Selenium-Binding Protein 1 in Prostate Cancer

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THESIS

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CONTRIBUTION OF AUTHORS

The introduction places my dissertation questions in the context of the larger field and highlights the significance of these questions. Parts of the introduction in Chapter 1 were previously published as a literature review in the *International Journal of Molecular Sciences (IJMS)*. Elhodaky, M., and Diamond, A. M. (2018) Selenium-Binding Protein 1 in Human Health and Disease. *Int. J. Mol. Sci.*, 19(11), 3437. DOI: 10.3390/ijms19113437. Both Alan M Diamond, who is my thesis advisor, and I contributed to the writing of this published literature review.

The results contain all my unpublished data representing a series of my own experiments directed at determining the biological and metabolic functions of SBP1 in prostate cancer cells, targeted at identifying the likely mechanism(s) of action as a tumor suppressor in this disease. Shrinidhi Kadkol helped perform the site-directed mutagenesis to generate the C57G-SBP1 construct. I used the generated construct to transfect PC-3 cells to conduct study experiments, as shown in figures 5, 7, and 8.

The discussion represents my analysis of the research presented in this thesis/dissertation. The overarching conclusions and future directions are also discussed.

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LIST OF ABBREVIATIONS

ACC	Acetyl-coA carboxylase
ACT	Anhydrochlortetracycline
AMP	Adenosine monophosphate
AMPK	AMP-activate protein kinase
ARE	Antioxidant response elements
AR	Androgen receptor
ATP	Adenosine triphosphate
β-actin	Beta-actin
BD	Behçet's disease
BP	Base pair
CAN	Chronic allograft nephropathy
CO_2	Carbon dioxide
CRISPR	Clustered regularly interspaced short palindromic repeats
CTRL	Control
CYP3A4	Cytochrome P450 3A4
Cys	Cysteine
DAPI	4′,6-diamidino-2-phenylindole
DHT	Dihydrotestosterone
DMS	Dimethylsulfide
DNA	Deoxyribonucleic acid
Dorso	Dorsomorphin
DOX	Doxycycline

eIF4E	Eukaryotic translation initiation factor 4E
ER	Estrogen receptor
ETC	Electron transport chain
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gly	Glycine
GPx1	Glutathione peroxidase 1
GSK3B	Glycogen synthase kinase 3 beta
HIF-1α	Hypoxia-inducible factor-1α
HIV	Human immunodeficiency virus
HNF4α	Hepatic nuclear factor 4-alpha
HSP60	Heat shock protein 60
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
IFNγ	Interferon gamma
KD	Knockdown
kDa	Kilodalton
KLK3	Kallikrein related peptidase 3
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
MTO	Methanethiol oxidase

Met	Metformin
MT	Methanethiol
NaHS	Sodium hydrogen sulfide
NES	Nuclear export sequence
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear magnetic resonance
NLS	Nuclear localization sequence
NS	Non-significant
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
p	*p value*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PIN	Prostate intraepithelial neoplasia
PSA	Prostate-specific antigen
PXR	Pregnane X receptor
RNA	Ribonucleic Acid
RPLP0	60S acidic ribosomal protein P0
RT-qPCR	Real time quantitative PCR
S^{79}	Serine-79
SBP1	Selenium-binding protein 1
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Se	Selenium
SEM	Standard error of the mean
shRNA	Short hairpin RNA
TBST	Tris-buffered saline with Tween
TGF-β	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
Thr^{172}	Threonine-172
VDU1	Von Hippel–Lindau protein-interacting deubiquitinating enzyme 1
Veh	Vehicle

SUMMARY

Selenium-binding protein 1 (SBP1) is a highly-conserved protein that was first discovered in mouse liver in 1989 by Bansal et al. due to its ability to bind selenium. SBP1 was found to be reduced in several cancer types compared to normal or benign tissues, including cancers of the thyroid, lung, stomach, liver, kidney, ovary, breast, prostate, colon, head and neck, bladder, and malignant melanoma. In addition to being lower in cancers, the degree of reduction of SBP1 expression in resected tissues is often predictive of how long a patient will be cancer-free and survive their disease. Reduced SBP1 levels have been correlated with poor survival in several types of carcinomas, including colorectal, gastric, nasopharyngeal, pulmonary, renal, and bladder cancers. Additionally, reduced levels of nuclear SBP1 were previously shown to be associated with a higher prostate cancer tumor grade and a greater likelihood of recurrence following prostatectomy. It is, therefore, likely that SBP1 may exert a tumor suppressor function in these tissues including prostate, and its loss or downregulation may facilitate the process of carcinogenesis.

Prostate cancer is the most common cancer, and the second leading cause of cancer-related death in men. The Krebs cycle of the normal prostate is inhibited in favor of the production of citrate energy, therefore distinguishing the energy metabolism of the normal prostate from that of other organs. This inhibition of Krebs cycle is generally relieved during prostate cancer progression, hence allowing a metabolic shift towards oxidative phosphorylation, a process that is crucial for prostate cancer cell survival and proliferation.

The broad goal of the research described in this thesis was the discovery of events that contribute to the progression of prostate cancer, and the role of SBP1 in this process. Using an *in silico* analysis, it was determined that the promoter region of *SBP1* contains putative binding sites

for the HNF4α transcription factor. The potential for HNF4α to regulate *SBP1* expression was supported by the evidence that HNF4α inhibition resulted in a dose-response increase in the levels of SBP1 mRNA and protein, therefore identifying HNF4α as a novel negative regulator of SBP1 expression in prostate cancer cells. Additionally, the elevated expression of *HNF4α* in prostate cancer compared to benign tissues may identify HNF4α as an oncogene in this disease. The consequences of altering the levels of SBP1 were investigated by ectopically expressing SBP1 in PC-3 prostate cancer cells. SBP1 over-expression in PC-3 cells attenuated their anchorage-independent growth and the migration in culture, both properties associated with transformation. One mechanism by which SBP1 impacts prostate cells is by altering cellular energy metabolism to become less reliant on OXPHOS, as evidenced by the reduction in oxygen consumption of cells when SBP1 is over-expressed. Evidence was generated that the reaction products of SBP1, H_2O_2 and H_2S, can activate AMPK, a major regulator of pathways of energy homeostasis. However, it remains to be determined whether the SBP1 reaction products activate AMPK directly or whether AMPK activation is a consequence of the reaction products suppressing mitochondrial OXPHOS and ATP production, which would subsequently activate AMPK. Uncovering the molecular events orchestrating the metabolic transformation occurring in prostate cancer is essential to future advances in understanding prostate cancer and identifying potential novel therapeutic targets.

I. INTRODUCTION

Parts of this chapter were previously published as a literature review in the *International Journal of Molecular Sciences (IJMS)*. Elhodaky, M., and Diamond, A. M. (2018) Selenium-Binding Protein 1 in Human Health and Disease. *Int. J. Mol. Sci.*, 19(11), 3437. DOI: 10.3390/ijms19113437

A. Background

Selenium (Se) is a non-metallic, essential trace element for many organisms, including humans. Se has long been recognized for its potential in cancer prevention, as evidenced by multiple animal, and human epidemiological studies that have reported an inverse association between Se exposure and cancer risk (1-8). Many mechanisms have been suggested for the chemopreventive effect of Se (9-11), including DNA hypomethylation (12), blocked cell cycle progression, enhanced cell death, decreased cell proliferation, increased glutathione peroxidase or thioredoxin reductases activities (13), modulated endoplasmic reticulum stress response (14), and enhanced DNA repair (15). Furthermore, Se has been found to play a key role in mammalian development (16) and immune function (17,18). Low levels of Se may be a contributing factor to several non-neoplastic pathologies, including male infertility (19), heart disease (20), inflammation (21,22), and neuromuscular disorders (23).

It is generally recognized that important cellular and organismal functions of Se are likely mediated by the action of selenoproteins constituting the mammalian selenoproteome (24). While the functions of many selenoproteins are still unknown, they likely have a significant role in human health and disease. Human selenoproteins are generally classified into three categories (11,25). The first category includes proteins in which Se is cotranslationally incorporated into the

elongating peptide as the amino acid selenocysteine in response to an in-frame UGA codon in the corresponding messenger RNA (26). The human selenoproteome contains 25 genes (25). The second category consists of proteins in which Se is incorrectly substituted for sulfur in sulfur-containing amino acids due to the similarity in structure between these two elements. The third category is composed of selenium-binding proteins that bind Se by an unknown mechanism. The studies reported in this thesis are primarily focused on one member of the latter category, selenium-binding protein 1 (SBP1, SELENBP1, hSP56).

B. SBP1 discovery

SBP1 was first discovered in mouse liver in 1989 by Bansal et al. using ^{75}Se labeling. Normal 6-week old female BALB/c mice were given a single intraperitoneal injection of ^{75}Se in the form of Na_2SeO_3. After 40 hours, animals were euthanized, and livers were harvested for preparation of liver cytosols, which were then used for a combination of gel filtration, ion-exchange chromatography, and SDS-PAGE techniques. This led to identification of a selenium-binding protein of apparent molecular weight of 56 kDa (27), which was designated as SBP1.

The full-length human *SBP1* cDNA clone was first described by Chang et al. in 1997 and determined to be 1668 base pair (bp) long with an open reading frame encoding 472 amino acids (28). *SBP1* is abundantly expressed in various human tissues, including liver, lung, prostate, colon, and pancreas, while moderate levels were detected in spleen, heart, and ovary. In contrast, its expression was barely detectable in thymus, testis, and peripheral blood leukocytes (29). SBP1 is a highly conserved protein. Flemetakis et al. reported that the predicted amino acid sequence of SBP1 is conserved in both plants and animals, ranging from 77 to 88% in plants, while the identity between the plants and mammalian proteins ranged from 57 to 60% (30). By comparison, this

degree of homology is higher than other conserved proteins, such as HSP60, γ-tubulin, apoptotic cell death 1 protein, and elF4E whose identities of the plant and human proteins are 44, 49, 48, and 52%, respectively (30). The homology between the mammalian *SBP1* of mice and humans is 86% (30), indicating that the potential fundamental cellular and molecular functions for SBP1 are also conserved across different species. SBP1 is very similar to another selenium-associated protein, selenium liver binding protein (AP-56, SBP2), whose sequence differs by only 14 residues from SBP1 and is encoded by a distinct gene (31). AP-56 is implicated in the detoxification of acetaminophen in the liver (31). Although these genes are regulated differently, their similarity may indicate a role for SBP1 in detoxification.

C. The role of Se in SBP1

The form of Se in SBP1 is currently unknown. Se is stably associated with SBP1, probably through a selenosulfide bond (perselenide), as indicated by the binding of Se to SBP1 being reversed by the addition of a reducing agent during SDS-PAGE (32). Based on structural and functional studies, it was suggested that one cysteine in SBP1 was the likely binding site for the Se molecule, the cysteine at position 57 (33). Converting cysteine 57 in SBP1 to a glycine and ectopically expressing that protein in human HCT116 cells, that do not express detectable SBP1 levels, indicated that the loss of the cysteine reduced the half-life of the protein, induced mitochondrial damage, and attenuated the degree of phosphorylation of signaling proteins such as p53 and GSK3β compared to the native protein expressed at similar levels (34).

The Se in SBP1 may facilitate its interaction with other proteins. SBP1 physically interacts with von Hippel–Lindau protein-interacting deubiquitinating enzyme 1 (VDU1), which plays a role in proteasomal protein degradation (32,35). This indicates that SBP1, via its interaction with

VDU1, may have a role in ubiquitination/deubiquitination-mediated protein degradation and detoxification pathways. When the Se moiety was dissociated from SBP1 by the addition of ß-mercaptoethanol, the interaction with VDU1 was completely blocked, indicating that Se may be essential for the interaction of these two proteins (32). While the Se moiety is likely required for its interaction with VDU1, the inclusion of Se in SBP1 does not appear to be essential for functioning as methanethiol oxidase (MTO), a recently-discovered novel human SBP1 enzyme activity that metabolizes sulfur-containing molecules (36).

D. SBP1 is a methanethiol oxidase

The enzymatic function of SBP1 was recently revealed by investigators examining the genetic determinants of extraoral halitosis, bad breath (36). The authors analyzed breaths and body fluids of five affected individuals with extraoral halitosis from three unrelated families using NMR spectroscopy and gas chromatography with a sulfur-specific detector. All patients exhibited elevated levels of methanethiol (MT), dimethylsulfide (DMS), dimethylsulfoxide, and dimethylsulfone in breaths and body fluids (36). The authors postulated that the accumulation of these compounds was due to a defect in a protein that oxidizes MT, leading to its accumulation in affected individuals. Methanethiol oxidases (MTOs) have not previously been reported in humans, but *SBP1* was identified as a candidate gene for extraoral halitosis by searching for human sequences that were similar to the gene encoding an MT-metabolizing protein previously recognized in methylotrophic bacteria, the *mtoX* gene. This effort revealed a 26% sequence identity and a homology of 54% between the two genes (36). Subsequent sequencing of *SBP1* in patients DNAs revealed four different biallelic mutations in the five patients (from three unrelated families), that were predicted to be pathogenic (1039G>T, 481+1G>A, 673G>T, and 985C>T).

Fibroblasts from these patients had significantly reduced SBP1 protein levels and undetectable MTO enzymatic activity compared to the control cells (36).

MTO converts MT to H_2O_2, formaldehyde, and hydrogen sulfide (H_2S), the latter is a gaseous signaling molecule with distinct functions at different cellular concentrations (37,38). At low concentrations, H_2S stimulates mitochondrial electron transport in mammalian cells, increasing oxygen consumption (39). At high concentrations, H_2S is toxic through the inhibition of mitochondrial respiratory-chain complex IV, and consequently reduces oxygen consumption (39). H_2S has been proposed as a therapy for multiple disorders by suppressing inflammation, affecting apoptotic pathways, increasing anti-oxidant defenses, and vasodilatation (37,40,41). It is quite conceivable that many of the consequences of SBP1 expression can be due to the effects on H_2S levels as well as the other products of the MTO-mediated reaction, on a broad spectrum of physiological endpoints.

E. SBP1 levels are reduced in cancer, and low levels are predictive of clinical outcome

One of the striking observations about SBP1 is the diversity of the types of cancers in which SBP1 was found to be reduced compared to normal or benign tissues (reviewed in (42)), including cancers of the thyroid (43), lung (44), stomach (45,46), liver (47), kidney (48), ovary (49-51), breast (52), prostate (53,54), colon (55,56), head and neck (57), bladder (58), and malignant melanoma (59). In addition to being lower in cancers, the degree of reduction of SBP1 in resected tissues is often predictive of how long a patient will be cancer-free and survive their disease (42). Reduced SBP1 levels have been correlated with poor survival in several types of carcinomas, including colorectal (55,60), gastric (46), nasopharyngeal (57), pulmonary (44), renal (48), bladder (58), and prostate (61) cancers. Recently, a search for genetic variations in

selenoprotein genes revealed that a polymorphism in the gene for SBP1, along with variations in the genes of selenocysteine encoding genes, were associated with prostate cancer aggressiveness at diagnosis (62). The exception to this pattern is ovarian cancer, where higher levels of SBP1 were associated with poor survival (50).

1. SBP1 levels and distribution in prostate cancer tissues

In addition to its levels, the distribution of SBP1 between cellular compartments may be relevant to cancer etiology. The associations between prostatic SBP1 levels, tumor grade, and disease recurrence following prostatectomy were investigated using a tissue microarray containing tissue from more than 200 prostate cancer patients who experienced biochemical recurrence (rising PSA) after prostatectomy and matched control patients whose cancer did not recur (61). Reduced SBP1 levels were associated with a higher likelihood of prostate cancer recurrence, as has been seen in other cancer types. However, a lower nuclear-to-cytoplasmic distribution of SBP1 was associated with a higher tumor grade (Gleason score) (61). These results indicate that sequestration of SBP1 in a particular cellular compartment may restrict access to relevant substrates, or the protein has different functions at these locations. Providing additional support for the role of SBP1 in prostate cancer, a study of 722 patients at Dana-Farber Cancer Institute identified an SBP1 polymorphism associated with an increased risk for aggressive prostate cancer among men with localized or locally advanced disease (62).

2. Is SBP1 a tumor suppressor?

The frequent loss of SBP1 in cancer and the association of reduced SBP1 levels with greater mortality could imply that SBP1 is a tumor suppressor. Alternatively, its loss or

downregulation may be a consequence of cancer development and progression, and the reduced levels represent a mere "bystander effect." Data supporting the direct role of SBP1 in cancer comes from studies where its levels are altered in cells and consequences relevant to transformation and tumorigenesis are revealed. Over-expressing SBP1 in colon and gastric cancer cells has generally yielded results consistent with a tumor suppressor function, including reduced growth in semi-solid media and decreased tumorigenicity in xenograft studies using immune-deficient mice (45,54,61,63,64). When over-expressed in lung cancer cells, SBP1 reduced proliferation and induced greater apoptosis compared to control cells only when the cells were challenged with H_2O_2 (47). Some of the phenotypic consequences of over-expressing SBP1 may be due to the downstream activation of the p53 tumor suppressor protein. Over-expression of SBP1 in HCT116 human colon cancer cells resulted in the increased phosphorylation of p53 (61). In addition to the phosphorylation of p53, SBP1 over-expression in the same cells resulted in the differential expression of 132 proteins, many are associated with energy metabolism and MAPK, Wnt, NF-κB, and Notch signaling (63). This same study reported that the expression of SBP1 resulted in the reduction of TWIST1, a critical regulator of the epithelial-mesenchymal transition and metastasis.

Consistent with over-expression data, either knocking down *Sbp1* or inactivating the gene using CRISPR/Cas9 editing in mouse lung cancer cells and injecting these cells into syngeneic hosts increased the size of tumors obtained compared to controls, although the number of tumors was not increased (65). Knockout mice that are null for *Sbp1* exhibit very limited pathology and do not develop tumors (66). However, examining the ovaries from these animals by gene expression microarrays indicated the increased expression of several genes associated with ovarian carcinogenesis, including *Notch1* and *Fas1* (66). Less clear is why the expression of tumor suppressor genes such as *Apc*, *RB1*, and *Trp53* was also enhanced in the ovaries from these mice.

Collectively, studies altering the levels of SBP1 provide substantial evidence that SBP1 serves as a tumor suppressor, and its loss or downregulation during cancer development contributes to disease development or progression.

3. Is SBP1 downregulation an early or late event in the process of tumorigenesis?

Given the data presented above indicating the frequent downregulation of SBP1 in cancers and its association with poor outcomes, it raises the issue of whether SBP1 loss occurs early in cancer development or late in the process, contributing to cancer progression. This issue was investigated by Zhang et al., who examined SBP1 levels in tissues classified as gastric cancer, precursor lesions, and matched controls of corresponding non-neoplastic epithelial tissues (67). SBP1 was reduced in most of the gastric cancer tissues compared to its abundant expression in matched non-neoplastic controls and precursor lesions, including tissues obtained from gastric ulcers, gastric polyps, as well as tissues presenting with chronic atrophic gastritis, intestinal metaplasia and dysplasia (67). SBP1 expression was similar in tissues with different levels of intestinal metaplasia or dysplasia, indicating that the reduction of SBP1 levels may be a late event associated with gastric carcinoma progression from normal gastric epithelium or premalignant lesions (67). These results are consistent with those of Kim et al. who observed much lower levels of SBP1 in colorectal carcinomas compared to matched controls of normal tissues and colon adenomas, supporting the notion that SBP1 loss is a late event during tumorigenesis (60). In addition, changes in the levels of proteins that occur during the progression of human squamous lung cancer were investigated using isobaric tags for relative and absolute quantitation labeling combined with 2D LC-MS/MS (68). SBP1 levels were determined by western blotting and immunohistochemistry and shown to be progressively lost during bronchial epithelial cancer progression (68).

In contrast to the data obtained examining gastric, bronchial, and colonic tissues, results have also been reported indicating that the reduction in SBP1 expression may be an early event in the evolution of some tumor types, including ovarian cancer (50) and uterine leiomyoma (69). Huang et al. identified SBP1 to be the most significantly reduced protein in ovarian cancer cell lines, including DOV13, OVCA429, OVCA882, TOV112D, and SKOV3 using a membrane proteome profiling analysis (50). However, relatively low levels of SBP1 were also observed in the immortalized human ovarian cell line, HOSE2089, indicating that the reduction of SBP1 may have occurred during the immortalization process (50). SBP1 expression was also approximately 4-fold lower in leiomyoma samples compared to normal myometrium, as determined by western blotting and immunohistochemistry (69). However, SBP1 levels were similar in tissues obtained from patients with proliferative secretory and atrophic endometrium in either leiomyoma or normal myometrium. These contrasting results may indicate distinct roles of SBP1 in the development of cancers of different origins.

F. Physiological roles for SBP1

The impact of SBP1 on normal biological processes and pathologies other than cancer may be due to roles in the modulation of cellular redox homeostasis. The SBP1 amino acid sequence contains two bis (cysteinyl) sequence motifs, Cys-X-X-Cys, at Cys5-X-X-Cys8, and Cys80-X-X-Cys83 shown to be a characteristic feature among several proteins which are involved in modulating the cellular redox state *in vivo* (70). In addition, SBP1 may also modulate the redox state of the extracellular environment. Experimental data in support of this come from a study where the knockdown of *SBP1* in MCF-7 breast cancer and HC116 colon cancer cells by siRNA resulted in increased levels of H_2O_2 and superoxide ion, leading to enhanced apoptosis when cells were exposed to selenite (71). The authors attributed this effect to the significant increase in

extracellular glutathione in the culture media. Changes in either the intracellular or extracellular environment can potentially impact a broad range of biological processes responsive to reactive oxygen in signaling pathways and contribute to the pathology associated with SBP1 dysregulation.

SBP1 has also been implicated in the late stages of intra-Golgi transport. Using an *in vitro* intra-Golgi cell-free transport assay, both endogenous and recombinant SBP1 (rSBP1) exhibited transport activity in the cell-free assay, and the addition of antibodies directed against SBP1 abolished this activity (72). These data indicate that SBP1 may be regulating vesicular intra-Golgi transport, particularly at the docking or fusions stages (72).

G. Roles for SBP1 in non-neoplastic diseases

Several studies have indicated a potential role for SBP1 in neurobiology. SBP1 has been localized at the tips of rapidly extending protrusions in T98G glioblastoma multiforme cells *in vitro* (73). Cell protrusive motility, which is tightly associated with actin filament polymerization, is an essential function for multiple cellular processes, including cell proliferation and migration. Monomeric G-actin, but not filamentous F-actin, was shown to be recruited to the SBP1-positive tip, indicating that the recruitment of SBP1 and G-actin at the cell margin precedes actin polymerization (73). In addition, SBP1 recruitment to the cell margin was observed to precede that of G-actin. The extension of the protrusion will stop when G-actin polymerizes to F-actin at the protruding edges; hence, SBP1 and G-actin disappear from these margins. SBP1 also localized to the growing tips of neurites in SH-SY5Y neuroblastoma cells *in vitro* (73), possibly indicating a role for SBP1 in neuronal cell outgrowth.

Changes in the levels of SBP1 in neuronal tissues may implicate the protein in several neuropathologies. *SBP1* mRNA was reported to be elevated in the frontal cortex of patients with

schizophrenia, indicating a potential specialized role in the pathophysiology of schizophrenia and the central nervous system (74-76). Genetic data have also implicated SBP1 in the risk of schizophrenia as two single nucleotide polymorphisms in the *SBP1* gene (rs2800953 and rs10788804) have been identified as susceptibility loci for schizophrenia in a family-wide association study (77). This, and a report of plasma SBP1 protein levels being decreased in patients with recent-onset schizophrenia (78), collectively indicate a potential specialized role for SBP1 in the pathophysiology of this disease. Whether these data indicate a role for SBP1 in the proper functioning of the central nervous system or the potential neuroprotective effect of Se against oxidative and excitatory brain damage (79) remains to be determined.

SBP1 may also be involved with the pathogenesis of glaucoma. Elevated levels of SBP1 have been associated with elevated ocular pressure (80). It was also identified as a differentially expressed gene in datasets comparing transcripts in glaucoma to normal control tissues, which has been verified in a rat model of acute elevated intraocular pressure (81). SBP1 was also identified as a novel target antigen in patients with Behçet's disease (BD) with uveitis, where an autoimmune response to retinal antigens is considered to be involved in the pathogenesis of the uveitis in those patients (82,83). What role SBP1 plays in these diseases has not yet been investigated.

H. The transcriptional regulation of SBP1

A greater understanding of the biological roles of SBP1 could be gained by examining how its expression is regulated. A subtractive hybridization approach was used to identify transcripts that were more abundant in the relatively fast-growing PC-3 human prostate cancer cells compared to slow-growing LNCaP cells (29). The low levels of *SBP1* mRNA in LNCaP cells was shown to be due to the downregulation of *SBP1* transcription as treatment of androgen-sensitive LNCaP

cells with dihydrotestosterone (DHT, the active form of androgen) reduced the levels of *SBP1* mRNA in a reversible, concentration-dependent manner (29). A more complicated picture was revealed by the analysis of the effect of androgen on normal ovarian epithelial cells obtained from the scraping of the ovary surface of patients with benign disease, an immortalized cell line, and ovarian cancer cell lines (50). Treatment of the primary and immortalized cells with DHT reduced the levels of *SBP1* mRNA, while SBP1 levels were increased in four tumor-derived cell lines by DHT treatment. The mechanism accounting for the differential response of these cell lines to DHT has not been resolved.

In addition to androgens, *SBP1* expression is also downregulated by estrogen treatment (17-β estradiol) in estrogen receptor (ER)-positive breast cancer cells, but not in ER-negative cells (52). The suppression of SBP1 expression by transforming growth factor-beta (TGF-β) was also observed using a rhesus monkey renal allograft model to identify molecules involved in the pathogenesis of chronic allograft nephropathy (CAN) (84). SBP1 was absent or markedly reduced in vascular smooth muscle cells in monkey kidney allografts with CAN. Testing growth factors previously associated with graft rejection, including IFNγ, TNFα, and PDGF, only TGF-β blocked the expression of SBP1 in the normal human vascular smooth muscle cell line, CRL-1999 (84). It is unlikely that the effects of androgens or estrogens on *SBP1* transcription is a direct consequence of the binding of the corresponding receptor to the *SBP1* promoter as there does not appear to be a consensus binding sequence for the receptor/transcription factor.

The mouse *Sbp1* gene has been identified as a direct target gene of the hypoxia-inducible factor-1α (HIF-1α) transcription factor in primary keratinocyte cell cultures (85). Scortegagna et al. examined HIF-1α gain of function during multistage murine skin chemical carcinogenesis in K14-HIF-$1\alpha^{Pro402A564G}$ transgenic mice. They concluded that HIF-1α was functioning as a tumor

suppressor, most likely by upregulating target genes, including *Sbp1*. Four hypoxia response elements were located within 1400 bp of the transcription start site of the human promoter region of *SBP1*, although the demonstration that these were bona fide response elements was not provided (85). HIF-1α is a central mediator of the cellular response to environmental stresses, such as hypoxia (86). It is over-expressed in many types of human cancer (87-89), and its over-expression is associated with treatment failure and increased mortality in some cancers including cancers of the cervix (90,91), breast (92,93), ovary (94), uterus (95), stomach (96), and brain (97). It is also associated with decreased mortality in other cancers, including those of the head and neck (98) and non-small-cell lung cancer (99). The consequences of the changes in HIF-1α levels are cancer-type specific, and the accompanying molecular alterations, such as SBP1 reduction/loss, can affect the balance between pro- and anti-apoptotic factors. A study by Huang et al. demonstrated that the decreased expression of SBP1 could lead to higher glutathione peroxidase 1 (GPX1) activity and reduced HIF-1α expression in hepatocellular carcinoma, indicating that SBP1 might exert its tumor suppressive function as a regulator of the tumor redox microenvironment (47).

In addition to the putative HIF-1α response elements in the *SBP1* promoter, two potential antioxidant response elements (ARE) with strong homology to the consensus ARE recognition motif are present in the promoter region of *SBP1* (100), although the functionality of these sequences as AREs has yet to be established. The presence of functional AREs in the promoter region of *SBP1* may account for the repression of transcription observed when the anti-oxidant selenoprotein GPX1 is ectopically expressed in colon carcinoma cell lines (100), as well as the reciprocal relationship observed in cells and tissues (42).

In some cases, epigenetic silencing by promoter methylation may be a mechanism by which the expression of *SBP1* is reduced in human colon cancers. Comparing DNA obtained from

colon cancer samples to DNA obtained from matched normal tissue indicated significantly more methylation in the promoter region of samples from the cancers (101). Hypermethylation of the *SBP1* promoter region was demonstrated in the human colon cell lines SW480, Caco-2, HT-29, and HCT1161 in which the extent of promoter methylation was associated with the degree of SBP1 protein levels. Moreover, treatment of these cells with 5-aza-deoxycytidine, a demethylation agent, decreased promoter methylation, and resulted in increased promoter activity and protein levels (101). In contrast, treatment of three different human colon cancer cell lines, LOVO, SNU-C4, and A549, with 5-aza-deoxycytidine did not result in increased SBP1 expression, nor was there any evidence of genetic loss at the *SBP1* locus (60). There was also a lack of evidence for either hypermethylation or genetic deletion, accounting for the low levels of *SBP1* observed in lung cancers (102). Furthermore, similar findings were seen in prostate cancer cell lines including, LNCaP, PC-3, and DU145, where the loss or downregulation of SBP1 does not appear to be due to any major deletion or rearrangement of the gene (29). While there is a consistent loss of SBP1 in many cancer types, there may be a multitude of ways in which tumor cells can achieve a reduction in SBP1 expression.

I. Prostate cancer metabolism

Prostate cancer is the most common cancer, and the second leading cause of death in men (103). However, a significant number of men undergo unnecessary prostatectomies or fail to receive sufficiently intensive treatment because the degree of tumor aggressiveness was unknown at the time of diagnosis. The prostate is a highly specialized organ, with one function being the accumulation and secretion of large amounts of citrate as a component of semen, thus supporting sperm health. Zinc accumulation in the prostate inhibits the mitochondrial aconitase enzyme that converts citrate to isocitrate that enters into the Krebs cycle to generate ATP by

oxidative phosphorylation (104). The organ relies on energy-inefficient aerobic glycolysis for its energy requirements (105,106). Alteration in this process is a hallmark of prostate cancer, in which, zinc levels decline dramatically, relieving the inhibition of aconitase. As a result, citrate re-enters the pathways that provide both energy (Krebs cycle/aerobic oxidation) and cellular building blocks (lipogenesis) to support cancer growth (105,107). In contrast, most solid tumors shift from oxidative phosphorylation, the primary energy source in normal tissues, to a heavy reliance on glycolysis. This phenomenon was first recognized by Otto Warburg in the 1920s and has been a focus of cancer biologists ever since. However, the unique metabolic changes typical of prostate carcinogenesis necessitate a different perspective in investigating the disease etiology for this organ.

As mentioned earlier, observations from cells derived from different tissue types support a tumor suppressor function for SBP1 (108). Here, the ability of SBP1 to impact properties of transformation and energy metabolism was investigated to understand the impact of its reduction in prostate cancer.

II. MATERIALS AND METHODS

A. Cells and culturing conditions

The LAPC-4 cell line was generously provided by Dr. L. Nonn laboratory (University of Illinois at Chicago, Chicago, IL). The PC-3 and LAPC-4 human prostate carcinoma cell lines were maintained in RPMI-1640 media (Gibco, #11875), whereas LNCaP human prostate carcinoma cell line was maintained in RPMI-1640 media (ATCC, #30-2001). All media were supplemented with 10% fetal bovine serum (Gemini Bio), 100 U/mL penicillin, and 100ug/mL streptomycin, and cells were maintained at 37°C with 5% CO_2. Cell lines were authenticated by Genetica DNA Laboratories (Burlington, NC). The inducible and constitutively-active SBP1 expression constructs were introduced to PC-3 TET-ON and PC-3 cells, respectively, via transfection using Continuum™ Transfection Reagent (Gemini Bio, #400-700). The same transfection reagent was also used for the transfection of plasmids into LNCaP cells. Infected and transfected cells were selected in 1ug/mL puromycin (Sigma-Aldrich) and 500ug/mL G418 (Sigma-Aldrich), respectively. Transfected cells were expanded and screened for SBP1 expression by western blotting and qRT-PCR using *SBP1* forward primer (5’-CCAAAGCTGCACAAGGTCAT-3’), *SBP1* reverse primer (5’- CATCCAGCAGCACAAAACCC-3’), *RPLP0* forward primer (5’-CCTCGTGGAAGTGACATCGT-3’), and *RPLP0* reverse primer (5’-CTGTCTTCCCTGGGCATCAC-3’). Expression of SBP1 was induced following incubation with 0.5ug/mL doxycycline or 0.05ug/mL anhydrochlortetracycline-HCl (Cayman Chemical, #19941) for 48-72 hours.

B. Real-time quantitative PCR

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, #74134) and reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, # 4368814), according to the manufacturer's instructions. RT-qPCR was performed with a QuantStudio 6 Flex Real-Time PCR System (ThermoFisher), using Fast SYBR™ Green Master Mix (ThermoFisher, #4385612). Fold changes were calculated by the ΔΔCT method, using RPLP0 as the control. In addition to SBP1 primers reported above, other primers used in RT-qPCR experiments include, *KLK3* forward primer (5'-CGAGAAGCATTCCCAACCCT-3'), *KLK3* reverse primer (5'-ACCCAGCAAGATCACGCTTT-3'), *CYP3A4* forward primer (5'-GTGGGGCCTTTGTCAGAACT-3'), and *CYP3A4* reverse primer (5'-TGGGCAAAGTCACAGTGGAT-3').

C. Plasmid construction

The doxycycline-inducible SBP1 expression construct, pRetroX-Tight-Pur-SBP1, was previously generated (61). To investigate the impact of nuclear versus cytoplasmic SBP1 localization, derivative expression constructs with SBP1 modified by the addition of the SV40 Large T Antigen nuclear localization sequence (NLS, PKKKRKV, 5'-CCAAAAAAGAAGAGAAAGGTA-3') or the HIV Rev Protein nuclear export sequence (NES, LPPLERLTL, 5'-TTGCCACCATTGGAGCGATTGACATTG-3') were created. These sequences were introduced into the 5' end of *SBP1* open reading frame using the following Not1-restriction-site-containing forward primers, (5'-GGCAGCAGCGGCCGCGCAGCAGCCACCATGCCAAAAAAGAAGAGAAAGGTAATGGCTACGAAATGTGGG -3') and (5'-

GGCAGCAGCGGCCGCGCAGCAGCCACCATGTTGCCACCATTGGAGCGATTGACATT GATGGCTACGAAATGTGGG-3') for SBP1-NLS and SBP1-NES, respectively. (109-111). The EcoR1-restriction-site-containing reverse primer (5'-TGCTGCGAATTCTGCTGCTCAAATCCAGATGTCAGAGC -3') was used for the generation of both derivative constructs. Successful cloning was verified by Sanger sequencing. The SBP1 shRNA and constitutively-active pCMV6-AC SBP1 expression constructs were purchased from OriGene Technologies, Inc. (#TG309566, #SC322261, respectively). The pCMV6-AC SBP1 plasmid (#SC322261, OriGene Technologies, Inc.) was used as a template for site-directed mutagenesis at cys57 of SBP1 using the Q5® Site-Directed Mutagenesis Kit (NEB, #E0554S), a forward primer (5'-TCCCCAGTATgGCCAGGTCAT-3'), and a reverse primer (5'-GACTTGGGGTCAACATCC-3'). The generated mutation of cys57 to gly57 (C57G) was verified by Sanger sequencing.

D. Immunofluorescence

Indicated cell lines were plated onto sterile Fisherbrand microscope cover glass slips (Fisher Scientific) placed in Corning Costar Flat Bottom 6-well Cell Culture Plates (Corning Inc.). The cells were allowed to grow to 80% confluence, washed three times with PBS, and fixed with 4% paraformaldehyde for 20 min. After fixation, the coverslips were transferred to a clean 6-well plate, and cells were again washed with PBS. Cells were then incubated with 0.1% saponin-TBST for 10 minutes at 37°C, after which they were washed three times in 0.1% saponin-TBST. Cells were then blocked for 30 minutes using a background sniper (BIOCARE Medical, Pacheco, CA). Following the blocking step, the cells were washed and incubated with SBP1 primary antibody (MBL, # M061-3) overnight at 1:150 diluted in Diamond Antibody Diluent (Cell Marque, Rocklin CA) in a humid chamber to prevent drying. Cells were then washed three times in 0.1% saponin-

TBST. Secondary antibody (Alexafluor-647) was then incubated at 1:200 in Diamond Antibody Diluent for one hour at room temperature in a dark, humid chamber. Cells were then washed three times in 0.1% saponin-TBST, after which they were washed three times in PBS. Cells were mounted using ProLong Gold Antifade reagent with DAPI (Invitrogen). Images were obtained using an LSM510UV confocal microscope (Zeiss).

E. Western blotting

Cells were harvested and lysed in 1x Cell Lysis Buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors. Lysates were mixed with NuPAGE LDS Sample Buffer (Life Technologies) and 10x Reducing Agent (Life Technologies) and boiled at 95°C for 10 minutes, after which lysates were loaded to 4–12% gradient Bis-Tris denaturing polyacrylamide gels (Life Technologies). After electrophoresis, proteins were transferred to an Immobilon-FL membrane (EMD Millipore) via electro-blotting. Membranes were incubated with antibodies overnight at 4°C. Antibodies against the following proteins were used: SBP1 at 1:2000 (MBL International), $pAMPK^{Thr172}$ at 1:1000, AMPKα at 1:1000, $pACC^{S79}$ at 1:1000, ACC at 1:1000, GAPDH at 1:10,000 (Cell Signaling Technology), and β-actin at 1:10,000 (Abcam, Cambridge, MA). An Odyssey® CLx imaging system (LI-COR Biosciences) was used to image and quantify protein bands.

F. Metabolic assays

Oxidative phosphorylation was examined by quantifying the oxygen consumption rate (OCR) using a Seahorse XF analyzer and Seahorse XF Cell Mito Stress Test Kits (Agilent Technologies, Inc.) according to the manufacturer protocol. In summary, mitochondrial respiration was determined in PC-3 cells using a Seahorse XF analyzer that measures parameters of

mitochondrial function by directly measuring the oxygen consumption rate (OCR) following the use of specific electron transport chain inhibitors, including oligomycin, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and rotenone/antimycin A. These inhibitors are injected sequentially, starting with oligomycin, which is an ATP synthase (Complex V) inhibitor and is injected first after acquiring basal measurements. The injection of oligomycin decreases the electron flow through the electron transport chain (ETC), causing a decrease in OCR, which is a reflection of mitochondrial ATP production (112). The second injection following oligomycin is FCCP, which is a potent uncoupler of OXPHOS. FCCP impacts ATP synthesis by disrupting the proton gradient across the mitochondrial membrane, enabling the electron flow to proceed uninhibited, and allowing the OCR to reach its maximal possible limit (Complex IV). This allows calculating spare respiratory capacity (SRC), which is the difference between maximal and basal respiration measurements. SRC reflects the cellular capacity to respond to cellular stresses or increased energy demands (112). The last injection is a combination of rotenone and antimycin A, which inhibits Complex I and III, respectively. This mixture turns off mitochondrial respiration, allowing to measure the non-mitochondrial respiration occurring by extra-mitochondrial cellular processes (112).

G. Cell proliferation and growth in semi-solid media

Proliferation was assayed by quantitation of cellular DNA using the FluoReporter® Blue Fluorometric dsDNA Quantitation Kit (ThermoFisher Scientific, Inc., #F-2962). Cells were plated at equal densities (5000 cells/well) on black, clear-bottom 96 well plates (Corning Inc.) and incubated at 37°C for three days, after which relative cell numbers were determined using the manufacturer protocol. Growth in semi-solid media was assayed by plating cells in triplicates in

0.6% agarose in media, according to a published protocol (113). Cells were imaged using an EVOS FL Imaging System (Invitrogen), and colonies were enumerated on day 21.

H. Wound healing assay

The wound-healing assay was used to evaluate cell migration. Cells were plated at equal densities on 6-well plates and incubated at 37°C until maximal confluency. Scratch wounds were generated by dragging a pipette tip through the cell monolayer in each well. The media was immediately replaced by 2mL of fresh media containing aphidicolin (Cayman Chemical, #14007) to inhibit cell proliferation. Cells were then imaged using an EVOS FL Imaging system and imaged at 24-hour intervals for up to three days. Changes in scratch widths (percentages of gap closure) were quantified by obtaining width measurements at the top, middle, and bottom of the scratches. Measurements were then averaged and used as a surrogate of cell migration.

I. Statistical analysis

GraphPad Prism software was used to perform statistical analysis. Two-tailed t-test statistical analyses were performed for all experiments, and data from at least three independent experiments are reported as means ± SEM. $p < 0.05$ was considered statistically significant.

III. RESULTS

A. SBP1 and energy metabolism

Given previous data indicating that ectopic expression of SBP1 can alter the expression of genes whose protein products are involved in energy metabolism, the effect of SBP1 over-expression on mitochondrial respiration of prostate cancer cells was investigated. A construct with SBP1 expression driven from a doxycycline-inducible promoter was introduced into the PC-3 human prostate cancer-derived cell line, selected as recipient cells as they express very low SBP1 levels. Transfected PC-3 cells exhibited robust induction of SBP1 following incubation with doxycycline (for three days), compared to the same cells exposed to only vehicle (Figure 1A). Ectopic SBP1 expression did not alter the proliferation of these cells relative to control cells (Figure 1B), similar to what was previously reported for SBP1-overexpressing HCT116 colon cancer-derived cells (61).

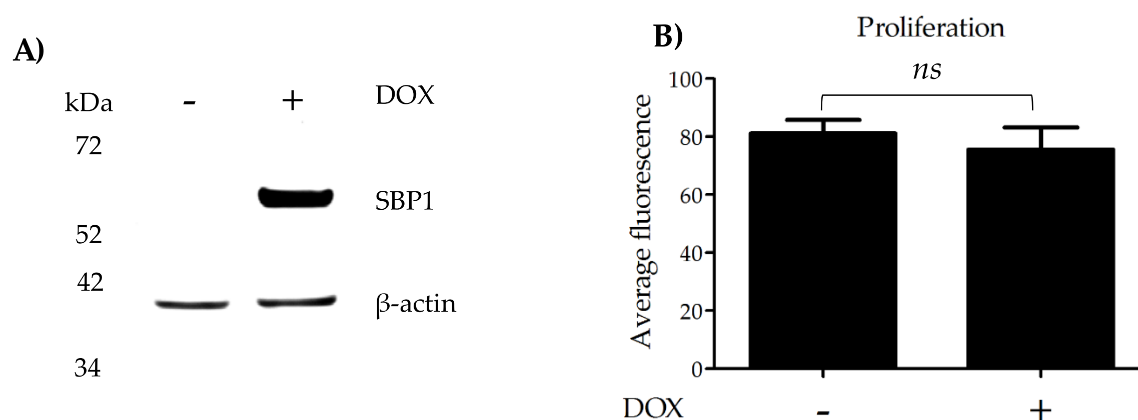


Figure 1. SBP1 over-expression does not affect the proliferation of PC-3 cells. A) SBP1 over-expression was successfully induced by doxycycline (DOX) in PC-3 cells. B) After three days of DOX-mediated induction of SBP1, fluorescence was quantified as an end-point for proliferation with a fluorometric dsDNA quantification assay. Data are represented in averages ± SEMs. *ns*, non-significant. n=3.

The effect of elevated SBP1 expression on mitochondrial respiration was determined in PC-3 cells. Expression of SBP1 in PC-3 cells significantly reduced mitochondrial ATP production (↓38%), maximal respiration (↓41%), and spare respiratory capacity (↓53%), therefore restricting the ability of cancer cells to respond to increased energy demand or to meet their metabolic needs under stress (Figure 2). SBP1 expression also reduced basal oxidative phosphorylation (OXPHOS, ↓39%) in a manner that is mimicking the healthy metabolism of normal prostate cells (Figure 2A), in which OXPHOS is inhibited, therefore providing evidence that SBP1 may be functioning as a metabolic tumor suppressor in prostate cancer cells.

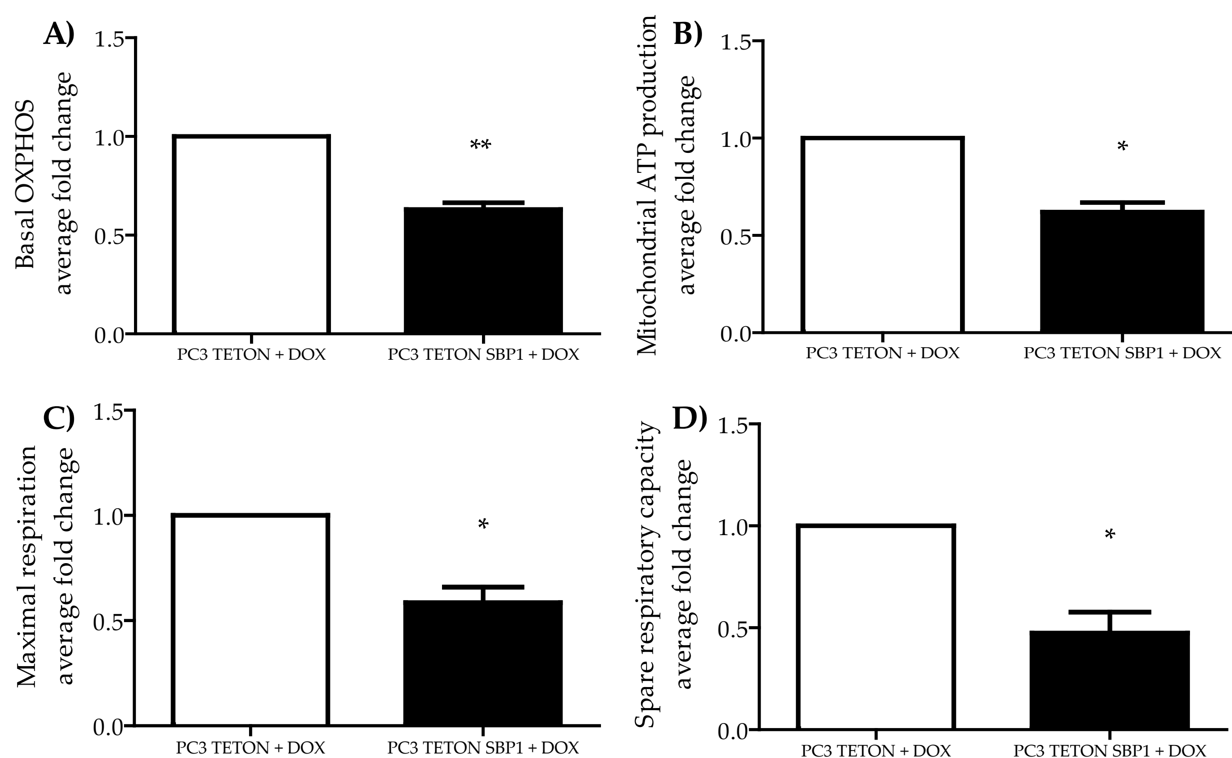


Figure 2. Over-expression of SBP1 alters energy metabolism in PC-3 cells. The basal OXPHOS (A), mitochondrial ATP production (B), maximal respiration (C), and spare respiratory capacity (D) are quantified in control and SBP1-overexpressing PC-3 cells incubated with vehicle or DOX 0.5ug/mL for 48hours. Data are normalized for DOX effect, and represented in averages ± SEMs. * $p < 0.05$, ** $p < 0.01$, n=3.

It was previously shown that SBP1 resides in both the nucleus and the cytoplasm in prostate epithelial cells, and the nuclear to cytoplasmic ratio was inversely associated with tumor grade (61). To investigate the impact of nuclear versus cytoplasmic SBP1 localization, derivative expression constructs with SBP1 modified by the addition of either the SV40 Large T Antigen nuclear localization sequence or the HIV Rev Protein nuclear export sequence were transfected into PC-3 cells, and targeting was visualized by immunofluorescence. These constructs successfully express and target SBP1 to the intended subcellular compartments (Figure 3A-B). When ectopically expressed in PC-3 cells, both targeted versions of SBP1 suppressed basal OXPHOS (SBP1-NLS:↓54%, SBP1-NES: ↓49%), mitochondrial ATP production (SBP1-NLS:↓58%, SBP1-NES: ↓52%), maximal respiration (SBP1-NLS:↓64%, SBP1-NES: ↓53%), and spare respiratory capacity (SBP1-NLS:↓71%, SBP1-NES: ↓57%) to a degree similar to the native SBP1 (Figure 3B-C). The similar degree of suppression of mitochondrial respiration by native and targeted SBP1 indicates that the SBP1-mediated reduction in OCR occurs independently of its subcellular localization.

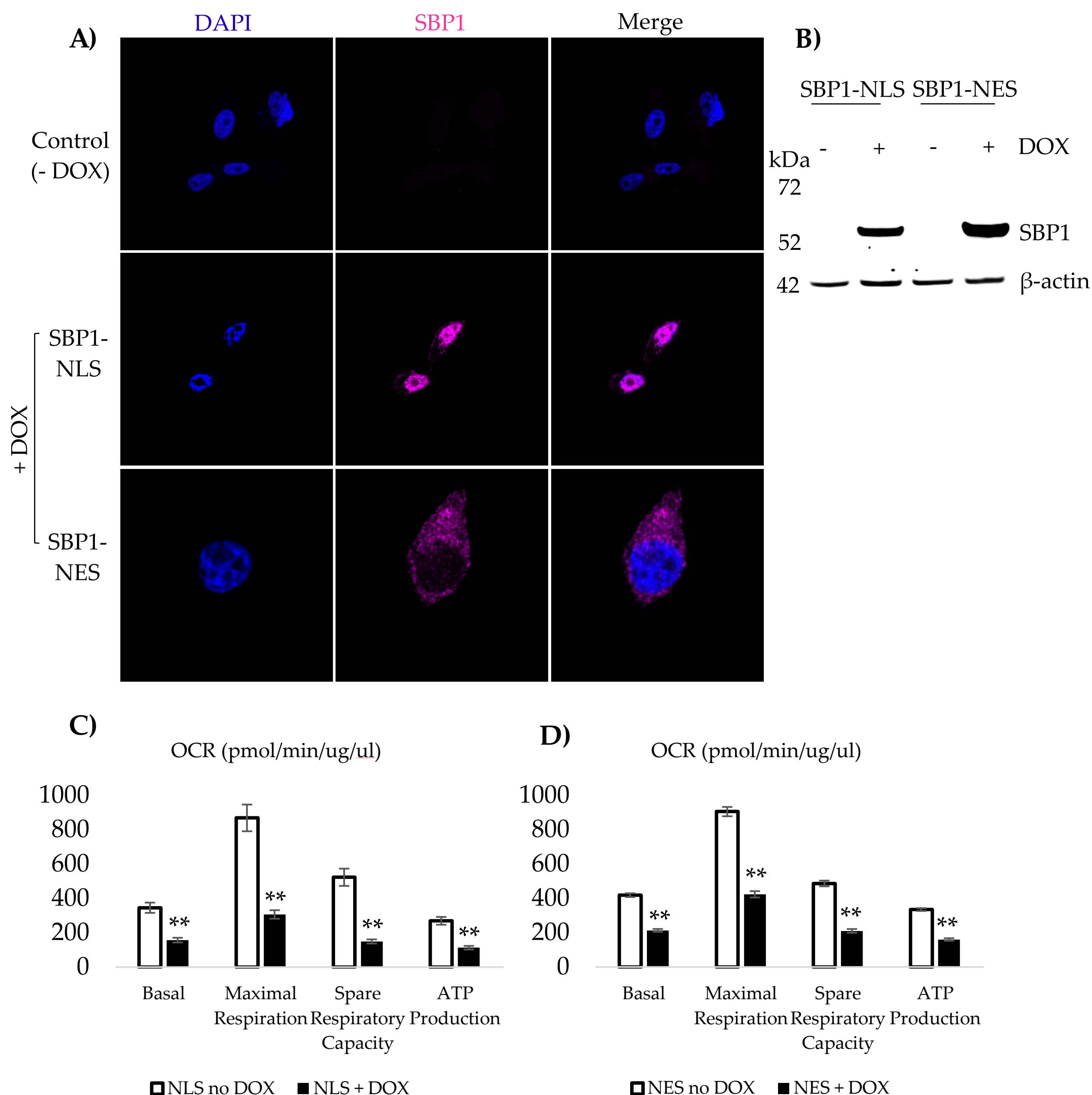


Figure 3. Targeting SBP1 to the nucleus or the cytoplasm. A) Immunofluorescence showing successful induction and targeting of SBP1 in the engineered PC-3 cells (magnification shown is 63x). SBP1-NLS is exclusively targeted to the nucleus, whereas SBP1-NES is exclusively excluded from the nucleus. B) Western blot showing successful induction of SBP1 in the engineered PC-3 cells. C-D) The basal OXPHOS, maximal respiration, spare respiratory capacity, and mitochondrial ATP production are quantified in control and PC-3 cells expressing engineered versions of SBP1 that either targeted to (C), or excluded from (D) the nucleus. Cells were incubated with vehicle or DOX at a concentration of 0.5ug/mL for 48hours. Data are represented in averages ± SEMs. ** $p < 0.01$. n=3.

The activation of two key regulators of glucose homeostasis by SBP1 was examined (Figure 4). AMP-activated protein kinase (AMPK) is activated by phosphorylation at Thr^{172} (114), and it stimulates glucose utilization and the uptake of fatty acids when ATP levels are low, favoring glycolysis and fatty acid oxidation over OXPHOS. Acetyl-CoA carboxylase (ACC) is inhibited by phosphorylation at Ser^{79} (115), and when active, it carboxylates acetyl-CoA to generate malonyl-CoA, the rate-limiting step for the generation of fatty acids (lipogenesis) as an alternative energy source. Stable ectopic expression of SBP1 in PC-3 cells resulted in a two-fold elevation of the phosphorylated form of AMPK compared to control cells (Figure 4A). Additionally, SBP1 expression caused a 20% increase in ACC phosphorylation, but it did not reach statistical significance. Compared to control cells, PC-3 cells that have their ectopic SBP1 expression controlled by a doxycycline-inducible construct also exhibited an increased AMPK phosphorylation after incubation with anhydrochlortetracycline, a doxycycline analog, for 48 hours (Figure 4B). SBP1 expression was silenced using an shRNA construct in LNCaP cells, a human prostate cancer cell line that produces significantly more SBP1 than PC-3 cells. As seen in figure 4C, reducing SBP1 levels by a mean of 75% in these cells resulted in about 46% reduction of AMPK phosphorylation at Thr^{172}, compared to cells transfected with a scrambled control shRNA construct. However, we did not observe a significant reduction in ACC phosphorylation in these cells.

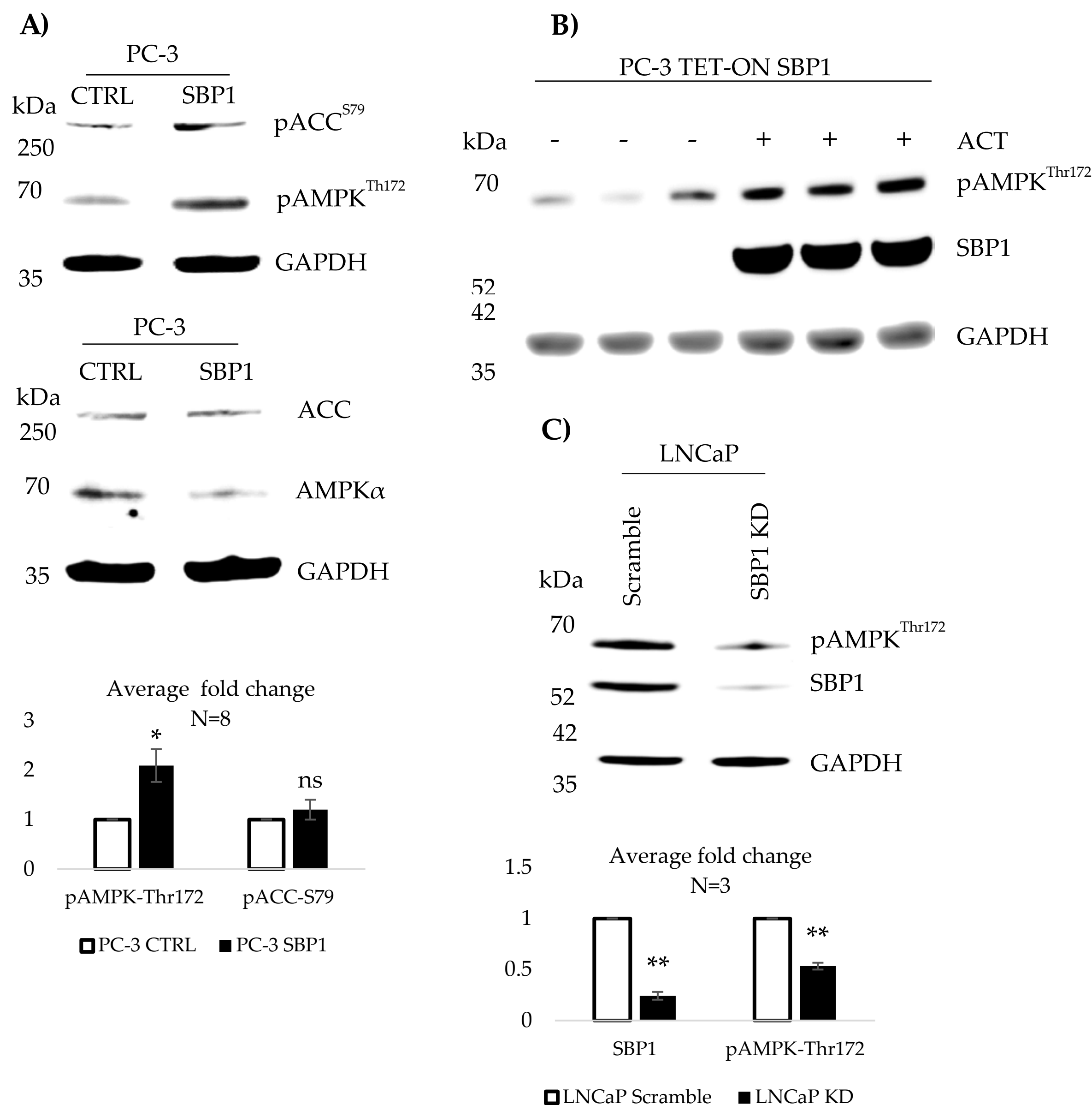


Figure 4. SBP1 activates AMPK in PC-3 cells. A) Representative western blot showing the phosphorylation of AMPK and ACC by ectopic expression of SBP1 in PC-3 cells. B) Western blot showing the activation of AMPK by ectopic expression of SBP1 in PC-3 cells from a DOX-induced promotor. Anhydrochlortetracycline, a DOX analog, was added to the media at 0.1ug/mL for 48 hours. The lanes represent independent three biological replicates. C) Representative western blot showing the reduction of pAMPK in LNCaP cells, in which SBP1 expression was silenced using an shRNA construct. Densitometric quantifications are shown below corresponding figures. *ns*, non-significant, $* p < 0.05$, $** p < 0.01$. Data are represented in averages ± SEMs.

B. The effects of SBP1 and selenium binding on cellular transformation

The frequent loss of SBP1 in prostate cancer could be a "bystander" effect during the process of carcinogenesis or may indicate a tumor suppressor function for SBP1. To address this, SBP1 was constitutively over-expressed in PC-3 cells, and transfectants. Both individual clones and a pool of transfectants, were examined for their ability to grow in semi-solid media, as anchorage-independent growth is a common feature of transformation. Ectopic expression of SBP1 significantly attenuated the ability of PC-3 cells to grow in semi-solid media by 47-90% compared to control vector-only transfected cells (Fig 5A-C), therefore indicating that SBP1 can suppress the transformation properties, and supporting a tumor suppressor function for SBP1 in prostate cancer cells.

In addition to growth in semi-solid media, the ability of tumor cells to migrate on a tissue culture plastic dish is a frequent surrogate for advanced or aggressive cancer cells. The migratory ability of SBP1-overexpressing cells was assessed using a wound healing assay (scratch assay). Cell monolayers were scraped with a pipette tip, and the migration of cells into the scratch was measured over time in the presence of the anti-proliferative agent, aphidicolin. Ectopic expression of SBP1 attenuated the migration of PC-3 cells into the scratched area by 30% after two days, relative to control vector-only transfected cells (Figure 6), therefore indicating that SBP1 can attenuate the migration of prostate cancer PC-3 cells *in vitro*.

What role the binding of selenium to SBP1 might provide to its function remains unknown. To determine whether selenium is essential for SBP1 functions revealed above, a derivative SBP1 construct was generated by site-directed mutagenesis to alter the cysteine 57 to glycine (C57G), the likely selenium-binding amino acid (33), and was transfected in PC-3 cells. The mutant SBP1 was ectopically expressed in PC-3 cells, and the cells were assayed for their ability to grow in

semi-solid media (soft agar). Mutated C57G-SBP1 and wild-type SBP1 expression reduced the growth of pool of transfected PC-3 cells in soft agar by 41% and 47%, respectively (Figure 5). Similarly, ectopic expression of mutant C57G-SBP1 attenuated the migration of PC-3 cells into the scratched area by 32% after two days, relative to control vector-only transfected cells, indicating that the likely selenium binding at cys57 does not affect the observed SBP1-induced attenuation of PC-3 cell migration and anchorage-independent growth *in vitro* (Figure 7).

The C57G-SBP1 was also able to enhance the activation of AMPK (Figure 8) to a similar extent as the wild type protein seen in figure 4, therefore indicating that the likely selenium binding at cys57 does not affect the observed SBP1-induced AMPK activation in prostate cancer PC-3 cells.

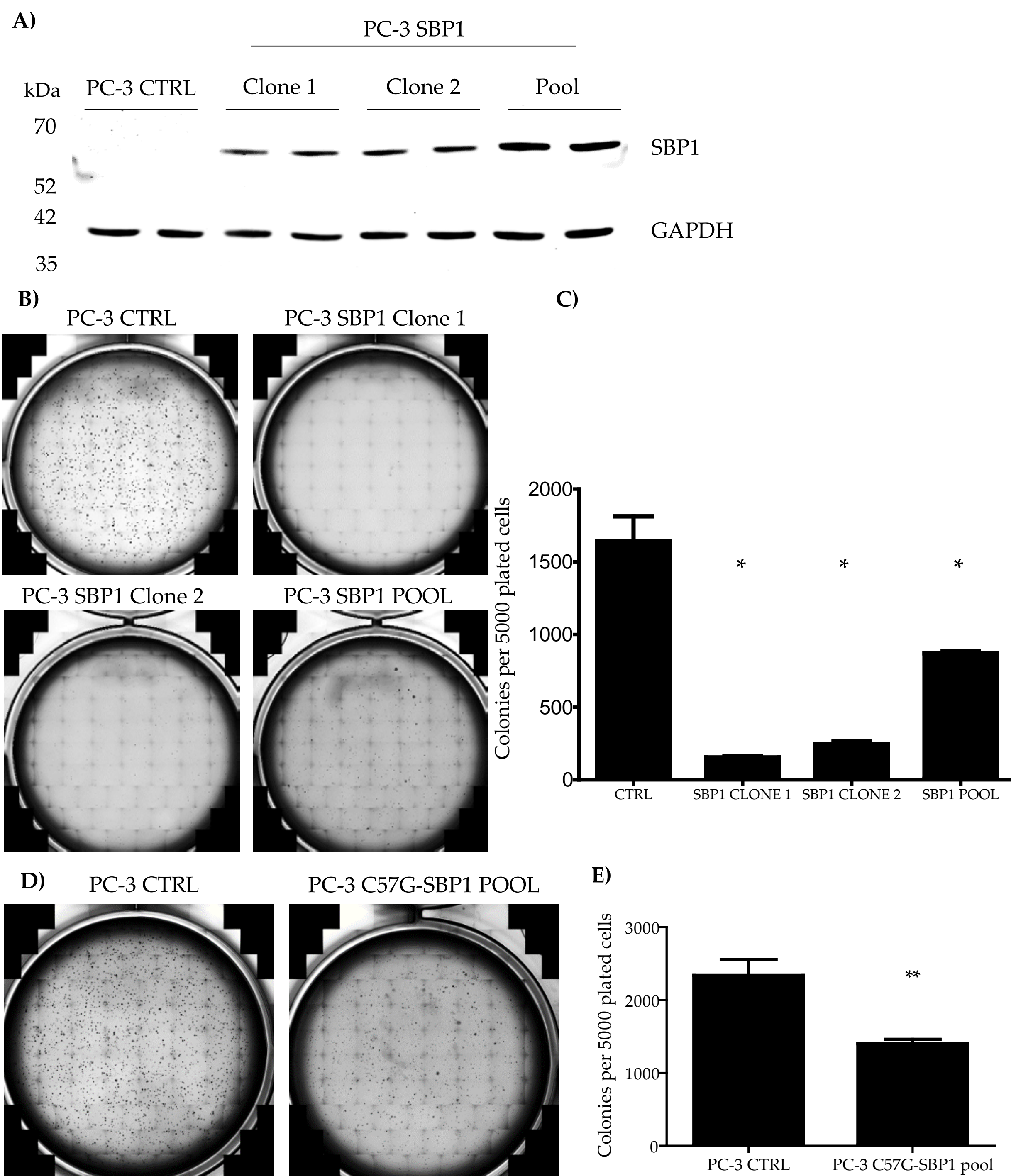


Figure 5. Ectopic expression of SBP1 attenuates anchorage-independent growth of PC-3 cells. A) A western blot showing high SBP1 levels in clones and pool of transfected PC-3 cells, compared to control vector-only transfected cells. B) Representative images of cell culture plates of transfected and control cells (refer to (C) for quantification of colonies). C) Quantification of average number of colonies formed per 5000 plated cells shown in B. D) Representative images of cell culture plates of transfected and control cells (refer to (E) for quantification of colonies). E) Quantification of average number of colonies formed per 5000 plated cells shown in D. * $p < 0.05$, ** $p < 0.01$. Data are represented in averages ± SEMs.

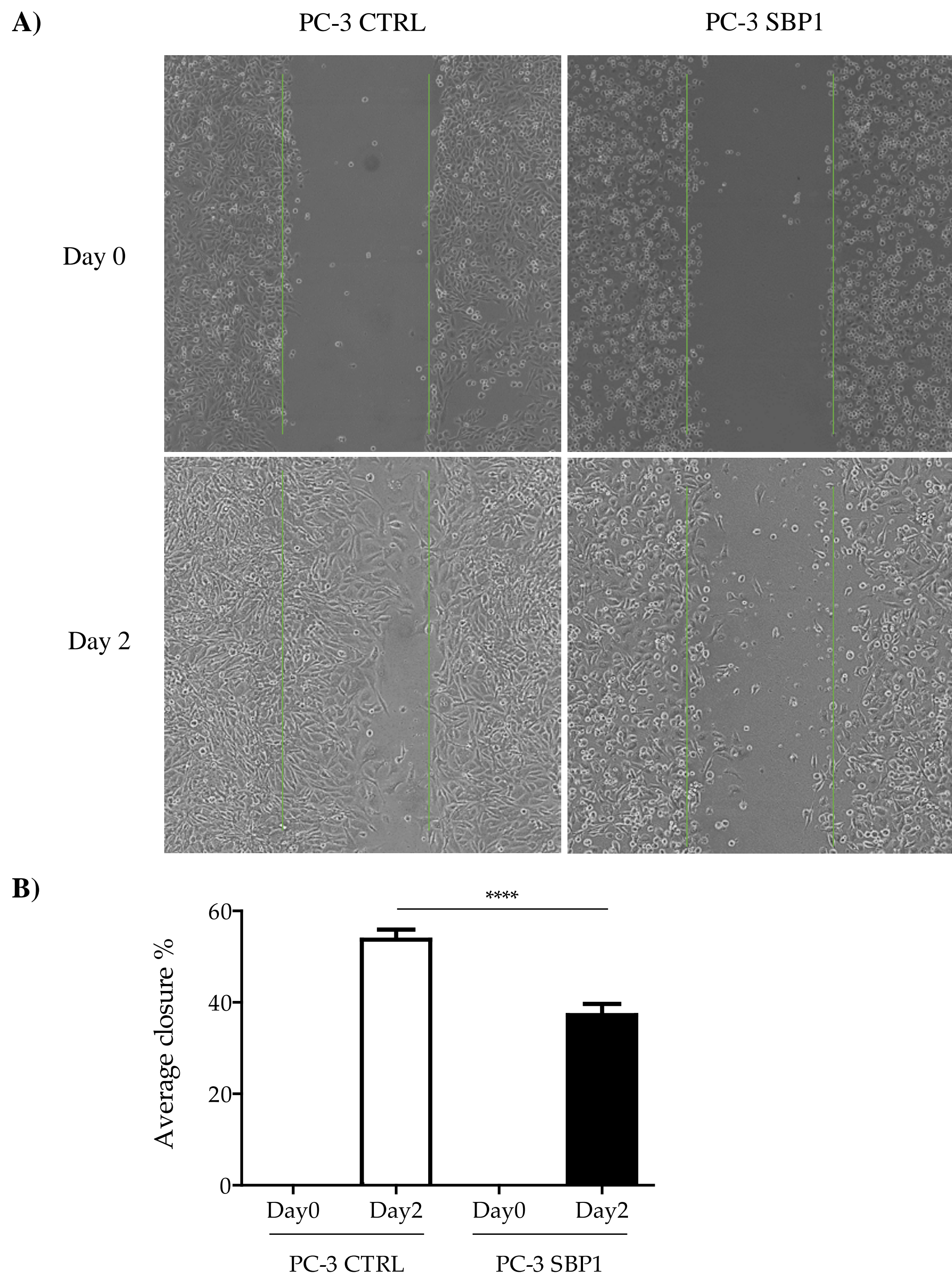


Figure 6. Ectopic expression of SBP1 attenuates the migration of PC-3 cells in a scratch assay. A) Representative images captured at day0 and day2 for control and SBP1-expressing PC-3 cells migrating into the scratched area. B) Quantification of data obtained from three independent experiments showing that SBP1 attenuates migration. Images and data were obtained using an EVOS FL Auto Imaging System (ThermoFisher). Data are represented in averages ± SEMs. **** $p < 0.0001$, n=3.

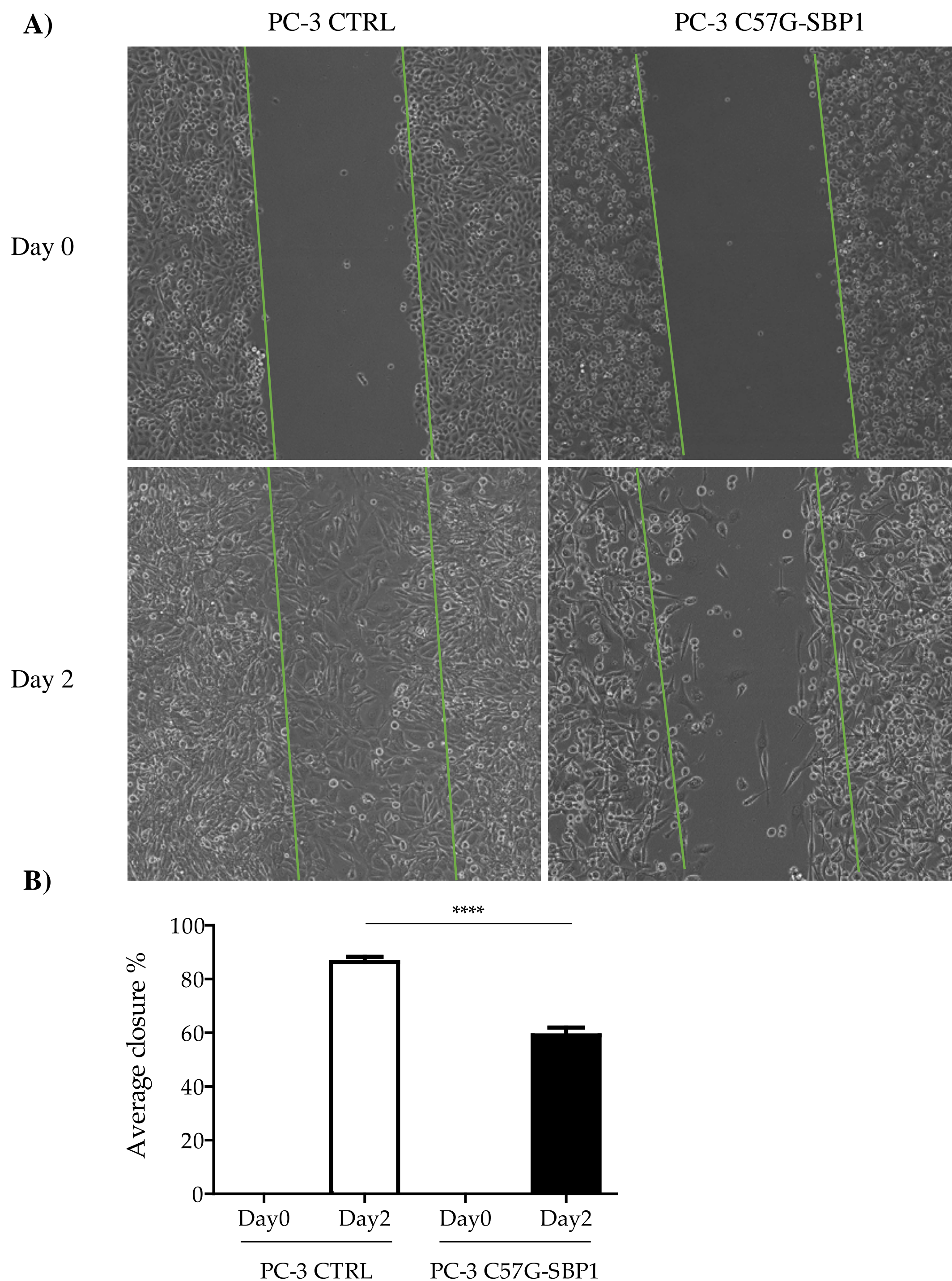


Figure 7. Ectopic expression of C57G-SBP1 attenuates the migration of PC-3 cells in a scratch assay. A) Representative images captured at day0 and day2 for control and C57G-SBP1-expressing PC-3 cells migrating into the scratched area. B) Quantification of data obtained from three independent experiments showing that SBP1 attenuates migration. Images and data were obtained using an EVOS FL Auto Imaging System (ThermoFisher). Data are represented in averages ± SEMs. **** $p < 0.0001$, n=3.

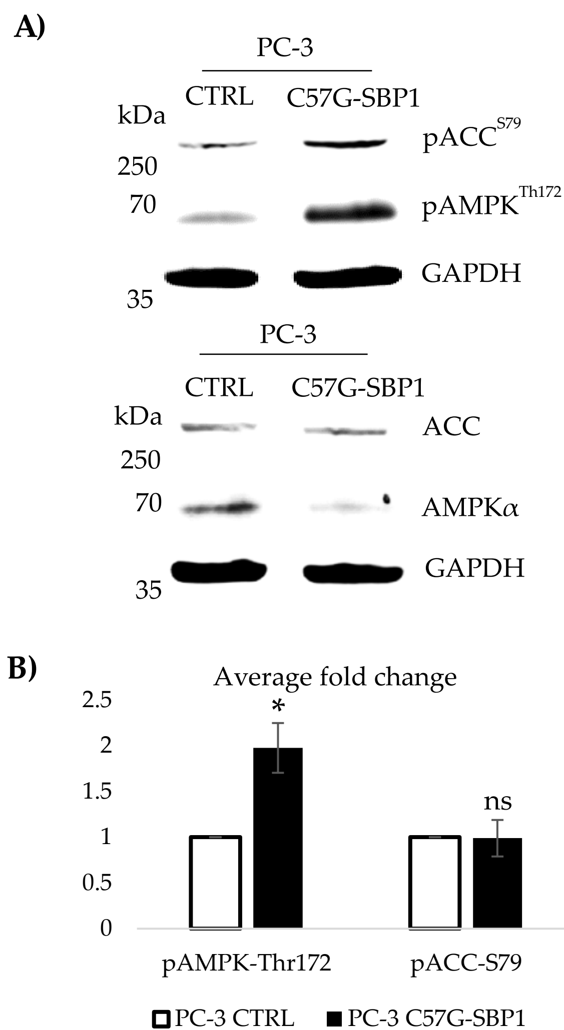


Figure 8. C57G-SBP1 activates AMPK PC-3 cells. A) Representative western blot showing the phosphorylation of AMPK and ACC by ectopic expression of C57G-SBP1 in PC-3 cells. B) Quantification of densitometries obtained from three independent experiments is shown. *ns*, non-significant, * $p < 0.05$. Data are represented in averages ± SEMs. n=3

C. The metabolic and biological function of the products of SBP1 enzyme activity

Since SBP1 can activate AMPK and attenuate cellular migration and growth in semi-solid media, we assessed whether AMPK activation contributes to these phenotypic changes associated with aggressive prostate cancer. Metformin has been shown to activate AMPK (116), and therefore AMPK activation was achieved by incubating PC-3 cells with 1mM metformin at the beginning of the scratch assay. Metformin-induced AMPK activation was verified by western blotting (Figure 9C). PC-3 cells exposed to metformin exhibited significantly attenuated migration into the scratched area by 32% after three days, relative to cells treated with vehicle only (Figure 9A-B), hence indicating that AMPK activation by metformin can inhibit the migration of prostate cancer PC-3 cells *in vitro*. Furthermore, SBP1-expressing PC-3 cells exhibited attenuated migration by 76% after incubation with metformin for three days, relative to cells treated with vehicle only (Figure 9A-B), hence indicating that AMPK activation by metformin potentiates the SBP1-induced attenuation of migration of prostate cancer PC-3 cells *in vitro*.

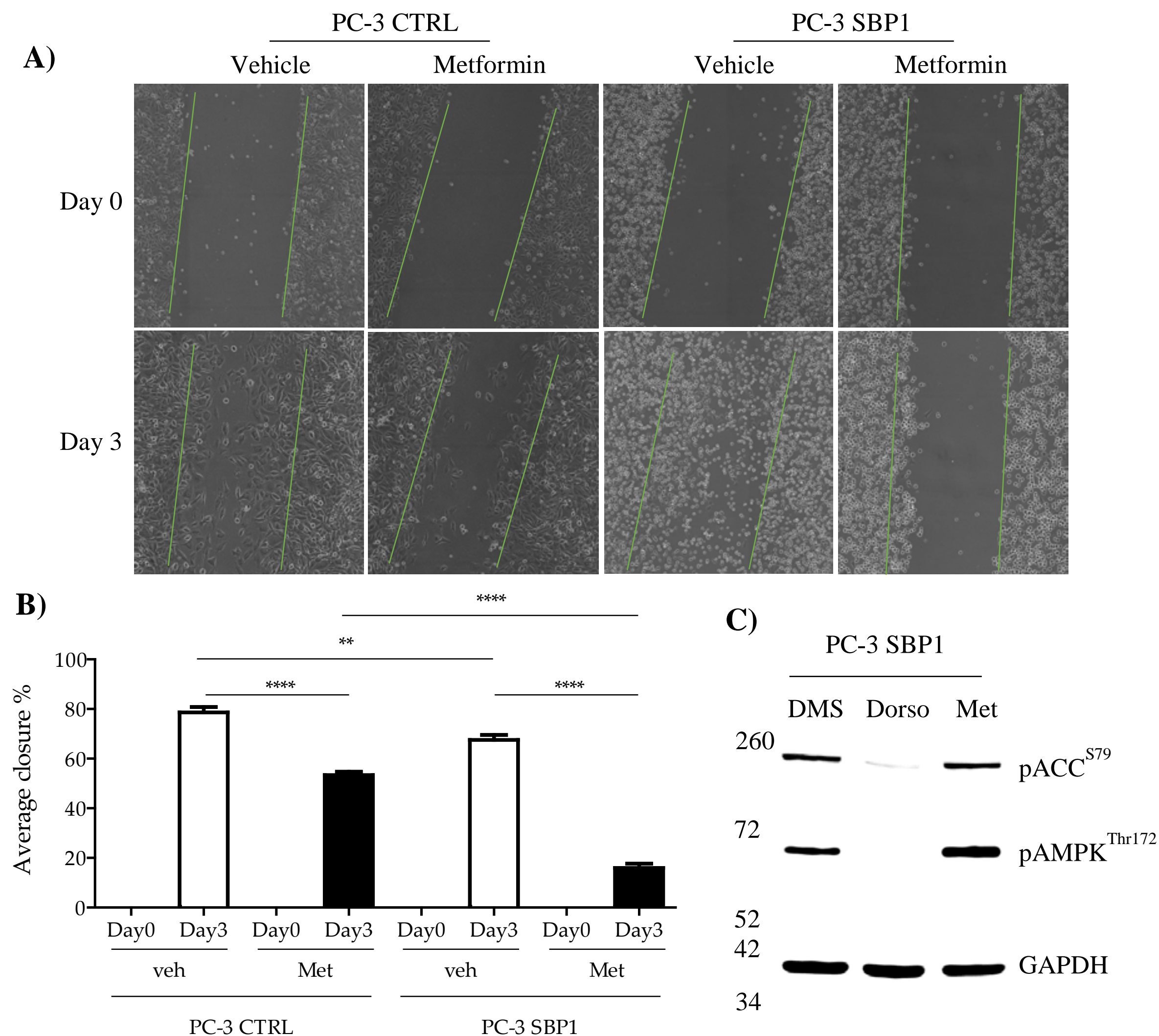
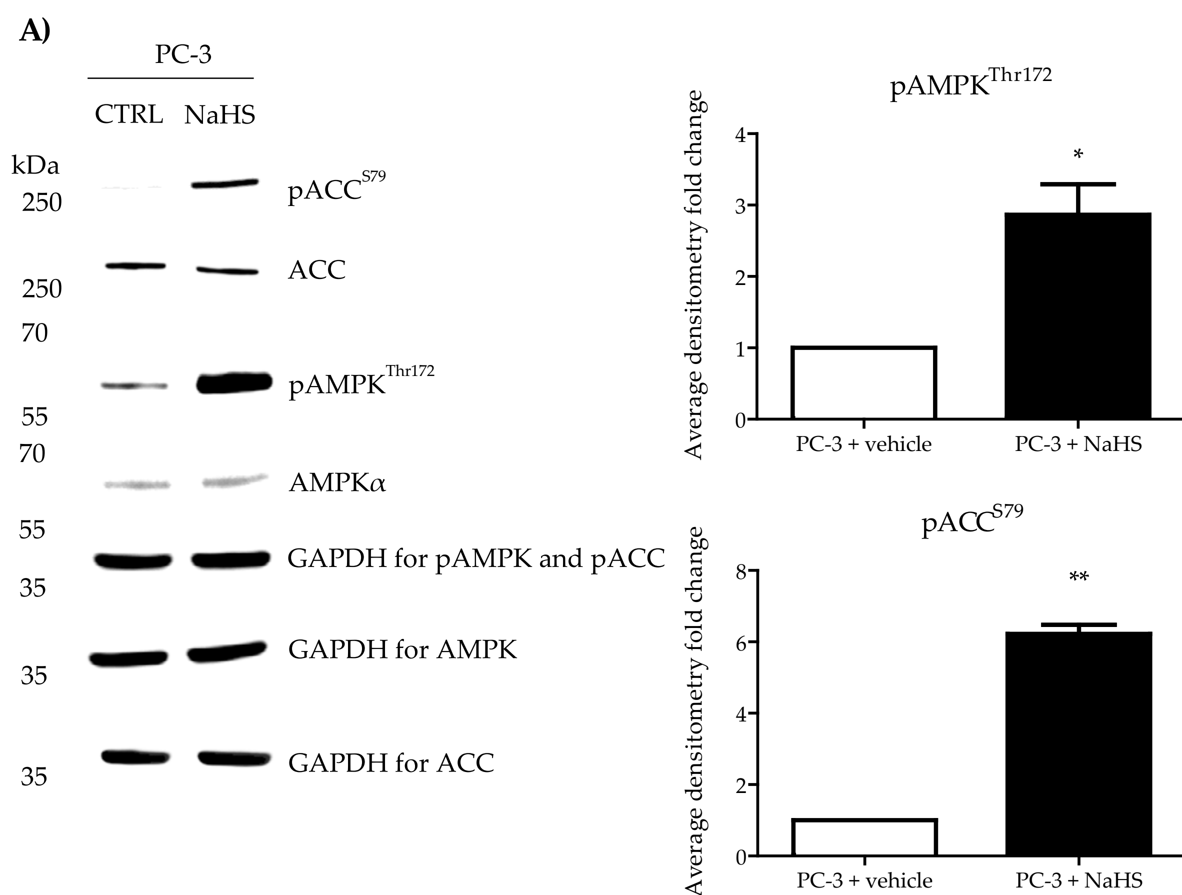


Figure 9. Pharmacological activation of AMPK attenuates migration of PC-3 cells. A) Representative images of PC-3 cells incubated with aphidicolin and metformin (Met, 1mM) showing the attenuation of migration in cells treated with Met, compared to control vehicle-only (veh) treated cells. B) Quantification of the data obtained from three independent experiments with the bars representing the width of the scratch. C) Western blot showing successful induction, and inhibition of AMPK by metformin 1mM, and dorsomorphin 10uM (Dorso), respectively. Data are represented in averages ± SEMs. ** $p < 0.001$; **** $p < 0.0001$, n=3.

SBP1 is a methanethiol oxidase (MTO), converting methanethiol to H_2O_2 and H_2S (36). Both reaction products are essential signaling molecules, with H_2S also being able to suppress mitochondrial respiratory complex IV at high concentrations (117-119). As seen in Figure 10, exposure of non-transfected PC-3 cells to either H_2O_2 or NaHS (H_2S donor) results in the phosphorylation of both ACC and AMPK, therefore emphasizing the role SBP1 may play by functioning as an MTO in inducing the phosphorylation of AMPK and ACC.



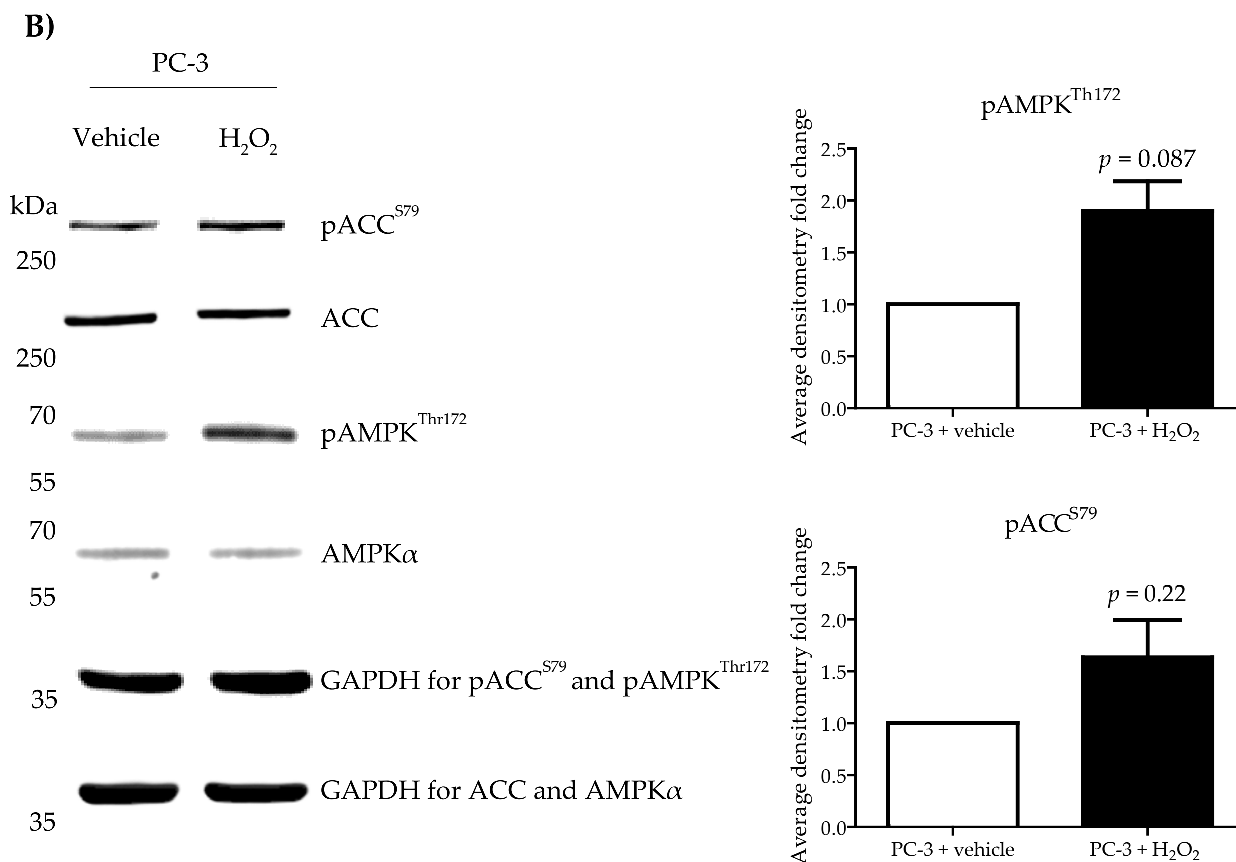


Figure 10. NaHS and H_2O_2 phosphorylate AMPK and ACC. A) Representative western blots showing that exposure of PC-3 cells to NaHS (1mg/mL for 15 minutes) or B) H_2O_2 (250uM for 1h) induced the phosphorylation of ACC and AMPK. Quantification of densitometries obtained from three independent experiments is shown next to the corresponding blot. Data are represented in average fold changes ± SEMs. * $P < 0.05$, ** $p < 0.01$. n=3.

D. The transcriptional regulation of SBP1

The mechanisms accounting for SBP1 downregulation in prostate cancer remain unknown. Hypermethylation of the SBP1 promoter region is present in colon cancers and colon cancer-derived cell lines (101) but not in other cell lines (60). No evidence of hypermethylation or genetic

deletion of SBP1 was detected in lung and prostate cancers (29,102). To investigate how SBP1 is downregulated in prostate cancer, an *in silico* analysis was performed using the SABiosciences platform (SABiosciences Corporation, Frederick, MD) to identify putative transcription factor binding sites in the *SBP1* promoter region (Figure 10A). The analysis revealed several consensus sequences recognized for hepatic nuclear factor 4-alpha (HNF4α), a transcription factor essential for liver development and differentiation (120), as well as a regulator of several enzymes involved in glucose and lipid metabolism (121,122). The Oncomine™ Platform (Thermo Fisher, Ann Arbor, MI) for analysis and visualization was used to examine *HNF4α* mRNA levels in prostate cancers (123). The analysis of 14 prostate cancer studies (124-137) indicated that *HNF4α* was significantly elevated in prostate carcinoma compared to benign tissues ($p = 0.03$) — the analysis can be accessed through the website provided in the Appendix.

To test the ability of HNF4α to regulate SBP1 expression, LNCaP cells, that express much higher SBP1 levels compared to PC-3 cells, were exposed to the HNF4α inhibitor, BI-6015 (Cayman Chemical). HNF4α inhibition by BI-6015 increased both SBP1 mRNA and protein levels in a dose-dependent manner, indicating that HNF4α suppresses *SBP1* expression in these cells (Figure 11B-D). To validate successful HNF4α inhibition by BI-6015, cells treated with BI-6015 exhibited a three-fold increase in *CYP3A4* mRNA expression, a known HNF4α target (Figure 11E) (138).

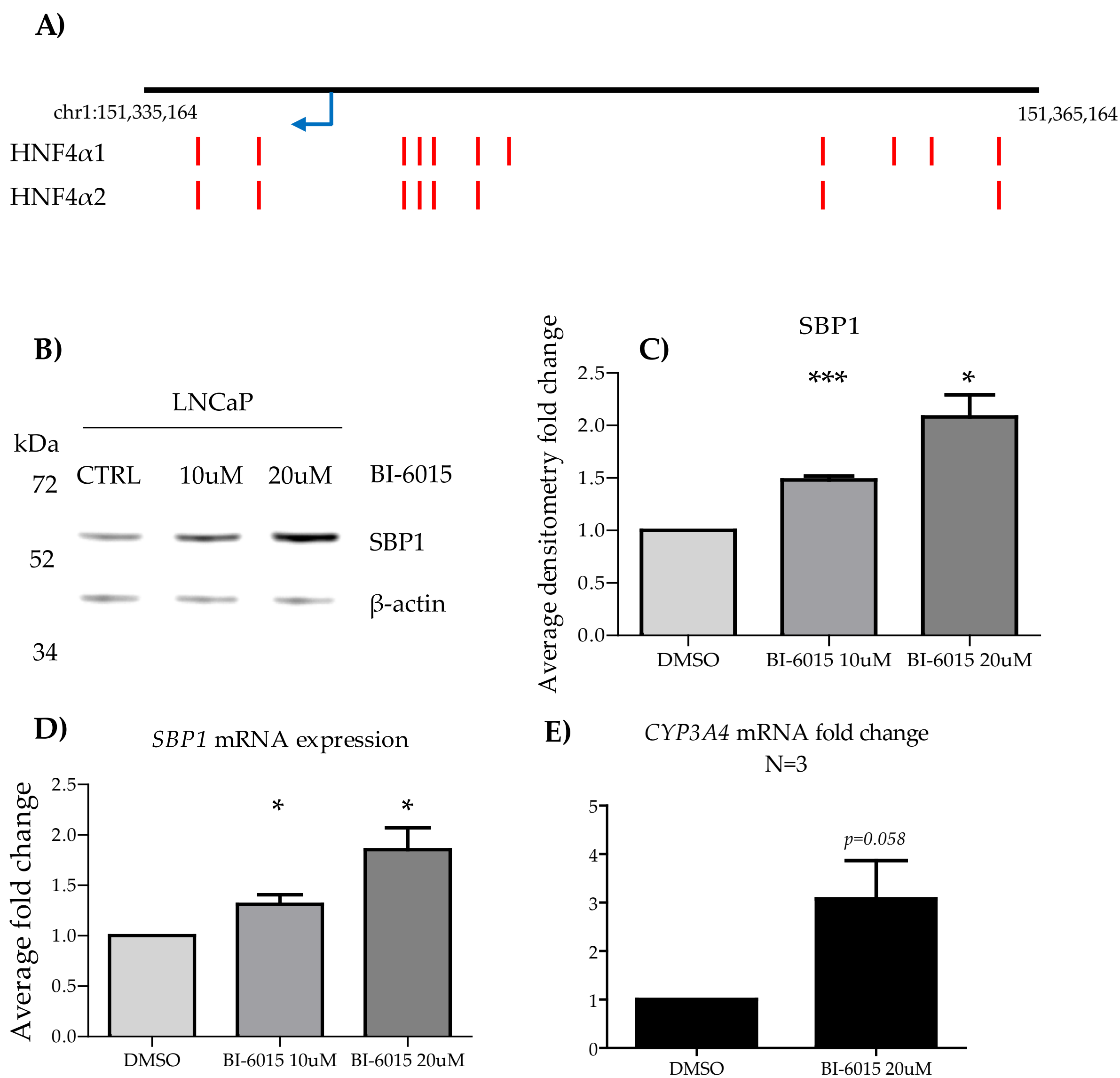


Figure 11. HNF4α suppresses SBP1 expression in LNCaP prostate cancer cells. A) An illustration showing the putative HNF4α transcription factor binding sites within the *SBP1* promoter. B-C) Representative western blot (B) and its densitometric analysis (C) showing a dose-dependent increase in SBP1 protein levels upon HNF4α inhibition by BI-6015, D) RT-qPCR demonstrating a significant dose-dependent increase in relative *SBP1* mRNA expression upon HNF4α inhibition by BI-6015 in LNCaP cells. E) RT-qPCR demonstrating an increase in relative *CYP3A4* mRNA expression upon HNF4α inhibition by BI-6015 in LNCaP cells. Data are represented in averages ± SEMs. * $p < 0.05$, *** $p < 0.001$, n=4.

IV. DISCUSSION

SBP1 loss has been implicated in the progression of cancers of many different tissue types (108). The evidence supporting this conclusion stems from observations of lower levels of SBP1 in cancers compared to benign tissues, or the association of lower SBP1 levels with poor clinical outcome. These data include tissue microarray analyses of prostate cancer samples that indicated that low SBP1 levels in the tumor tissues were associated with an increased risk of prostate cancer recurrence following prostatectomy (61). There are reports of cell culture or xenograft studies where over-expressing SBP1 inhibited phenotypes related to cellular transformation or tumorigenicity (64,65,101), but none of these studies specifically used prostate-derived cancer cells. The data presented here show that over-expressing SBP1 in human PC-3 prostate carcinoma cells attenuated their anchorage-independent growth and migration *in vitro*, two conventional assays of transformation. These data support the evidence that SBP1 is functioning as a tumor suppressor in prostate cancer, a disease with the magnitude of its metabolic landscape is yet to be defined.

The prostate is a highly specialized organ, with one function being the accumulation and secretion of large amounts of citrate as a component of semen, thus supporting sperm health. Zinc accumulation in the healthy prostate inhibits the mitochondrial aconitase enzyme that converts citrate to isocitrate, which then enters into the Krebs cycle to generate ATP by OXPHOS (104). Prostate tissue relies on energy-inefficient aerobic glycolysis for its energy requirements, and the alteration in this process is a hallmark of prostate cancer, where zinc levels decline dramatically, relieving the inhibition of aconitase (105,106). As a result, citrate re-enters pathways that provide both energy (Krebs cycle/aerobic oxidation) and cellular building blocks (lipogenesis) to support

cancer cell growth (105,107). In contrast to what occurs during prostate cancer, most solid tumors shift from OXPHOS, the primary energy source in normal tissues, to a heavy reliance on glycolysis. This phenomenon was first recognized by Otto Warburg in the 1920s and has been a focus of cancer biologists ever since. However, the unique metabolic changes typical of prostate carcinogenesis necessitate a different perspective in understanding the etiology for this disease. The molecular events involved in this transition are not well understood, but are potential therapeutic targets, particularly in aggressive disease that is no longer responsive to other treatments. Here, we have identified the loss of SBP1 as a possible contributor to this metabolic transformation. In this study, over-expressing SBP1 inhibited OXPHOS in prostate cancer cells, therefore mimicking the metabolic phenotype of the healthy prostate, in which OXPHOS is also inhibited. It also provides evidence that SBP1 may exert a metabolic tumor suppressor function in the healthy prostate, and its loss or downregulation may facilitate the metabolic transformation observed in prostate cancer, a process that is crucial for cell survival and proliferation. It is particularly striking to see that SBP1 is still capable of suppressing OXPHOS regardless of its subcellular localization, whether nuclear or cytoplasmic, therefore denoting a potentially shared mechanism that suppresses mitochondrial respiration. Perhaps if this suppression is mediated via H_2S generated by SBP1 MTO enzymatic activity, it is possible that H_2S generated by the nuclear SBP1 is capable of crossing nuclear membrane and affecting the mitochondria. In fact, the high concentration of H_2S was shown to suppress mitochondrial respiratory complex IV (117-119).

Another possible explanation may involve AMPK, which is an intracellular energy sensor and has a crucial role in maintaining energy homeostasis. AMPK is a heterotrimer consisting of a catalytic α subunit and two non-catalytic β and γ subunits that exist in several isoforms (139-143). The $\alpha 2$ subunit isoform has been shown to be preferentially found in the nucleus (144).

Additionally, both α and β subunits were found to shuttle between the nucleus and the cytoplasm (145). It is, therefore, likely that the nuclear SBP1 may interact and activate AMPK in the nucleus. However, this is yet to be investigated. Uncovering the molecular and metabolic switches that regulate the energy metabolism in healthy and cancerous prostate cells may be crucial in advancing our understanding of this metabolic shift and discovering new therapeutics for prostate cancer patients.

A high AMP/ATP ratio will activate AMPK to restore intracellular energy balance. Several studies have found a beneficial effect of metformin, an AMPK activator, in reducing prostate cancer incidence and improving overall survival (146-151). Metformin has also been shown to inhibit the proliferation of prostate cancer cells (152-155). In this study, we demonstrate that SBP1 is capable of activating AMPK, and the metformin-induced AMPK activation reduced the migration of prostate cancer cells, therefore providing additional evidence for the potential mechanism(s) by which SBP1 may be functioning as a tumor suppressor in prostate cancer cells. This is further supported by the evidence that both H_2O_2 and H_2S, products of SBP1 functioning as an MTO (36), were also capable of activating AMPK. As mentioned earlier, high concentration of H_2S can suppress mitochondrial respiratory complex IV (117-119), hence inhibiting OXPHOS, although it has not been particularly tested in this study. The functional importance of H_2S in the pathobiology of the prostate cancer cells has been recognized (156,157), and several studies have shown that impaired sulfide metabolism is involved in patients with prostate cancer (158-160). Studies have also shown that H_2S and/or sulfide-containing compounds inhibit the survival of prostate cancer cells *in vitro* and *in vivo* (161,162). H_2S can also repress androgen receptor (AR) transactivation, which is post-translationally modified by H_2S through S-sulfhydration (163).

Prostate cancer is a disease that is mainly driven by AR signaling, which involves a complex interplay of a network of signaling molecules (164,165). AR was found to regulate many genes involved in the metabolism of prostate cancer cells (166-170). Additionally, AR was shown to suppress *SBP1* expression in LNCaP cells (29), and we also observed a dihydrotestosterone-induced suppression of *SBP1* in LAPC-4 cells (Figure 12), therefore suggesting a potential AR-mediated mechanism of the suppression of SBP1 levels seen in prostate cancer (61).

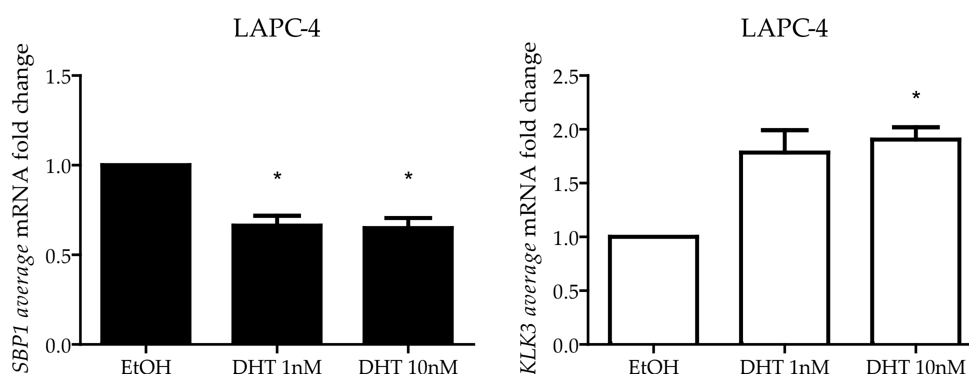


Figure 12. Dihydrotestosterone (DHT) suppresses *SBP1* expression in LAPC-4 cells. RT-qPCR demonstrating a reduction in relative *SBP1* mRNA expression after 24h DHT treatment, following 24h of serum-starvation. *KLK3* was used as a positive control for DHT action. Data are represented in averages ± SEMs. * $p < 0.05$. n=3.

Additionally, we identified HNF4α as a novel negative transcriptional regulator of *SBP1* expression, and the elevated expression of *HNF4α* in prostate cancer compared to benign tissues

using the Oncomine^{TM} platform may identify HNF4α as an oncogene in this disease. HNF4α is a transcription factor essential for liver development and differentiation (120), as well as a regulator of several enzymes involved in glucose and lipid metabolism (121,122). However, its role in prostate cancer development and progression is yet to be established. Here, we provide evidence that elevated HNF4α expression may underlie the observed SBP1 loss seen in prostate cancer. Interestingly, our preliminary data in LAPC-4 and LNCaP cells show an increase in *HNF4α* relative mRNA expression after treatment with DHT following a period of serum starvation (data not shown), therefore, indicating a potential cross-talk between HNF4α and AR signaling pathways, which remains to be further studied. AMPK has been also shown to repress the transcriptional activity of HNF4α by directly phosphorylating it on serine 304 (171), therefore adding additional dimension to the regulation of HNF4α, and hence SBP1. Furthermore, the inhibition of AMPK by exposing PC-3 cells to dorsomorphin caused a significant increase in both SBP1 mRNA and protein levels (data not shown), which may indicate a regulatory feedback loop between AMPK and SBP1.

The only published study that implicates HNF4 in prostate cancer reports that the PXR-HNF4 (69789A>G) SNP is associated with the risk of higher PSA levels in prostate cancer patients (172). A different study reported that the same SNP (rs7643645) modifies a binding site for HNF4 located within the Pregnane X receptor (*PXR)* gene promoter, hence resulting in a lower *PXR* and *CYP3A4* mRNA levels together, with a decrease in CYP3A4 basal activity (138). CYP3A4 is cytochrome P450 enzyme involved in testosterone catabolism (173-175), and the lower CYP3A4 activity seen in patients with this SNP may result in higher circulating testosterone levels, which may increase the risk of prostate cancer development and progression.

Low levels of dietary selenium have been associated with prostate cancer risk in several studies (176), and reduced protein stability may be one of several possible mechanisms by which reduced levels of SBP1 contribute to prostate cancer risk. Although the nature of the selenium residue in SBP1 is yet to be determined, the binding of selenium to SBP1 is sufficiently stable to remain bound through its isolation by gel filtration, ion-exchange chromatography, and SDS-PAGE (27). It was predicted that selenium binds SBP1 at its cys57 residue (33), and mutagenesis of that cysteine did not measurably alter its MTO activity (36). Similarly, in this study, mutation of the potential selenium binding site at cys57 did not change the ability of SBP1 to activate AMPK, or to attenuate cancer migration or anchorage-independent growth of prostate cancer cells. These results imply that selenium binding does not affect SBP1 function as a tumor suppressor. The impact of the binding of selenium to SBP1 may be to stabilize the protein, as a mutation at cys57 was found to reduce the protein's half-life in HCT116 colon cancer-derived cells (34). However, this is yet to be investigated in prostate cells.

V. CONCLUSION AND FUTURE DIRECTIONS

In conclusion, the broad goal of the research described in this thesis was the discovery of the metabolic and biological roles of SBP1 in prostate cancer. It was previously reported that SBP1 levels were significantly reduced in prostate cancer compared to benign tissue obtained from adjacent areas (53). Using an *in silico* analysis, it was determined that the promoter region of *SBP1* contains putative binding sites for the HNF4α transcription factor. The potential for HNF4α to regulate *SBP1* expression was supported by the evidence that HNF4α inhibition resulted in a dose-response increase in the levels of SBP1 mRNA and protein, therefore identifying HNF4α as a novel negative regulator of SBP1 expression in prostate cancer cells. Additionally, the elevated expression of *HNF4α* in prostate cancer compared to benign tissues may identify HNF4α as an oncogene in this disease. The consequences of altering the levels of SBP1 were investigated by ectopically expressing SBP1 in PC-3 prostate cancer cells. SBP1 over-expression in PC-3 cells attenuated their anchorage-independent growth and the migration in culture, both properties associated with transformation. One mechanism by which SBP1 impacts prostate cells is by altering cellular energy metabolism to become less reliant on OXPHOS, as evidenced by the reduction in oxygen consumption of cells when SBP1 is over-expressed. Evidence was generated that the reaction products of SBP1, H_2O_2 and H_2S, can activate AMPK, a major regulator of pathways of energy homeostasis. However, it remains to be determined whether the SBP1 reaction products activate AMPK directly or whether AMPK activation is a consequence of the reaction products suppressing mitochondrial OXPHOS and ATP production, which would subsequently activate AMPK. Uncovering the molecular events orchestrating the metabolic transformation

occurring in prostate cancer is essential to future advances in understanding prostate cancer and identifying potential novel therapeutic targets.

Based on the obtained data, a model is proposed for the role of SBP1 in prostate cancer etiology (Figure 13). It is hypothesized that SBP1 negatively regulates OXPHOS in the healthy prostate cells by the production of H_2O_2 and H_2S and consequential activation of AMPK. The reduction of SBP1 levels in prostate cancer can occur due to increased binding of HNF4α, acting as a transcriptional inhibitor to the *SBP1* promoter. Consequently, there is a reduction in H_2O_2 and H_2S-mediated signaling, inhibition of AMPK, and stimulation of OXPHOS and building blocks of biomolecules needed for tumor growth and progression. Other effects of SBP1 loss in tumor cells remain to be discovered.

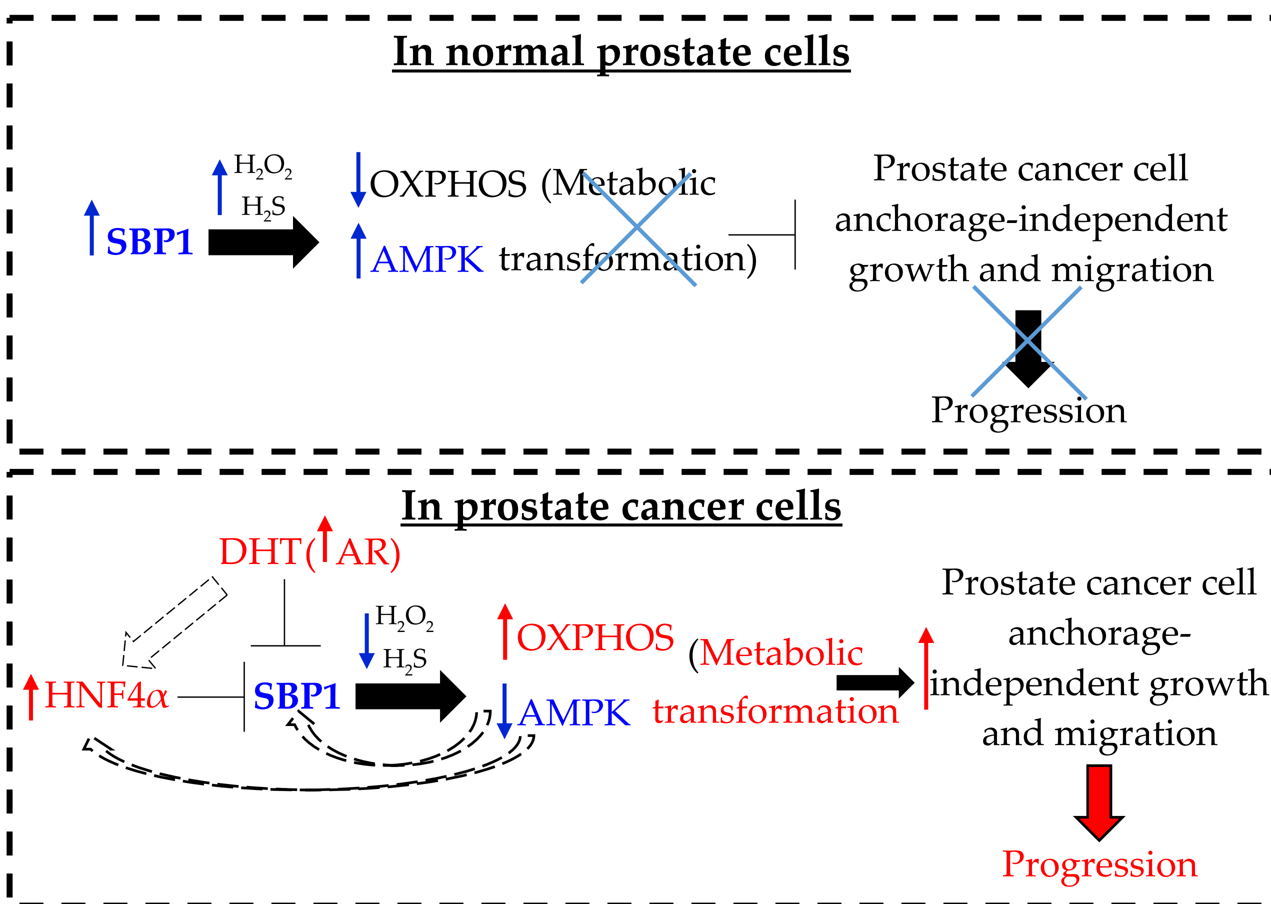


Figure 13. Illustration of the key findings of the study. The figure illustrates key study observations for a better depiction of *SBP1* transcriptional regulation and the role of SBP1 loss in the metabolic transformation that accompanies prostate cancer progression. Proteins and processes in blue are thought to function as tumor suppressors and are commonly lost/downregulated in prostate cancer, whereas those in red are thought to function as oncogenes and are commonly upregulated in prostate cancer.

The studies presented in this thesis provide a foundation for future studies to extend these observations to human and mouse tissues. For example, a current gap of knowledge in prostate cancer treatment is the lack of tools that would help identify and differentiate patients with indolent

versus aggressive prostate cancers. A case-control study may be performed by obtaining prostate cancer tissues of different stages to assess the clinical utility of HNF4α/SBP1/AMPK, whether independently or combined, to differentiate indolent and aggressive cancers. Furthermore, it would be crucial to compare the performance (non-inferiority or superiority) of these markers to PSA in predicting patients' clinical outcomes.

Additionally, the Hi-Myc male mice are known to develop murine prostate intraepithelial neoplasia (PIN) within three months and a high frequency of invasive epithelial prostate lesions after six months (177,178). These mice can be utilized to investigate whether the inhibition of HNF4α, using its specific inhibitor BI-6015, can slow the development or progression of prostate cancer lesions, which will help establish HNF4α as an oncogene in this disease.

An *Sbp1* knockout mouse, C57BL/6N-Selenbp1$^{tm1b(KOMP)Wtsi}$, was generated by the US National Institutes of Health Knockout Mouse Production and Phenotyping Project by deleting critical coding regions. It has no overt phenotype but exhibits dramatically reduced MTO activity in erythrocytes, liver, kidney, muscle, and brain mirroring effects seen in individuals with *SBP1* mutations (36,66). Prostate tissues were not examined in these studies. Based on our results, it is anticipated that these *Sbp1*$^{-/-}$ mice have altered energy metabolism in prostate tissue. Prostates obtained from *Sbp1*$^{-/-}$ and wild-type control mice will be studied by subjecting them to metabolic analyses, including measuring OCR under basal and stress conditions. Additionally, submitting these prostates for quantitative proteomics analysis will help validate our observations, and identify the global and key metabolic changes occurring in these tissues due to *Sbp1* loss.

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APPENDIX

Mostafa Elhodaky <melhod2@uic.edu>

Re: MDPI Contact Form: Requesting permission to re-use my IJMS published article

1 message

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support@mdpi.com <support@mdpi.com> Wed, Sep 18, 2019 at 8:56 PM
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> Mostafa Elhodaky Ph.D. Candidate (GY-4), Department of Pathology University of Illinois at Chicago 909 S. Wolcott Ave. | Chicago, IL
> 60612 | MC847 Email: melhod2@uic.edu

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3-4 minutes

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https://software.oncomine.com/resource/main.html#ac:1863,1998,2438,8158,1879,5481,1983,5905,1990,1887,1895,1894,1947,1878;dso:foldChangeOverex;ec:[2,3];et:over;g:3172;pg:1;pvf:3483,3521,14782,150004;scr:datasets;th:g100.0,p0.050,fc0.0;v:18

VITA

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EDUCATION

University of Illinois at Chicago, *Chicago, IL*
Ph.D., Pathology, *2020*

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M.D., *2011*

PEER-REVIEWED PUBLICATIONS

1. **Elhodaky, M.**, and Diamond, A. M. (2018) Selenium-Binding Protein 1 in Human Health and Disease. *Int J Mol Sci* **19**, 3437
2. Iriondo, O., Liu, Y., Lee, G., **Elhodaky, M.**, Jimenez, C., Li, L., Lang, J., Wang, P., and Yu, M. (2018) TAK1 mediates microenvironment-triggered autocrine signals and promotes triple-negative breast cancer lung metastasis. *Nat Commun* **9**, 1994-1994
3. Forte, V. A., Barrak, D. K., **Elhodaky, M.**, Tung, L., Snow, A., and Lang, J. E. (2016) The potential for liquid biopsies in the precision medical treatment of breast cancer. *Cancer Biol Med* **13**, 19-40

PROFESSIONAL EXPERIENCE

2011 - 2012	Medical Intern, Tanta University, Tanta, Egypt
2012 - 2012	Primary Care Physician, Ministry of Health, Cairo, Egypt
2012 - 2013	Teaching Assistant, Tanta University Faculty of Medicine (Department of Forensic Medicine and Clinical Toxicology), Tanta, Egypt
2012 - 2013	Resident Physician, Tanta University Emergency Hospital (Department of Forensic Medicine and Clinical Toxicology), Tanta, Egypt
2013 - 2014	Visiting Research Fellow, Children's Hospital Los Angeles, Los Angeles, CA
2014 - 2014	Clinical Laboratory Technician , PAREXEL International, Glendale, CA
2014 - 2016	Research Technician II, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA

FELLOWSHIPS AND AWARDS

2019 - 2020	CCTS Pre-doctoral Education for Clinical and Translational Scientists Fellowship, University of Illinois College of Medicine
2019	Chancellor’s Student Service and Leadership Award, University of Illinois at Chicago
2018	Travel award, Graduate Student Council, University of Illinois, Chicago, IL
2004 - 2011	Excellent Student Award, Tanta University, Egypt

LEADERSHIP, CERTIFICATIONS, AND PROFESSIONAL MEMBERSHIPS

2019 - 2020	Representative of the Department of Pathology, UIC Graduate Student Council (GSC), Chicago, IL
2019	ECFMG Certification
2019 –	Member, American Society for Clinical Pathology
2019 –	Member, United States and Canadian Academy of Pathology
2019 –	Member, Chicago Pathology Society
2019 –	Member, American Urological Association
2019 –	Associate Member, American Association for Cancer Research

ABSTRACTS AND PRESENTATIONS

1. **Elhodaky, M.**, Hong, L., & Diamond, A. (June, 2019). Selenium-binding protein 1 reduces oxygen consumption and activates AMPK in prostate cancer cells. Oral Presentation presented at: The American Society of Nutrition Annual National Conference; Baltimore, MD
2. **Elhodaky, M**., Ekoue, D., Ansong, E., Hong, L., Nonn, L., & Diamond, A. (October, 2018). The role of selenium-binding protein 1 (SBP1) in the metabolism of human prostate cancer cells. Poster presented at: The Annual Research Symposium of the Graduate Education in Medical Sciences Program, University of Illinois at Chicago; Chicago, IL
3. **Elhodaky, M.**, Ekoue, D., Ansong, E., Hong, L., Nonn. L, & Diamond, A. (June, 2018). Selenium-binding protein 1 (SBP1) alters the metabolism of human prostate cancer cells (P26-013). Poster presented at: The American Society of Nutrition Annual National Conference; Boston, MA
4. Iriondo, O., **Elhodaky, M.**, Liu, Y., Lee, G., Lang, J.E., Wang, P., & Yu, M. (April, 2017). TAK1 mediated IL1 expression as autocrine signaling to promote breast cancer metastasis (Abstract 4857). Poster presented at: The American Association for Cancer Research Annual National Meeting; Washington, D.C., DC
5. Iriondo, O., Liu, Y., **Elhodaky, M.**, Li, L., Lang, J.E., Wang, P., Yu, M. (April, 2016). TAK1: a potential target in the treatment of breast cancer metastasis (Abstract nr 918). Poster presented at: The American Association for Cancer Research Annual National Meeting; New Orleans, LA
6. Iriondo, O., Li, L., **Elhodaky, M.**, & Yu, M. (May, 2015). Influence of hypoxia signaling in the tumorigenicity of circulating tumor cells. Poster presented at: The Annual Symposium of Stem Cell and Regenerative Medicine Department, University of Southern California; Santa Barbara, CA
7. Iriondo, O., Liu, Y., **Elhdoaky, M.**, Li, L., Lang, J., Wang, P., & Yu, M. (May, 2015). TAK1 is a potential target in the treatment of breast cancer metastasis. Poster presented at: The Annual Symposium of Stem Cell and Regenerative Medicine Department, University of Southern California; Santa Barbara, CA