index. The Ki-67 index is a parameter that measures cancer proliferation and aggressiveness. Ki-67 is a protein in cells that increases with cell division.
Using a staining process, the percentage of tumor cells that are positive for Ki-67 can be measured. Since PTOV1 overexpression has a significant association with the Ki index, PTOV1 may have participated in active prostate proliferation\(^{19}\).

In vitro studies showed partial colocalization of PTOV1 from the cytoplasm to the nucleus in quiescent PC cells after serum stimulation at the early S phase. These observations are seen when PC3 cells are transiently transfected with PTOV1. PTOV1 transfection has led PC3 cells to enter the S phase and increased the levels of cyclin D1, suggesting that PTOV1 transfection may increase PC3 cell proliferation. Further PTOV1 has been shown to be involved in PC cell growth and metastasis in vivo\(^8\). Genomic data sets that are publicly available show high levels of PTOV1 in aggressive prostate cancer sites and other tumor types as well, suggesting that this may be due to the high rate of PTOV1 amplification at those tumor sites. Amplification frequencies above 18% are known as the highest frequencies, which can be seen in metastatic cells and neuroendocrine prostate tumors. Collectively all these data suggest that there is an association between PTOV1 expression and aggressive features of prostate cancer\(^{20,21}\).

Apart from PTOV1 overexpression in prostate cancer, several studies have shown PTOV1 overexpression in several different cancer types as well, such as breast, liver, colon, bladder, kidney, pancreas, cerebral gliomas, and ovary\(^{22,23}\). Previous studies show that there is an association between PTOV1 overexpression and high-grade malignancy in hepatocellular carcinoma, epithelial ovarian cancer, breast cancer, and clear cell renal carcinomas. PTOV1 overexpression also correlates with clinical and pathological characteristics, tumor aggressiveness, unfavorable prognosis, and poor survival in several cancer types. 94% of breast cancer samples expressed PTOV1 protein, whereas 49.1% expressed it to a higher degree. Patients who have higher expression levels of PTOV1 and lower expression levels of PTOV1 have a survival of 78 months and 115 months, respectively\(^{22}\). In
laryngeal squamous cell carcinoma, (LSCC) PTOV1 overexpression increased throughout the clinical stage. Additionally, some studies demonstrated that PTOV1 overexpression combined with human papillomavirus (HPV) provides more prognostic information for LSCC than HPV alone, suggesting that PTOV1 can be used as an early prognostic marker for LSCC as well.\textsuperscript{24-26} Highly aggressive breast cancer cells, high-grade urothelial carcinoma, show higher PTOV1 expression in the nucleus compared to the cytoplasm. This is similar to prostate cancer, suggesting that nuclear localization of PTOV1 protein may be important to cancer progression, proliferation, and aggressiveness. These data collectively suggest that PTOV1 and its transcription regulation functions may play a role in cancer progression in different types of cancers.\textsuperscript{8,22} Additionally, mutations in the PTOV1 gene can be observed in several tumor types and the majority of mutations are located in the A domain of the PTOV1 gene. R117L and R117Q mutation can be found in LSCC and prostate cancer respectively and these mutations have a medium predicted functional score, implying that these mutations may alter the PTOV1 functions, thus leading to cancer progression and survival.

1.2.5: Role of PTOV1 as a transcriptional regulator

Mediator of RNA polymerase II transcription subunit 25 (MED25) is an enzyme in humans that is encoded by the MED25 gene. MED25 is responsible for the transcription of most RNA polymerase II-dependent genes. MED25 has 3 subunits, is a component of the eukaryotic RNA polymerase II complex, and regulates eukaryotic transcription. PTOV2 was also recently found to correspond to subunit 25 of the Mediator complex. While PTOV1 has two structurally similar tandem repeats, PTOV2 only has one PTOV domain required for CBP binding. Additionally, PTOV and PTOV1 have conserved amino acid residues that are responsible for TAD binding in MED25.\textsuperscript{24-27} This implies that the PTOV domain in both proteins (PTOV1 and PTOV2) acts as an activator binding domain. On the other hand, PTOV1 does not bind with the mediator complex but MED 25 does.\textsuperscript{28} The PTOV domain
located in MED25 and PTOV1 has different biological functions. The reason for this functional difference may be due to differentially grouped amino acids around charged regions of the domains, as well as their different protein interaction patterns.

Vitamin C and its derivatives are known as Retinoic acids (RA). Retinoic acids are useful chemotherapeutics used in cancer treatments because of their functions in cell growth, proliferation, and apoptosis. In the presence of RA, retinoic acid receptor (RAR) binds to the element known as RARE and acts as a transcription factor with the association of different coregulators such as MED 25 to regulate gene expression. MED 25 regulates RAR by forming the MED25-CBP-RAR complex. Since both PTOV1 and MED25 have PTOV domains that are responsible for CBP binding, both compete for CBP binding through their conserved domains. These two proteins regulate the transcriptional function of RAR by regulating CBP location in chromatin. These observations collectively suggest that the PTOV domain in MED25 and PTOV1 re-modulate the chromatin and pre-initiation complex, which is responsible for the CBP recruitment to the transcription machinery.

In addition, there is some evidence that suggests that the PTOV domain of MED25 is responsible for the binding of the TAD domain, which is in several other transcription factors. Examples of these transcription factors are ERM/ETV5, a PEA3 member of ETS-related transcription factors, the nuclear receptor Hepatocyte Nuclear Factor 4 alpha, the transcription factor ATF6 alpha, a master regulator of the endoplasmic reticulum (ER) stress response genes, the retinoic acid receptor (RAR) STAT6 and chromatin remodelers. Since PTOV1 does not interact with the mediator complex, it may hinder the PTOV domain in MED25 when it is overexpressed.

The Notch pathway is a signaling pathway that is responsible for tissue homeostasis, cell proliferation, cell growth, and cell differentiation. HES1 and HEY1 are two genes that are located
downstream of the notch signaling pathway. Notch receptor activation stimulates its entrance to the nucleus and behaves as a transcription factor for HES1 and HEY1. When the Notch is absent, a transcription suppressor complex forms on promoters of these genes, which includes SMRT/NCoR and HDAC1 units. Changes in Notch signaling lead to the progression of different cancer types and its activity has been studied in prostate cancer in both in vivo and in vitro models. There is evidence that overexpression of PTOV1 suppresses the downstream genes of the Notch signaling pathway such as HES1 and HEY1 by interacting with the SMRT/NCoR and HDAC1 components of the transcription suppresser complex. Interestingly, repression of the transcription of downstream targets by PTOV1 can be inhibited by overexpression of CBP, suggesting that CBP can activate HES1 transcription. These data suggest that PTOV1 is a negative co-regulator of the Notch signaling pathway. In human prostate cancer cell lines downregulation of PTOV1 leads to the upregulation of HES1 and HEY1 genes.

Moreover, the Wnt signaling pathway is also important in cancer progression and cell proliferation. Previous studies have shown that overexpression of PTOV1 promotes the Wnt signaling pathway as well as increased cellular proliferation and cancer progression. Dickkopf-1 (DKK1) is a negative regulator of the Wnt signaling pathway. Recent studies have shown that overexpression of PTOV1 upregulates beta-catenin and its nuclear translocation, leading to its increased transcriptional activity and downregulation of DKK1 in breast cancer cell lines. Collectively all these data suggest that PTOV1 acts as a regulator of transcription activity for the Wnt/beta-catenin signaling pathway. In conclusion, all these observations suggest that PTOV1 is a regulator of transcription for several genes such as RAR, HES1, HEY1, and Dickkopf-1.
1.2.6: PTOV1 and c-jun translation

C-jun is an important transcription factor that regulates the AP-1 complex and regulates several functions such as apoptosis, cell proliferation, and cell survival. The AP-1 complex is well established in cancer progression and cell metastasis. Previous studies have shown that overexpression of PTOV1 in primary prostate cancer and metastatic cancer cells upregulates C-jun translation and promotes cancer growth. Besides that, C-jun plays a role in regulating the phosphatidylinositol 3-Kinase/Akt/mTOR signaling pathway and acts as a transcription factor. Since overexpression of PTOV1 upregulates C-jun translation and its downstream genes, PTOV1 can be assumed as a positive regulator of tumor cell proliferation and progression. Recent studies have shown that PTOV1 interacts with a protein called RACK1, which is a regulator of cell signaling and ribosomal function. More specifically PTOV1 requires RACK1 protein to bind with 40s ribosomes implying that it may have a role in upregulating translation, hence stimulating protein synthesis, including C-jun. However, the exact mechanism is not well understood.
CHAPTER 2: 14-3-3 AND HUWE1 COORDINATELY REGULATE PTOV1

2.1 Introduction

Initially, PTOV1 was observed as an overexpressed mRNA transcript in primary prostate cancer samples\(^4\). Previous studies have shown an association between PTOV1 overexpression and cellular proliferation, cellular survival, tumor growth, and cancer progression in mouse xenograft models\(^{17,45–51}\). These data led to the conclusion that PTOV1 is a protein that is important for cell growth and that PTOV1 inhibition using siRNA can lead to cell death\(^{50,52,53}\). Thus, PTOV1 has been proposed as a potential therapeutic target in cancer\(^{44}\). However, the mechanisms that regulate PTOV1 function are not well understood.

Our long-term goal was to determine how PTOV1 promotes cellular survival and therefore cancer development and progression. To accomplish this goal, we were seeking to understand the regulation of PTOV1 and how its interaction with 14-3-3\(\zeta\) contributes to the oncogenic functions of PTOV1.

Our central hypothesis was that the prosurvival activity of PTOV1 is promoted by phosphorylation of PTOV1 at S36 and S53, which induces binding with 14-3-3\(\zeta\). This binding subsequently sequesters PTOV1 in the cytoplasm and protects it from proteasomal degradation. This hypothesis has been formulated based on preliminary data showing that disruption of the PTOV1-14-3-3\(\zeta\) interaction through S36A or S53A mutations leads to accumulation of PTOV1 in the nucleus. These mutations also lead to a reduction of total cellular PTOV1 protein levels, which can be rescued by inhibition of proteasomal function. The rationale for these experiments was that understanding the mechanisms regulating PTOV1 function and degradation will provide tools that may allow for better therapeutic targeting of PTOV1-driven cancers.
Here we employed different experimental strategies to better understand the upstream regulation of PTOV1 and how it leads to cellular proliferation and cancer progression. We initially identified PTOV1 as a new 14-3-3 interactor using a proteomics approach. 14-3-3 is a phosphor-binding protein, which binds to the phosphorylated motif on targeted proteins and modulates their activity. 14-3-3 proteins can bind to diverse signaling proteins such as phosphatases, kinases, and transmembrane receptors. 14-3-3 can bind to more than 200 proteins and can regulate their activity. The human 14-3-3 is a phosphor binding protein and is a member of a protein family that consists of seven structurally similar isoforms. Several of the isoforms of the 14-3-3 family are known to play a role in the development of aggressive cancer phenotypes. The effect of 14-3-3 binding to phosphorylated proteins can vary, depending on the identity of the protein. Such regulatory effects of binding include altering the conformation, localization, or protein interaction of the targeted protein. 14-3-3 proteins have been shown to play important roles in cancer cell signaling both in oncogenic and tumor-suppressive pathways. 14-3-3s are not enzymes, but they play an important role by binding to different types of binding partners and modulating their functions. An important fact is that 14-3-3 interaction with the target protein depends on one or two serine (S) or threonine (T) phosphorylations within loosely conserved motifs on the binding partner. Since 14-3-3s are phosphorylation-dependent proteins, they recruit upstream kinases and their signaling pathways to regulate or mediate any specific effects on their target protein. These effects include sequestration of target proteins, activation or inhibition of enzyme activity of the target protein, or even scaffolding of protein-protein interactions, depending on the binding partner. Our preliminary data shows that 14-3-3 protein is responsible for the regulation of PTOV1. However, the mechanism of 14-3-3 binding with PTOV1 and how it regulates PTOV1 is unclear. Here we are trying to provide insights into the mechanism in which 14-3-3 may regulate PTOV1.
Further, we have found that HUWE1 is another interacting partner of PTOV. We confirmed this interaction by performing co-immunoprecipitation (co-IP) experiments and found that HUWE1 is the E3 ligase responsible for the degradation of PTOV1. HUWE1 is one of many E3 ubiquitin-protein ligases. The main function of E3 ligases is the ubiquitination and subsequent proteasomal degradation of target proteins. There are some other functions as well, including the regulation of apoptosis by catalyzing the polyubiquitination and degradation of MCL1, mediating DNA polymerase beta (POLB) monoubiquitination at 'Lys-41', 'Lys-61' and 'Lys-81' residues\textsuperscript{62}, which leads to base-excision repair, etc. Ubiquitination of tumor suppressors such as p53, core histones such as H1, H2A, H2B, H3 and H4, and MFN2 can negatively regulate mitochondrial fusion, neural differentiation, and proliferation regulation. Further, regulation of polyubiquitination of CDC6, isoforms of PA2G4, and ubiquitination of NR1D1 mediate their degradation\textsuperscript{62–69}. Our contribution here was to characterize the mechanism of PTOV1 regulation by 14-3-3 and HUWE1, as well as establishing PTOV1 as a cancer-driver and target for cancer therapy.

Here we were able to discover a mechanism by which 14-3-3 and the E3 ligase, HUWE1 may contribute to regulating the localization, degradation, and function of PTOV1. This data would be the first data to give shreds of evidence about the regulation of PTOV1 itself, its function, and its stability at the protein level. Furthermore, these data may identify pathways that could provide insight into inhibiting the oncogenic function of PTOV1.
2.2 Results

2.2.1 Phosphorylation of PTOV1 at S36 and S53 is required for binding to 14-3-3.

To determine the phosphorylation-dependent 14-3-3-binding partners, we performed coimmunoprecipitation (co-IP) LC-MS/MS proteomics in cells expressing HA-14-3-3ζ WT or the non-phospho binding mutant HA-14-3-3ζ K49E. Oncogene PTOV1 was the only interactor that pulled down with 14-3-3ζ WT, but not with the K49E mutant. A previously done 14-3-3 LC-MS/MS study had also shown PTOV1 among many interactors, but its significance and regulation remained unexplored\(^7\). To validate the observed data, we also performed the converse LC-MS/MS experiment with GFP-PTOV1 as bait, suggesting a PTOV1 interaction with 6 of the 7 14-3-3 isoforms, not including the σ isoform. We further validated the PTOV1-14-3-3 interaction by coIP immunoblotting (Figure 2-1A). Similar to PTOV1, 14-3-3 z overexpression correlates with higher risk prostate cancers (Figure 2-1B).

To determine the PTOV1 phosphorylation sites which are responsible for 14-3-3 binding, we first selected a list of S and T residues using a combination of consensus sequence analysis via 14-3-3 site prediction algorithms\(^71\), disorder prediction with IUPRED\(^72,73\), and the frequency of high-throughput detection of site-specific phosphorylations using phosphosite.org\(^44\) (Figure 2-1C). Using these analyses, we discovered that S36, S53, and S109 are responsible for 14-3-3-docking site phosphorylations. S36 and S53 phosphorylation sites are conserved from mouse to human (Figure 2-1D) CoIP immunoblotting confirmed that S36 and S53, but not S109, are essential for PTOV1 binding to 14-3-3 (Figure 2-1E).
Figure 2-1-1: Phosphorylation of PTOV1 at S36 and S53 is required for binding to 14-3-3. A) FLAG-PTOV1 was expressed in HEK-293T cells, followed by IP on FLAG-resin and immunoblotting for 14-3-3 and FLAG. A representative image from three biological replicates is
shown. B) Positive correlation between RNA expression levels of the YWHAZ gene and Gleason scores for 485 patients from The Cancer Genome Atlas. C) Composite graph of IUPRED disorder score and the high-throughput identification frequency of phosphorylations (phosphosite.org) across the PTOV1 amino acid sequence. D) Alignments of mammalian PTOV1 sequence surrounding S36 and S53. E) GFP-PTOV1 was expressed in HEK-293T cells, followed by IP on GFP-Trap resin and immunoblotting for 14-3-3 and GFP. The right panel shows quantification (LI-COR infrared imaging) of 14-3-3 coIP signal normalized to GFP (coIP) and expressed as a fraction of the WT GFP-PTOV1 coIP signal from four biological replicates. Data collected from Dr. Katie Pennington

2.2.2 Loss of 14-3-3 binding leads to an accumulation of PTOV1 in the nucleus.

Our previous coIP experiments consistently showed that mutation of S36A/S53A phosphorylation sites leads to loss of 14-3-3 binding, which eventually leads to a low recovery amount of PTOV1 protein inside cells. Therefore, we hypothesized that this observation may be due to either a decrease in total cellular levels of PTOV1 or due to PTOV1 colocalization to a different part of the cell. Previous studies have shown that 14-3-3 can mediate cellular localization of an interacting protein and we also showed that PTOV1 is translocated into the nucleus at the beginning of the S phase\textsuperscript{54}. As the next step, we wanted to see how the 14-3-3-PTOV1 interaction affected the subcellular localization of PTOV1 based on these observations.

To monitor PTOV1 localization in the cell, we used confocal microscopy and imaging flow cytometry. For this experiment, we stably expressed either GFP-or FLAG-tagged PTOV1 WT or PTOV1 S36A into PC3 cells. We observed PTOV1 WT is mainly distributed in the cytosol and PTOV1 WT levels in the nucleus are relatively low (Figure 2-2A-B). Conversely, the PTOV1 S36A 14-3-3 deficient mutant is mainly located in the nucleus (Figure 2-2A-B). Based on coIP results we showed that an S53 phospho mutant would also cause a similar shift of PTOV1 protein into the
nucleus, (Figure 2-2b) but the S109 mutant does not. Collectively all these data suggest that loss of 14-3-3 binding, by mutation of the phosphorylation sites, results in the loss of 14-3-3 binding, eventually leading to PTOV1 nuclear accumulation.

**Figure 2-2: Loss of 14-3-3 binding leads to an accumulation of PTOV1 in the nucleus.** A) PC3 cells stably expressing FLAG-PTOV1 WT or S36A were analyzed by confocal imaging, deconvolved by Hyugens software, and assessed for cytosolic and nuclear localization of PTOV1. The right panel shows the Pearson coefficient (Leica software) of PTOV1 colocalization with DAPI. B) PC3 cells stably expressing GFP-PTOV1 (WT or indicated mutants) were analyzed by imaging flow cytometry for nuclear localization of PTOV1 as a function of overlap with propidium iodide (PI) nuclear stain.
The right panel shows quantification of PTOV1/PI colocalization expressed as a log transformation of Pearson coefficient (Imaris software).

2.2.3 Loss of 14-3-3 binding destabilizes PTOV1 protein.

Previous observations suggest that loss of 14-3-3 binding shifts PTOV1 localization into the nucleus, but this observation alone does not support the fact that 14-3-3 may also control PTOV1 stability. On the other hand, whole-cell imaging and immunoblot experiments show consistently lower levels of PTOV1 S36A signal. To further study this observation, we decided to do cycloheximide (CHX)-chase experiments using FLAG-PTOV1 WT or FLAG-PTOV1 S36A PC3 stable cells. We observed from this experiment that PTOV1 S36A 14-3-3 binding deficient mutant tends to degrade quicker in comparison to PTOV1 WT. This observation implies that PTOV1 protein stability may depend on 14-3-3 binding and that it may protect PTOV1 from degradation (Figure 2-3A). Next, we wanted to find out the mechanism of how 14-3-3 controls the stability of PTOV1. To do this, we first wanted to check whether PTOV1 is degraded through proteasomal or lysosomal degradation pathways. First, I treated PC3 cells stably expressing PTOV1 WT and PTOV1 S36A with MG132, which is a proteasome inhibitor, and with bafilomycin, which is a lysosome inhibitor. I then checked PTOV1 levels in the cytosol and confirmed that PTOV1 is degraded through the proteasome and not through the lysosome (Figure 2-3B). We also validated previously observed data using Imaging flow cytometry to analyze PC3 cells stably expressing GFP-PTOV1 WT and mutants to confirm that PTOV1 protein levels increase when they are treated with MG132. All this data suggests that PTOV1 degradation is inhibited by proteasomal inhibition. These data suggest that 14-3-3 binding prevents PTOV1 degradation, whereas the loss of 14-3-3 binding increases the degradation of PTOV1 through the proteasome.
Figure 2-3: Loss of 14-3-3 binding destabilizes PTOV1 protein. A) PC3 cells stably expressing FLAG-tagged PTOV1 WT or S36A were treated with cycloheximide (CHX) over the indicated time course, followed by immunoblotting for FLAG (PTOV1) and alpha-Tubulin. The bottom panel shows quantification of an immunoblot signal for FLAG normalized to alpha-Tubulin and expressed as a fraction of the signal at time 0. Error bars represent SEM and p-values were calculated with a t-test comparing WT and S36A signal at a given time point. B) PC3 cells stably expressing FLAG-PTOV1 WT or indicated mutants were treated with DMSO (D) 10 uM MG132 (M) or 100 nM bafilomycin (B) for 4 hours. The bottom panel shows quantification of immunoblot FLAG (PTOV1) immunoblot signal expressed as a fraction of the signal upon DMSO treatment. Error bars represent the standard deviation of the mean and a two-tailed student t-test was used to calculate p-values.
2.2.4 HUWE1 interacts with PTOV1 and controls PTOV1 stability.

Next, we wanted to check the upstream cellular mechanism that controls PTOV1 degradation. Using our previous proteomics data and looking at the peptide count, we found that the E3 ligase HUWE1 is a top interactor of PTOV1 in comparison with other interactors. We then validated this hypothesis using PTOV1 coIPs with endogenous HUWE1 (Figure 2-4A). To check whether HUWE1 is the responsible kinase that regulates PTOV1 degradation, we depleted HUWE1 with siRNA in PC3 cells stably expressing GFP-PTOV1 S36A for imaging flow cytometric analysis and FLAG-PTOV1 WT or FLAG-PTOV1 S36A for immunoblotting. In both experiments, the knockdown of HUWE1 resulted in increased PTOV1 protein levels in both WT and S36A mutants (Figure 2-4B). Furthermore, we confirmed that the reason for the increased PTOV1 protein levels is due to slower kinetics of PTOV1 degradation in HUWE1-depleted cells (Figure 2-4C-D).
Figure 2-4: HUWE1 interacts with PTOV1 and controls PTOV1 stability. A) HEK-293T cells overexpressing GFP or GFP-PTOV1 (or mock-transfected) were subject to IP on GFP-Trap resin, followed by immunoblotting for HUWE1 and GFP. PC3 cells stably expressing FLAG-PTOV1 WT or S36A were transfected with siRNA against HUWE1 or a control sequence (non-specific), followed by immunoblotting for FLAG (PTOV1) and indicated proteins. The right panel shows quantification of PTOV1 (FLAG) signal normalized to alpha-Tubulin and expressed as a fraction of control siRNA treatment. Error bars represent SD and p-values were calculated using a two-tailed student t-test from 3 biological replicates. C) PC3 cells stably expressing FLAG-PTOV1 WT were transfected with siHUWE1 or control for 48 hours, then treated with chx as in figure 4A. Cells were harvested at time points that were determined empirically to visualize PTOV1 WT degradation. Lower panel shows quantification of signals from three biological replicates. D) PC3 cells stably expressing FLAG-PTOV1 S36A were treated and analyzed as in panel C. Quantification represents three biological replicates.
CHAPTER 3: PTOV1 REGULATION BY SGK2

3.1 Introduction

Our preliminary data suggests that PTOV1 is regulated by 14-3-3, and targeted for proteasomal degradation by HUWE1, an E3-ubiquitin ligase. 14-3-3 regulates the fate of its substrates by binding to specific phosphorylated sites of the target protein. Our central hypothesis was that the pro-survival activity of PTOV1 is promoted by phosphorylation of PTOV1 at serines 36 and 53, which induces binding of 14-3-3 and subsequently both sequesters PTOV1 in the cytoplasm and protects it from proteasomal degradation. In an attempt to understand the mechanisms of PTOV1 regulation, we aimed to identify the upstream kinase responsible for the PTOV1-14-3-3 interaction. We did so by screening small-molecule kinase inhibitors and monitoring S36 phosphorylation with a custom phospho antibody. We then used siRNA knockdown to confirm positive hits from the screen.

Through these experiments, we found that glucocorticoid-induced kinase 2 (SGK2) is responsible for phosphorylation of PTOV1 at S36 and S53 and, as a result, mediates 14-3-3 $\zeta$ binding.

SGK2 kinase is a member of the glucocorticoid-induced kinase family, a member of the AGC kinase group. The AGC family has known 60 family members, some of which play roles in cancer progression and cell survival, such as AKT, S6K, and RSK proteins. SGKs family consists of two other kinases apart from SGK2, known as glucocorticoid-induced kinase 1 (SGK1) and glucocorticoid-induced kinase 3 (SGK3). SGK family proteins regulate several transporters, channels, and pumps in cultured epithelial cells. For instance, the major Na$^+$ ion transporter in the intestine is the Na$^{(+)}$/H$^{(+)}$ exchanger 3 (NHE3). SGK1 and SGK3 are known to mediate NHE3 by interacting with NHE Regulatory Factor 2 (NHERF2) via Dexamethasone (Dex) in epithelial cell lines. Studies have shown that NHERF2 and SGK1-null mice possess attenuated NHE3 regulation compared to wild-type mice $^{77,78}$. Conversely, the function of SGK2 in NHE3 regulation is not well known. Previous
data has shown that SGK1, SGK2, and SGK3 have similar biochemical properties and structures. SGK1 and SGK3 share 80% similarity in their kinase domains, 44-68% similarity in their non-catalytic C-terminal domains, and 25% similarity in their N-terminal domains. SGK2 does not have any identified similarities with the other isoforms (SGK1 and SGK3); while the roles of SGK1 and SGK3 have been well-studied, the function of SGK2 is poorly understood. 74–76.

Recent studies have shown that SGKs play a role in cancer progression through regulation of the PI3-K/AKT pathway, suggesting that they may have oncogenic properties. The phosphoinositide 3-kinase (PI3-K) signaling pathway regulates diverse biological processes, including cell growth, proliferation, and survival. Components of the PI3-K signaling pathway have been mutated or dysregulated, in some cancers. The PI3-K signaling pathway is mainly regulated by protein kinase B/v-akt murine thymoma viral oncogene homolog (PKB/AKT) signaling, but SGK family members may also regulate PI3-K signaling

SGKs have similar substrate specificity to AKT family members, and, like AKTs, are known to regulate cell proliferation and survival. Previous studies have suggested that SGK3 mediates AKT-independent oncogenic signaling; furthermore, another study has shown that SGK2 mediates PT-induced cell death in different cancer types via autophagy inhibition in epithelial ovarian cancer. 79–81 Despite this finding, SGK2 is generally thought to promote cell survival, as depletion of the SGK2 in different cancer cell lines has been shown to induce apoptosis. 82–85 Another study shows SGK2 has a substrate which is a VTPase subunit, suggesting that SGK2 may play a role in autophagy control and acidification of the lysosome. 84. Importantly, SGK1 is known to phosphorylate 14-3-3 binding sites on target proteins. 82,86. On the whole, the cellular functions of SGK2 remain unclear. However, there are a very small number of studies investigating the function of SGK2. Herein we have demonstrated the role of SGK2 in PTOV1 regulation in the PC3 prostate cancer cell line.
3.2 Results

3.2.1 SGK2 emerged as a common hit between the S36 and S53 sites.

We first did a biased approach in which we used kinases that phosphorylate at S36 and S53. Examples for those kinases are CAMKII, AKT, and PKC, etc. This approach was not successful and was not able to find specific kinase or kinases that specifically phosphorylate S36 and S53 phosphorylation sites. So, we instead used an unbiased method in which biotin-tagged peptides which encompasses PTOV1 phosphorylate sites S36 and S53 and performed an in vitro radiometric assay using 245 different human kinases which are members of the serine/threonine (S/T) kinase family (Figure 3-1).
Figure 3-1: In vitro screening of S/T kinases identifies a subset of kinases that phosphorylate S36 of PTOV1. An N-terminally biotinylated PTOV1 peptide surrounding S36 was incubated in a streptavidin-coated FlashPlate and subject to a radiometric kinase assay with 245 individual serine/threonine (S/T) protein kinases. To account for signals from kinase autophosphorylation, each assay (with peptide) was normalized to control with the kinase but no peptide.

From the in vitro radiometric assay, we identified a possible subset of kinases responsible for PTVO1 regulation among 245 kinases of the S/T family. This small group of kinases includes PBK, PKC-d, SGK2, and DYRK1A. We observed that SGK2 kinase showed higher mean kinase activity compared to other kinases at varying concentrations of PTOV1 S36 peptides. At 1 uM concentration of peptide, SGK2 displayed the highest mean kinase activity; with no peptide present, it showed the lowest kinase activity. All the other kinases, such as SGK1, PBK, PKC-d, showed lower kinase activity than SGK2. These data collectively suggest that recombinant SGK2, but not SGK1, PBK, or PKC-d, phosphorylates S36 in vitro (Figure 3-2).
3.2.2 SGK2 phosphorylate PTOV1 at s36

*In vitro* radiometric assays showed that SGK2 is the responsible kinase for PTOV1 phosphorylation. We next validated SGK2 mediation of PTOV1 in cell-based assays. We attempted to generate phospho-specific antibodies for both sites but were only able to recover specific phospho-antibodies for S36. Thus, we decided to focus on our validation experiments to check only S36 phosphorylation.
To check whether there is any phosphorylation signal difference when treated with an SGK2 inhibitor, we treated PTOV1 WT and PTOV1 S36 stably-expressed PC3 cells with an SGK2 inhibitor called GSK (with DMSO as control) and monitored the pS36 signal. Previously, we observed that the 14-3-3-binding deficient phosphor-null mutant completely abrogated 14-3-3 binding. From validation results, we observed that PTOV1 S36 expressed PC3 cells showed almost no phosphorylation signal compared to our control. Notably, PTOV1 WT PC3 cells showed a lower phosphorylation signal compared to control when treated with SGK2 inhibitor, confirming that SGK2 is the responsible kinase for the phosphorylation of PTOV1 (Figure 3-3).

**Figure 3-3: SGK2 Phosphorylates PTOV1.** PC3 cells stably expressing FLAG-tagged WT or S36A mutants of PTOV1 were treated with indicated SGK2 inhibitor (GSK 650393) for 48 hours at 100 nM, followed by IP on FLAG resin and immunoblotted for pS36 and FLAG. The right panel shows quantification of pS36 signal normalized to FLAG signal and expressed as a fraction of WT from
three biological replicates. Error bars represent SEM, and p-values were calculated using a two-tailed student t-test.

To confirm these findings, we analyzed PTOV1 S36 phosphorylation in cells depleted of SGK2 via siRNA knockdown. We treated PC3 cells stably expressing FLAG-tagged WT or the S36A mutant form of PTOV1 with the indicated siRNAs, followed by an IP on FLAG resin and immunoblotting for pS36. We observed that. As predicted by our kinase screen, SGK2 depletion by RNAi reduced pS36 PTOV levels. Together, our data suggest that SGK2 is the upstream kinase responsible for PTOV1 phosphorylation at S36 (Figure 3-4).
**Figure 3-4: Depletion of SGK2 by SiRNA reduces pS36 signal.** Upper panel shows an immunoblot validation of SGK2 siRNA (signal shown is endogenous SGK2) in PC3 cells. PC3 cells stably expressing FLAG-tagged PTOV1 WT or the S36A mutant were transfected with indicated siRNAs, followed by IP on FLAG resin and immunoblotting for pS36 (PTOV1) and FLAG.

### 3.2.3 SGK2 inhibition disrupt the 14-3-3 binding

Finally, we performed another cell-based experiment confirming our findings on SGK2 regulation of PTOV1 via S36. We treated PC3 cells with SGK2 inhibitors and measured 14-3-3 signal and binding by IP. Similar to our previous results, PTOV1 WT PC3 cells, when treated with SGK2 inhibitor, show a significantly lower level of 14-3-3 signal compared to the control. In contrast, when SGK2 was inhibited, it prevented PTOV1 phosphorylation at S36 and subsequent 14-3-3 binding. These data suggest that SGK2 phosphorylates PTOV1 at S36 site and facilitates 14-3-3 binding. Together, our data suggest a model in which SGK2 phosphorylates PTOV1 at S36 (and likely S53, based on *in vitro* data) to promote 14-3-3 binding (Figure 3-5,3-6).
**Figure 3-5: SGK2 inhibition reduces the 14-3-3 binding with PTOV1.** PC3 cells stably expressing WT or the S36A mutant forms of GFP-tagged PTOV1 were treated with indicated SGK2 inhibitor (GSK 650393) for 48 hours at 100 nM, followed by IP on GFP resin and immunoblotting for pS36 (PTOV1) and GFP. The below panel shows quantification (LI-COR infrared imaging) of pS36 signal normalized to GFP (coIP) and expressed as a fraction of WT from three biological replicates. Error bars represent SEM and p-values were calculated using a two-tailed student t-test.
Figure 3-6: Schematic model of SGK2-mediated phosphorylation of PTOV1 and 14-3-3 binding.
CHAPTER 4 - DISCUSSION

We initially focused on elucidating the cellular functions of 14-3-3 through studying phosphor-mediated binding of 14-3-3 to its target proteins. 14-3-3 is a putative signaling hub that regulates a variety of oncogenes and tumor suppressors to mediate cell growth, survival, and adaptation. As the phosphorylation of specific residues on the target protein is necessary for 14-3-3 binding, this implicates the presence of upstream kinases mediating 14-3-3 binding to its substrates. By studying the regulation of the 14-3-3 interactome, we hope to elucidate the role of 14-3-3 in cancer and disease progression.

Our study shows that SGK2 is the upstream kinase that is responsible for phosphorylating PTOV1 at S36. Based on our data, we have derived a model wherein SGK2 phosphorylates PTOV1 at S36 and S53 to promote 14-3-3 binding.

![Figure 3-6: Schematic model of SGK2-mediated phosphorylation of PTOV1 and 14-3-3 binding.](image)

Previous studies have shown that SGK1 and SGK3 are activated downstream of the PI3-K signaling pathway. Since SGK2 shares some substrate similarity and similar homology with AKT, and AKT is...
downstream of the PI3K pathway, we hypothesized that SGK2 may activate downstream of the PI3K signaling pathway. The fact that mitogenic activation of PI3-K in the G1 phase and SGK2 activation downstream of this pathway and our suggested mechanisms may explain the cell cycle-dependent PTOV1 shuttling between nucleus and cytoplasm. To investigate the role of PI3-K signaling in PTOV1 regulation, I treated PTOV1 WT and PTOV1 S36 stably expressing PC3 cells with a PI3K inhibitor and monitored the pS36 signal. I observed that PTOV1 WT PC3 cells exhibited a lower pS36 signal compared to control, but also noticed a reduction in total PTOV1 signal. These observations may be explained by an increase of PTOV1 degradation and reduction in PTOV1 transcription in response to PI3-K inhibition. In the future, our lab may focus on obtaining a better understanding of the upstream signaling that controls SGK2 and its temporal regulation over the cell cycle.

Early studies showed that PTOV1 stays in the cytosol in the G1 phase and shuttles into the nucleus during the S phase. We showed that sequestration of PTOV1 in the cytoplasm is regulated by 14-3-3, and when PTOV1 is not bound to 14-3-3, it is degraded. The 14-3-3-mediated cytoplasmic sequestration of PTOV1 leads to an increased cJun translation at the G1 phase, leading to cell cycle progression. cJun is a transcription factor that regulates the AP-1 complex and several cellular functions such as apoptosis, proliferation, and survival. Additionally, cJun plays a role in regulating the phosphatidylinositol 3-Kinase/Akt/mTOR signaling pathway and acts as a transcription factor. cJun increases the cyclin D1 protein expression and plays a major role in cell cycle progression through the G1 phase. Previous studies have shown that cJun protein inhibition or deletion leads to low levels of cyclin D1, which promotes Rb activation and ultimately leads to G1 arrest. Furthermore, studies have shown that overexpression of PTOV1 increases the cyclin D1 protein expression. These observations may explain why we were not successful in making PTOV1 viable.
KO cell lines. Additionally, when 14-3-3 is not bound to PTOV1 it is targeted for proteasomal degradation. This process reduces PTOV1 levels in the cytosol, which in turn silences cytosolic expression of cJun and cyclin D1.

Another important interactor of PTOV1 is HUWE1, is an E3-ubiquitin ligase that is responsible for the targeting of PTOV1 for degradation. Our data show that 14-3-3 binding deficient mutants colocalize to the nucleus and are targeted for degradation via HUWE1-mediated ubiquitination, whereas PTOV1 WT is sequestered in the cytoplasm. This implies that the loss of 14-3-3 binding increases the interaction between PTOV1 and HUWE1, which leads to HUWE1-dependent degradation of PTOV1 via the proteasome. HUWE1 is a large E3 ligase that comprises 482kDa bases and belongs to the HECT domain family\(^9\). HUWE1 is known to ubiquitinated a variety of substrates, including p53\(^5\), Mcl-1\(^9\), c-Myc\(^9\), Chk1\(^9\), and H2AX\(^9\), in order to mark them for degradation. We initially supposed that HUWE1 is normally oncogenic, but the nature of its downstream substrates revealed a different image. For instance, depletion of HUWE1 leads to upregulation of p53 and Mcl-1 proteins which are known as tumor suppressors and play opposite roles in cellular growth and survival. PTOV1 is also a HUWE1 substrate, but unlike p53 and Mcl-1, it is an oncogene. This raises questions in our understanding of HUWE1. Specifically, we wonder how HUWE1 may cooperate with PTOV1 to regulate cell growth and survival. Our data suggest that depletion of HUWE1 elevates PTOV1 protein levels, which, in turn, promotes the expression of cJun, a pro-growth translational target of PTOV1\(^5\).

Collectively, our data suggest a model in which SGK2 phosphorylates PTOV1 at S36 and S53 and facilitates 14-3-3 binding at these sites. This interaction in turn sequesters PTOV1 in the cytosol, which promotes PTOV1-mediated expression of cJun. Upon loss of 14-3-3 binding, PTOV1 localizes
to and accumulates in the nucleus, where it is subject to being targeted for proteasomal degradation by HUWE1.

Figure 4-1: Model of SGK2-, 14-3-3- and HUWE1-mediated regulation of PTOV1 localization, stability, and function.

In conclusion, our data provide the first mechanism of regulation for the poorly understood oncogene, PTOV1, and shed new light on the SGK2 kinase. Furthermore, this mechanism adds to our field’s understanding of the expanding biology of 14-3-3. In many instances, 14-3-3 proteins interact with different binding partners and sequester them in the cytosol to protect them from degradation. For example, 14-3-3 interacts with the pro-apoptotic Bcl-2 family protein Bad and sequesters it in the cytosol. This prevents Bad from pro-survival Bcl-2 proteins to inhibit cell death. In another case, 14-3-3s sequestered PRAS40 and TSC2 to allow for Rheb-mediated activation of mTORC1.

Furthermore, 14-3-3s also sequesters FOXO and YAP/TAZ transcription factors in the cytoplasm to inhibit their nuclear function. In striking parallel, PTOV1 shuttles between the cytoplasm and nucleus and is sequestered in the cytoplasm by interaction with 14-3-3. We can hypothesize that PTOV1 interaction with 14-3-3 in the cytoplasm may divide the cytosolic and nuclear functions of PTOV1 by
sequestration. Thus, PTOV1 expands the concepts and theories behind 14-3-3 regulation and also illustrates the value of 14-3-3 as a tool to discover functional phosphorylations, kinase-substrate relationships, and mechanisms that could be exploited therapeutically.
CHAPTER 5 - MATERIALS AND METHODS

5.1 Plasmids, cloning

Table 5-1: Details of Plasmids

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<th>Plasmid</th>
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<td>HA-14-3-3 zeta</td>
<td></td>
<td>Cite</td>
</tr>
<tr>
<td>peGFP PTOV1</td>
<td>Gift of Dr. Attila Németh</td>
<td>(cite [<a href="https://www.ncbi.nlm.nih.gov/pubmed/26156556">https://www.ncbi.nlm.nih.gov/pubmed/26156556</a>])</td>
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<td>pLV hUbC FLAG-dCas9 T2A GFP</td>
<td>Addgene</td>
<td>53191</td>
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<tr>
<td>pLV hUbC PTOV1 T2A GFP</td>
<td>Subcloned from pLV hUbC FLAG-dCas9 T2A GFP</td>
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<tr>
<td>pLV hUbC GFP-PTOV1</td>
<td>Subcloned from peGFP-PTOV1 into</td>
<td></td>
</tr>
</tbody>
</table>
Point mutations, as described in the text, were cloned using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA) per manufacturer’s protocol using primer sequences as indicated in the following table. Clones were confirmed with sequencing by Eton Bioscience (San Diego, CA, USA). Plasmids were maxiprepped using GeneJET Plasmid Maxiprep Kit (Thermo Fischer Scientific, Waltham, MA, USA) per the manufacturer’s protocol.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<td>S36A For</td>
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<td>S53A For</td>
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<td>S53A Rev</td>
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<td>S109A For</td>
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5.2 Confocal Microscopy

Cells were seeded onto acid-etched coverslips and incubated for 48 hours before fixation. Cells were fixed for 10 minutes with 1% or 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100/PBS for 10-15 minutes. Samples were then blocked with SEA BLOCK Blocking Buffer (Thermo Scientific, Waltham, MA, USA) for 1 hour at room temperature and subsequently incubated with primary antibodies at 4C overnight. Cells were washed with 0.1% Tween/PBS (PBS-T) and incubated with secondary antibodies in PBS for 45 minutes to 1 hour at room temperature. Cells were washed with PBS-T and subsequently stained with 1.43 uM DAPI for 5 minutes. The coverslips were then mounted with Prolong Diamond Antifade Mountant (Thermo Scientific, Waltham, MA, USA) and allowed to cure overnight at room temperature while protected from light. Images were acquired on a LEICA TCS SP8 confocal microscope fitted with a HC PL APO 63X/1.40 Oil CS2 objective and a HyD detection system (Leica Microsystems, Wetzlar, Germany).

5.3 Confocal microscopy data analysis

For data integrity, samples for each set were seeded, fixed, and stained at the same time to have the same conditions. Furthermore, laser power and image resolution were kept the same for each set. All images were processed using Huygens Essential express deconvolution tool and Pearson's coefficients were calculated using the colocalization analyzer tool in the same software. For the colocalization calculation, threshold intensity values were set at 10% of the highest intensity value for each image. Furthermore, the Pearson’s calculation was limited to individual cells using a trace tool found in the Huygens software. Pearson’s coefficient averages and significance were calculated in GraphPad Prism 8 with the Welch’s t test correction.

5.4 Cell culture and gene expression
HEK293T and PC3 cells were purchased from ATCC (Atlanta, GA, USA). Lenti-X 293T cells were purchased from Clontech (Mountainview, CA, USA). HEK 293T and Lenti-X 293T cells were maintained in DMEM and PC3 cells in DMEM/F12 media, each supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were incubated at 37°C and 5% CO2.

For transient expression experiments, HEK 293T cells were seeded at 15-20% confluence and grown overnight. Cells were then transfected in complete media with 8 ug plasmid/ 10 cm dish as indicated using 40 ug PEI-MAX transfection reagent (Polysciences, Warrington, PA, USA) using standard protocols. Media was changed 6-12 hours post-transfection. Cells were harvested 48 hours following transfection for downstream applications.

Lentiviral constructs were produced in Lenti-X 293T cells via transfection of the indicated transfer vectors for either FLAG-PTOV1 or GFP-PTOV1, along with psPAX2 and pMD2.G in a 4:2:1 ratio. To generate PC3 cells stable expressing the indicated PTOV1 constructs, PC3 cells were seeded at 20% confluence and grown overnight. Cells were then transduced with the appropriate lentiviral supernatants in the presence of polybrene. The media was changed after 24 hours, and cells were selected by FACS for GFP-expression 2-3 days post-transduction.

For knockdown experiments, PC3 cells were transfected with pooled ON-TARGETplus siRNA reagents (Horizon Discovery, Cambridge, UK) for each indicated target using Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). Cells were seeded at 20% confluence and grown overnight. The cells were washed 2X with PBS, after which OPTI-MEM (Thermo Fisher Scientific, Waltham, MA, USA) was added to the cells. The siRNAs were incubated at 100 nM with the RNAiMAX reagent at room temperature for 20 minutes before addition to cells. The cells were incubated with the siRNA-RNAiMAX complex in the OPTI-MEM for 4
hours. FBS was then added to the cells, and they incubated another 8 hours before the media was changed for complete DMEM/F12. Cells were harvested for downstream applications 48 hours later.

5.5 CoIP, Immunoblotting, and antibodies

Cell dishes were placed on ice, the media was aspirated, and cold PBS was added. Cells were scraped from the dishes in the cold PBS and transferred to 15 mL centrifuge tubes. Dishes were washed an additional time with PBS to collect residual cells. Cells were then pelleted at 1200 xg at 4°C and the supernatant discarded. Cell pellets were resuspended in Amanda’s CoIP Buffer (insert recipe) and transferred to 1.5 mL microcentrifuge tubes. Samples were rotated at 4°C for at least 15 minutes, passed through a 25-gauge needle, and then pelleted to remove debris. The clarified lysates were then incubated with pre-washed beads (HA, FLAG, or GFP-Trap, as indicated) at 4°C for 1 hour overnight. Beads were washed 3X with PBS.

For immunoblot analysis, the beads were resuspended in 1X SDS sample buffer and boiled for 5 min to elute. Samples were then loaded onto Criterion TGX 4-15% precast polyacrylamide gels (BIO-RAD, Hercules, CA, USA) and ran at 150V for 1 hour. Following electrophoresis, the gels were rinsed with distilled water and soaked in 20% EtOH. The samples were then transferred to nitrocellulose membranes using the iBlot transfer system (Thermo Fisher Scientific, Waltham, MA, USA) at 20V for 6 minutes. Following the transfer, the membranes were blocked in 1:1 PBST: Intercept Blocking Buffer for 1 hour at room temperature. Primary antibodies were diluted from 1:500 to 1:5,000 in 1:1 PBST: Intercept Blocking Buffer and incubated overnight at 4°C. Secondary antibodies were diluted 1:10,000 in 1:4 PBST: Intercept Blocking Buffer and incubated for 1 hour at room temperature. Blots were imaged and quantitated using a LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).
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### 5.6 Protein-protein interaction proteomics

For LC-MS/MS analysis, samples were eluted from beads with 6 M guanidine and boiled for 5 min. Eluted samples were transferred to fresh microcentrifuge tubes twice to minimize bead carry-over. Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol for the microplate assay. Samples were then reduced with 5 mM DTT at 55°C for 15 min. After cooling, samples were alkylated with 15 mM iodoacetamide (MilliporeSigma, St. Louis, MO, USA) for one hour in the dark. Samples were then loaded onto 30 kD centrifugal filters (VWR, Radnor, PA, USA) and washed.
twice with 6M guanidine and twice with 10 mM ammonium bicarbonate. Protein samples were then
digested with mass spectrometry grade trypsin on the filter at 1:50 (w/w) ratio at 37°C with shaking
overnight. The next day, the digested protein samples were eluted from the filter and washed down
with 10 mM ammonium bicarbonate. The eluate was transferred to mass spec vials, and vacuum dried
until dry. The samples were then resuspended in OrbiA solvent (3% acetonitrile, 0.1% formic
acid). Quantitative LC-MS/MS was performed on 1 ug of each sample using a Fusion Lumos mass
spectrometer (Thermo).

The LC-MS/MS data were analyzed using PEAKS analysis software (Bioinformatics Solutions,
Waterlook ON, Canada) using the SwissProt Homo sapien database. The database search included a
fixed carbamidomethyl modification on Cys and variable modifications, including Meth oxidation,
Asn/Gln deamination, and Ser/Thr/Tyr phosphorylation. Relative peptide abundance was calculated
using the area under the curve (AUC) data for the indicated targets for each sample.

5.7 Imaging flow cytometry

To assess PTOV1 nuclear localization, GFP-PTOV expressing PC3 cells were trypsinized, washed,
and fixed in 1% PFA for 10 min at 4°C. Cells were then permeabilized in 70% ethanol at 4°C
overnight. Before the acquisition, cells were washed and incubated in PBS containing 1ug/ml
propidium iodide (PI) and 100ug/ml RNAse for 30 minutes at room temperature. Cells were
transferred to ice before acquisition on the ImageStream MKII (Luminex Corporation, Austin, TX,
USA) Sample preparation was performed identically for experiments involving NES-GFP-PTOV
transfected HEK cells. For MG132 and HUWE1 knockdown experiments, data was acquired using
live PC3 cells expressing GFP-PTOV resuspended in PBS+2% FBS without fixation,
permeabilization, or staining.
All analyses were performed in IDEAS software (Luminex Corporation). Nuclear localization was assessed using the default nuclear localization tool. PC3 cell images were spectrally compensated using single color controls before analysis. Cell images were gated by gradient RMS, and then by size and aspect ratio to include only single, in-focus cells. To measure nuclear localization, a log-transformed Pearson’s correlation coefficient (i.e. similarity score) was calculated for each image using the appropriate channels for PI and GFP.

5.8 Cellular fractionation

NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA) was used for all nuclear and cytosolic fractionation experiments. PC3 Cells were washed with PBS twice and harvested with trypsin-EDTA, centrifuged at 500 g for 5 min. Cells were washed again by resuspending the pellet in PBS, and pelleted by centrifugation at 500 g for 2-3 min. Cytosolic and nuclear fractions were then isolated according to the manufacturer’s protocol.

5.9 Drug treatments

Lysosomal and proteasomal degradation experiments utilized MG132 (Selleckchem, Houston, TX, USA cat. S2619) and Bafilomycin (Cayman Chemical Company, Ann Arbor, Michigan, USA cat. 11038). Cells were treated for 2 hours with either 10 uM MG132, 100 nM Bafilomycin, or a similar volume of vehicle control (DMSO) for 2 hours before harvest.

PTOV1 degradation rates were assessed using cycloheximide (Cayman Chemical Company, cat 14126). Cells were treated with cycloheximide at 50 ug/ml and then harvested at predefined time points between 0 and 6 hours.

SGK2 inhibition experiments were performed using GSK 650394 (Tocris, Minneapolis, MN, USA cat. 3572). Cells were treated with 10 uM GSK 650394 for 48 hours before harvest.
5.10 Kinase screening

245 purified Ser/Thr kinases were evaluated for activity against peptides encompassing S36 and S53 of PTOV1 via the radiometric KinaseFinder assay (ProQinase GmbH). In short, the peptides above were reconstituted in 50 nM HEPES pH 7.5 at 200 μM stock solution. Reaction buffer (60 mM HEPES-NaOH pH 7.5, 3 mM MgCl2, 3 mM MnCl2, 3 μM Na-orthovanadate, 1.2 mM DTT, 1 μM ATP/[γ-33P]-ATP), protein kinase (1-400 ng/50 μL) and PTOV1 peptides (1 μM) were distributed into 96-well, V-shaped polypropylene microtiter plates (assay plate). All PKC assays (except the PKC-mu and the PKC-nu assay) additionally contained 1 mM CaCl2, 4 mM EDTA, 5 μg/ml phosphatidylserine and 1 μg/ml 1.2-dioleyl-glycerol. The MYLK2, CAMK1D, CAMK2A, CAMK2B, CAMK2D, CAMK4, CAMKK2, and DAPK2 assays additionally contained 1 μg/ml calmodulin and 0.5 mM CaCl2. The PRKG1 and PRKG2 assays additionally contained 1 μM cGMP. One well of each assay plate was used for a buffer/substrate control containing no enzyme. The assay plates were incubated at 30°C for 60 minutes. Subsequently, the reaction cocktails were stopped with 20 μl of 4.7 M NaCl/35 mM EDTA. The reaction cocktails were transferred into 96-well streptavidin-coated FlashPlate® HTS PLUS plates (PerkinElmer, Boston MA), followed by 30 min incubation at room temperature on a shaker to allow for binding of the biotinylated peptides to the streptavidin-coated plate surface. Subsequently, the plates were aspirated and washed three times with 250 μl of 0.9% NaCl. Incorporation of radioactive 33P was determined with a microplate scintillation counter (Microbeta, Perkin Elmer). For evaluation of the results of the FlashPlate® PLUS-based assays, the background signal of each kinase (w/o biotinylated peptide) was determined in parallel. Kinases of interest were selected from the screen described above to repeat at three peptide concentrations (1 μM, 0.5 uM, and 0.25 uM) in triplicate.
5.11 Gene-expression data analysis

We downloaded RNA-Sequencing data for prostate-cancer patients from The Cancer Genome Atlas\textsuperscript{95}. These data had previously been aligned to version hg19 of the human reference genome\textsuperscript{96} and summarized as gene-level read counts using the Rsubread software\textsuperscript{97,98}. In addition, we downloaded clinical data for these patients and extracted Gleason scores. After identifying patients for whom we had both gene-expression data and Gleason scores, data for 485 patients remained. Using these data, we evaluated the relationship between Gleason scores and log2-transformed expression levels of \emph{PTOV1}, \emph{YWHAZ}, or \emph{YWHAE}. To download and parse the data, we wrote a script in the Python programming language (https://python.org). To generate the graphics, we used the R statistical software (version 3.6) and the ggplot2 package (version 3.3.1). The scatter plots use regression lines to show correlation trends and 95% confidence intervals to indicate uncertainty.
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