Received: 30 March 2020 | Accepted: 21 May 2020

DOI: 10.1002/pros.24028

ORIGINAL ARTICLE

The Prostate WILEY

Selenium-binding protein 1 alters energy metabolism in prostate cancer cells

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Funding information
National Institutes of Health, Grant/Award Numbers: R01CA193497, R21CA182103

Abstract

Objective: The broad goal of the research described in this study was to investigate the contributions of selenium-binding protein 1 (SBP1) loss in prostate cancer development and outcome.

Methods: SBP1 levels were altered in prostate cancer cell lines and the consequences on oxygen consumption, expression of proteins associated with energy metabolism, and cellular transformation and migration were investigated. The effects of exposing cells to the SBP1 reaction products, H_2O_2 and H_2S were also assessed. In silico analyses identified potential HNF4α binding sites within the *SBP1* promoter region and this was investigated using an inhibitor specific for that transcription factor.

Results: Using in silico analyses, 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 data indicating that HNF4α inhibition resulted in a dose-response increase in the levels of SBP1 messenger RNA and protein, identifying HNF4α as a novel negative regulator of SBP1 expression in prostate cancer cells. The consequences of altering the levels of SBP1 were investigated by ectopically expressing SBP1 in PC-3 prostate cancer cells, where SBP1 expression attenuated anchorage-independent cellular growth and migration in culture, both properties associated with transformation. SBP1 overexpression reduced oxygen consumption in these cells and increased the activation of AMP-activated protein kinase (AMPK), a major regulator of energy homeostasis. In addition, the reaction products of SBP1, H_2O_2, and H_2S also activated AMPK.

Conclusions: Based on the obtained data, it is hypothesized that SBP1 negatively regulates oxidative phosphorylation (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.

KEYWORDS
HNF4α, hSP56, prostate cancer metabolism, SBP1, SELENBP1, selenium-binding protein 1

1 | INTRODUCTION

Selenium, an essential trace element, was considered a strong candidate for cancer prevention following decades of experimental studies demonstrating that low, nontoxic levels of dietary selenium could reduce the incidence of a wide variety of cancer types in rodents.[1-3] These data, and human epidemiological studies demonstrating an inverse association between selenium in the diet and prostate cancer risk,[4,5] provided motivation to initiate human supplementation trials designed to determine whether selenium could reduce the risk or progression of prostate cancer.[5] In these trials, the selenium supplements provided no benefit. The discrepancies between the randomized controlled trials, animal experiments, and human observational studies have been discussed in several publications.[5-7]

Selenium-binding protein 1 (SBP1, SELENBP1, and hSP56) is a highly conserved protein that was first discovered in mouse liver in 1989 by Bansal et al[8] due to its ability to bind selenium[75]. SBP1 levels are frequently lower in cancers of different types as compared to the corresponding healthy tissues, and lower levels often correlate with worse clinical outcomes (reviewed in[9]). Observations from cells derived from different tissue types support a tumor suppressor function for SBP1.[9] Ectopic expression of SBP1 in a variety of cancer cell lines reduced their growth in semisolid media and decreased tumorigenicity in xenograft models.[10] Others have shown that overexpression of SBP1 alters signaling pathways regulated by MAPK, Wnt, NFκB, and Notch.[11] A biochemical function of SBP1 was only recently resolved as it was discovered that SBP1 mutations resulted in extraoral halitosis, bad breath, and determined that SBP1 is a methanethiol oxidase (MTO) that converts methanethiol to hydrogen peroxide (H_2O_2) and hydrogen sulfide (H_2S).[12] Both of these products are critical signaling molecules, with the latter being able to suppress mitochondrial respiratory complex IV at high concentrations.[13-15]

SBP1 levels are also lower in prostate cancer as compared to adjacent benign tissue.[10] Both the nuclear levels of SBP1 and the nuclear to cytoplasmic ratio were inversely proportional to tumor grade, and tumors in the lowest quartile of SBP1 were more than twice as likely to recur as those of any other quartile.[10] 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.[16] It is, therefore, likely that SBP1 exerts a tumor suppressor function in the prostate, and its loss or downregulation may facilitate carcinogenesis.

In the prostate, the Krebs cycle is inhibited in favor of the production of citrate, therefore, distinguishing the energy metabolism of the normal prostate from that of other organs. This inhibition is generally relieved during prostate cancer progression, allowing a metabolic shift towards oxidative phosphorylation (metabolic transformation), a process that is crucial for prostate cancer cell survival and proliferation. SBP1 has been implicated in the regulation of energy metabolism as a quantitative proteomic analysis of cells ectopically expressing SBP1 indicated altered levels of proteins involved in lipid and glucose metabolism.[11] Here, we investigate the ability of SBP1 to impact properties of transformation and energy metabolism in human prostate-derived cancer cells to understand the impact of SBP1 reduction or loss during prostate cancer progression.

2 | MATERIALS AND METHODS

2.1 | Cells and culturing conditions

The PC-3 human prostate carcinoma cell line was maintained in RPMI-1640 media (Gibco), and LNCaP human prostate carcinoma cell line was maintained in RPMI-1640 media (American Type Culture Collection). All media were supplemented with 10% fetal bovine serum (Gemini Bio), 100 U/mL penicillin, and 100 µg/mL streptomycin, and cells were maintained at 37°C with 5% CO_2. Cell lines were authenticated by Genetica DNA Laboratories (Burlington, NC). The constitutively-active and inducible SBP1 expression constructs were introduced via transfection using Continuum Transfection Reagent (Gemini Bio) into PC-3 cells, and PC-3 cells that were previously infected with the tetracycline trans-activator (TETON) construct,[10] respectively. The same reagent was also used for the transfection of plasmids into LNCaP cells. Transfected cells were selected in 500 µg/mL G418 (Sigma-Aldrich), and expanded and screened for SBP1 expression by Western blot analysis and quantiative real-time polymerase chain reaction (qRT-PCR) using *SBP1* forward primer (5′-CCAAAGCT GCACAAGGTCAT-3′), *SBP1* reverse primer (5′- CATCCAGCA GCACAAAACCC-3′), *RPLP0* forward primer (5′-CCTCGTGGAA GTGACATCGT-3′), and *RPLP0* reverse primer (5′- CTGTCTTCC CTGGGCATCAC-3′). Ectopic expression of SBP1 was induced following incubation with 0.5 µg/mL doxycycline or 0.05 µg/mL anhydrochlortetracycline-HCl (Cayman Chemical) for 48 to 72 hours.

2.2 | Real-time quantitative polymerase chain reaction

Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) and reverse-transcribed with High-Capacity cDNA Reverse Transcription Kit (ThermoFisherScientifice), according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed with a QuantStudio 6 Flex Real-Time PCR System (ThermoFisherScientific), using Fast SYBR Green Master Mix (ThermoFisherScientific). Fold changes were calculated by the $\Delta\Delta C_t$ 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′).

2.3 | Plasmid construction

The doxycycline-inducible SBP1 expression construct, pRetroX-Tight-Pur-SBP1, was previously generated.[10] 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′-TTGCCACCATTGGAGC GATTGACATTG-3′) were created. These sequences were introduced into the 5′ end of *SBP1* open reading frame using the following *Not*1-restriction-site-containing forward primers, (5′-GGCAGCAGCG GCCGCGCAGCAGCCACCATGCCAAAAAAGAAGAGAAAGGTAATG GCTACGAAATGTGGG-3′) and (5′-GGCAGCAGCGGCCGCGCAGC AGCCACCATGTTGCCACCATTGGAGCGATTGACATTGATGGCTAC GAAATGTGGG-3′) for SBP1-NLS and SBP1-NES, respectively.[17-19] The *Eco*R1-restriction-site-containing reverse primer (5′- TGCTGCG AATTCTGCTGCTCAAATCCAGATGTCAGAGC -3′) was used for the generation of both derivative constructs. Successful cloning was verified by Sanger sequencing. SBP1 shRNA and constitutively-active pCMV6-AC SBP1 expression constructs were purchased from OriGene Technologies, Inc. The pCMV6-AC SBP1 plasmid (OriGene Technologies, Inc) was used as a template for site-directed mutagenesis at cys57 of SBP1 using the Q5 Site-Directed Mutagenesis Kit (NEB), 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.

2.4 | Immunofluorescence

Indicated cells were plated onto sterile Fisherbrand microscope cover glass slips (ThermoFisher Scientific) and placed in Corning Costar Flat Bottom six-well Cell Culture Plates (Corning Inc). The cells were allowed to grow to 80% confluence, washed three times with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde for 20 minutes. After fixation, the coverslips were transferred to a clean six-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) 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 1 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 4′,6-diamidino-2-phenylindole (Invitrogen). Images were obtained using an LSM510UV confocal microscope (Zeiss).

2.5 | Western blot analysis

Cells were harvested and lysed in 1× Cell Lysis Buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors. Lysates were mixed with NuPAGE LDS Sample Buffer (Life Technologies) and 10× Reducing Agent (Life Technologies) and boiled at 95°C for 10 minutes, after which lysates were loaded to 4 to 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, 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.

2.6 | 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 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, causing a decrease in OCR, which is a reflection of mitochondrial ATP production.[20] The second injection following oligomycin is FCCP, which is a potent uncoupler of oxidative phosphorylation (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.[20] 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 the measurement of non-mitochondrial respiration occurring by extramitochondrial cellular processes.[20]

2.7 | Cell proliferation and growth in semisolid media

Proliferation was assayed by the quantitation of cellular DNA using the FluoReporter Blue Fluorometric dsDNA Quantitation Kit (ThermoFisher Scientific, Inc). Cells were plated at equal densities (5000 cells/well) on black, clear-bottom 96-well plates (Corning Inc) and incubated at 37°C for 3 days, after which relative cell numbers were determined using the manufacturer protocol. Growth in semisolid media was assayed by plating cells in triplicates in 0.6% agarose in media, according to a published protocol.[21] Cells were imaged using an EVOS FL Imaging System (Invitrogen), and colonies were enumerated on day 21.

2.8 | Wound healing assay

The wound-healing assay was used to evaluate cell migration. Cells were plated at equal densities on six-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 2 mL of fresh media containing aphidicolin (Cayman Chemical) to inhibit cell proliferation. Cells were then imaged using an EVOS FL Imaging system at 24-hour intervals for up to 3 days. Changes in scratch widths 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.

2.9 | Statistical analysis

The 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 mean ± standard error of mean. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | SBP1 alters oxygen consumption in PC-3 cells

Given previous data indicating that ectopic expression of SBP1 can alter the expression of genes whose protein products are involved in energy metabolism,[11] the effect of SBP1 overexpression 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 3 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.[10]

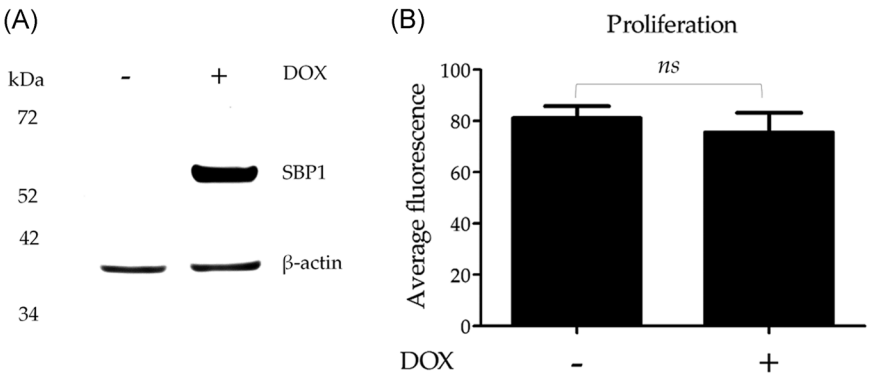

FIGURE 1 SBP1 overexpression does not affect the proliferation of PC-3 cells. A, Western blotting analysis indicating the overexpression of SBP1 when induced by doxycycline (DOX) in PC-3 cells. The migration of molecular weight markers is shown to the left of the figure. B, After 3 days of DOX-mediated induction of SBP1, double stranded DNA quantification was quantified as a surrogate for proliferation. Data are represented as averages ± standard error of mean. SBP1, selenium-binding protein 1; *ns*, nonsignificant. n = 3

The effect of elevated SBP1 expression on mitochondrial respiration in PC-3 cells was determined. Overexpression of SBP1 in PC-3 cells significantly reduced basal OCR (basal OXPHOS), OCR following the injection of oligomycin (mitochondrial ATP production), and OCR following the injection of FCCP (maximal respiration), and spare respiratory capacity (Figure 2).

3.1.1 | SBP1-mediated reduction in OCR occurs independently of its subcellular localization

SBP1 was previously shown to reside in both the nucleus and the cytoplasm in prostate epithelial cells, and the nuclear to cytoplasmic ratio was inversely associated with tumor grade.[10] To investigate the impact of nuclear vs 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 targeted SBP1 to the intended subcellular compartments (Figure 3A,B). When expressed in PC-3 cells, both nuclear-targeted and -excluded SBP1 reduced all parameters of mitochondrial respiration to a similar extent as 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.

3.1.2 | SBP1 activates AMPK, a critical regulator of energy and glucose homeostasis

AMP-activated protein kinase (AMPK) stimulates glucose utilization when ATP levels are low, favoring glycolysis,[22] and is activated by phosphorylation at Thr^{172}. Stable SBP1 expression in PC-3 cells resulted in a two-fold elevation of the phosphorylated (active) form of AMPK compared to control cells (Figure 4A). Compared to control cells, PC-3 cells that express SBP1 from a doxycycline-inducible construct also exhibited an increased AMPK phosphorylation after incubation with anhydrochlortetracycline (ACT), a tetracycline analog, for 48 hours (Figure 4B). ACT was used instead of doxycycline (DOX) due to the reported ability of DOX to affect glycolytic metabolism.[23] SBP1 expression was silenced using a 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 75% in these cells resulted in a 46% reduction of AMPK phosphorylation at Thr^{172}, compared to cells transfected with a scrambled control shRNA construct.

3.2 | SBP1 suppresses 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 issue, SBP1 was constitutively overexpressed in PC-3 cells. Both individual clones and a

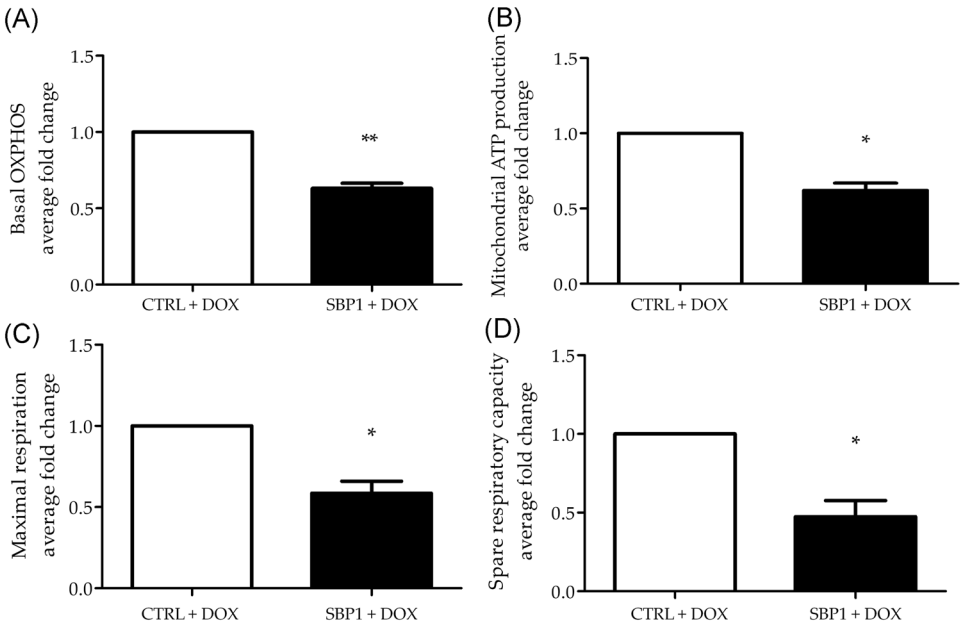

FIGURE 2 Overexpression of SBP1 alters oxygen consumption in PC-3 cells. The oxygen consumption rate (OCR) following the specific inhibitors indicated in the text was measured using the Seahorse platform. The basal OXPHOS (A), mitochondrial ATP production (B), maximal respiration (C), and spare respiratory capacity (D) are deduced from the resulting OCR in control and SBP1-overexpressing PC-3 cells incubated with vehicle or DOX 0.5 μg/mL for 48 hours. Data are represented as averages ± standard error of mean. OXPHOS, oxidative phosphorylation; SBP1, selenium-binding protein 1; $^{*}P < .05$, $^{**}P < .01$, n = 3

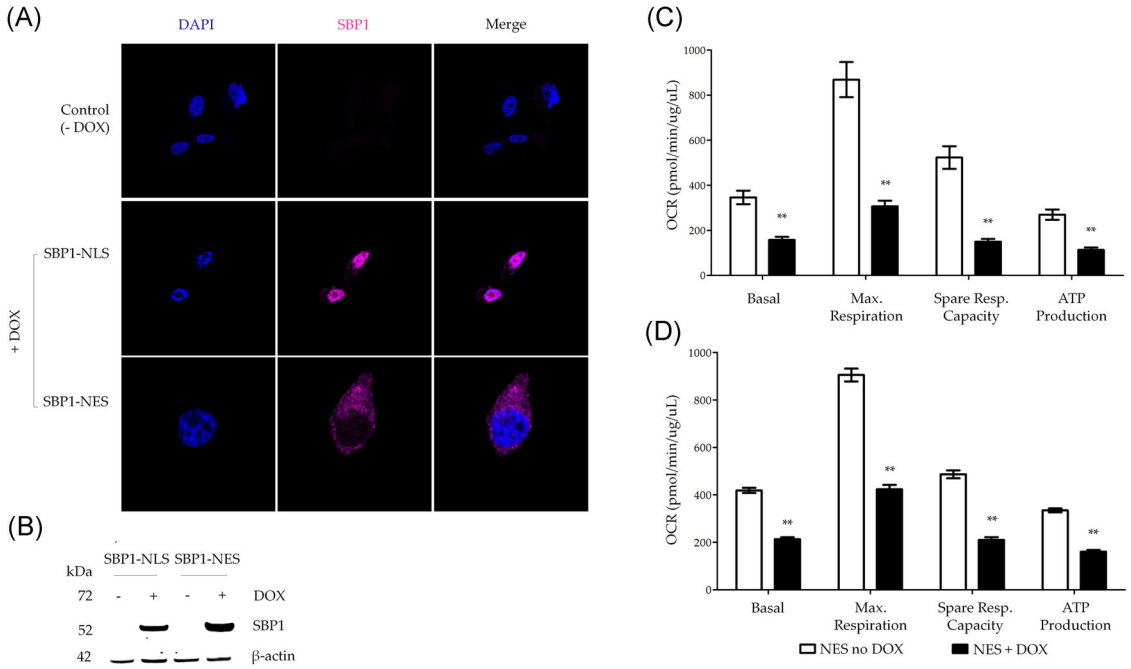

FIGURE 3 Targeting SBP1 to the nucleus or the cytoplasm has the same effect on oxygen consumption. A, Immunofluorescence showing successful induction and targeting of SBP1 in PC-3 cells. Nuclear-targeted SBP1 (SBP1-NLS) and nuclear-excluded SBP1 (SBP1-NES) is visualized using anti-SBP1 antibodies (red), and nuclei are stained with DAPI (blue). B, Western blot analysis indicating induction of the SBP1-NLS and SBP1-NES in PC-3 cells. C, D, Basal OXPHOS, maximal respiration, spare respiratory capacity, and mitochondrial ATP production are quantified in control and PC-3 cells expressing SBP1-NLS (C) or SBP1-NES (D). Cells were incubated with vehicle or DOX at a concentration of 0.5 μg/mL for 48 hours. Data are represented as averages ± standard error of mean. DAPI, 4′,6-diamidino-2-phenylindole; DOX, doxocycline; OXPHOS, oxidative phosphorylation; SBP1, selenium-binding proetin 1; $^{**}P < .01$. n = 3 [Color figure can be viewed at wileyonlinelibrary.com]

pool of transfectants were examined for their ability to grow in semisolid 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 semisolid media, compared to control vector-only transfected cells (Figure 5A-C).

In addition to growth in semisolid 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 antiproliferative agent, aphidicolin. Ectopic expression of SBP1 attenuated the migration of PC-3 cells into the scratched area by 30% after 2 days, relative to control vector-only transfected cells (Figure 6).

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, the likely selenium-binding amino acid,[24] to glycine (C57G). The mutant SBP1 was ectopically expressed in PC-3 cells, and the cells were assayed for their ability to grow in semisolid media (soft agar). Mutated and wild-type SBP1 expression reduced the growth 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 2 days, relative to control vector-only transfected cells (Figure 6). The C57G-SBP1 was also able to enhance the activation of AMPK (Figure 7) to a similar extent as the wild type protein, as seen in Figure 4.

3.3 | The metabolic and biological function of the products of SBP1 enzyme activity

Since SBP1 can activate AMPK and attenuate cellular migration and growth in semisolid media, we assessed whether AMPK activation could contribute to these phenotypic changes associated with aggressive prostate cancer. AMPK activation was achieved by incubating PC-3 cells with 1 mM metformin at the beginning of the scratch assay.[25] Metformin-induced AMPK activation was verified by Western blot analysis (Figure 8C). Migration into the scratched area was attenuated by 32% after 3 days in PC-3 cells exposed to metformin, relative to cells treated with vehicle only (Figure 8A,B), hence indicating that AMPK activation by metformin can inhibit the migration of prostate cancer PC-3 cells in vitro. Furthermore, Migration was attenuated by 76% after 3 days in SBP1-expressing PC-3 cells exposed to metformin, relative to cells treated with vehicle only (Figure 8A,B), indicating that AMPK activation by metformin potentiates the SBP1-induced attenuation of migration of prostate

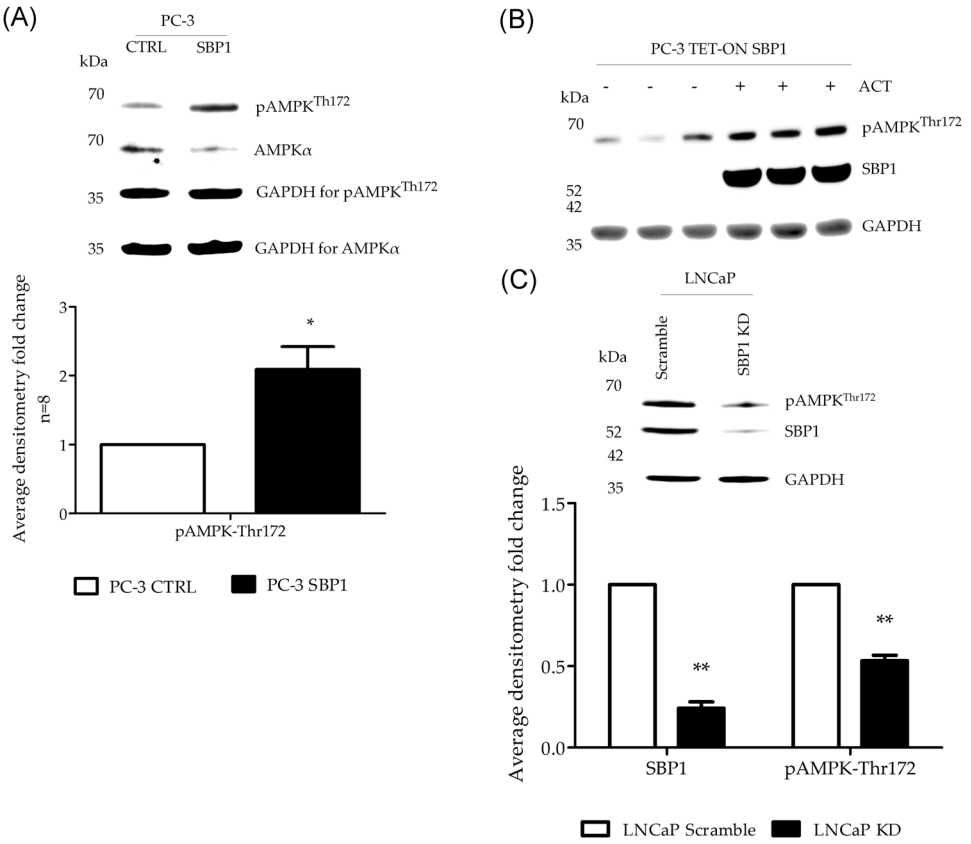

FIGURE 4 SBP1 activates AMPK in PC-3 cells. A, Representative Western blot analysis showing the effect of SBP1 expression on the nonphosphorylated (inactive) and phosphorylated forms (active) of AMPK. B, Western blot analysis showing the levels of pAMPK achieved by ectopic expression of SBP1 in PC-3 cells from a DOX-inducible promotor. ACT, a DOX analog, was added to the media at 0.1 μg/mL for 48 hours. The lanes represent three independent replicates. C, Representative Western blot analysis showing the reduction of pAMPK in LNCaP cells, in which SBP1 expression was silenced using a short hairpin RNA construct. Densitometric quantification of the bands relative to the GAPDH loading control are shown below the corresponding figures as the averages from three independent experiments. AMPK, AMP-activated protein kinase; DOX, doxocycline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *ns*, nonsignificant, SBP1, selenium-binding protein 1; $^{*}P < .05$, $^{**}P < .01$. Data are represented in averages ± standard error of mean

cancer PC-3 cells in vitro. It is, therefore, possible that the observed SBP1-induced attenuation of PC-3 cellular migration is mediated, at least partially, by AMPK activation.

SBP1 is an MTO, converting methanethiol to H_2O_2 and H_2S.[12] Both reaction products are essential signaling molecules, with H_2S also being able to suppress mitochondrial respiratory complex IV at high concentrations.[13-15] As seen in Figure 9, exposure of PC-3 cells to either H_2O_2 or NaHS (H_2S donor) results in the activation of AMPK. The above results collectively indicate that SBP1 may suppress transformation-related properties, at least partially, by producing H_2O_2 and H_2S, which activate AMPK and suppress mitochondrial respiration.

3.4 | The transcriptional regulation of SBP1

The mechanisms by which SBP1 levels are reduced in prostate cancer remain unknown. Hypermethylation of the *SBP1* promoter region occurs in colon cancers and colon cancer-derived cell lines[26] but not in other cell lines.[27] No evidence of hypermethylation or genetic deletion of *SBP1* was detected in lung and prostate cancers.[28,29] 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 by hepatic nuclear factor 4-alpha (HNF4α), a transcription factor essential for liver development and differentiation[30] as well as a regulator of several enzymes involved in glucose and lipid metabolism.[31,32]

To test the ability of HNF4α to regulate SBP1 expression, the androgen receptor-responsive LNCaP prostate cancer-derived cells were exposed to the HNF4α inhibitor, BI-6015 (Cayman Chemical). HNF4α inhibition increased both SBP1 mRNA and protein levels in a dose-dependent manner (Figure 10B-D). Successful HNF4α inhibition by BI-6015 was verified by demonstrating that BI-6015-treated cells exhibited a threefold increase in the levels of the mRNA of *CYP3A4*, a known HNF4α target (Figure 10E).[33] The Oncomine Platform (Thermo Fisher, Ann Arbor, MI) for analysis and visualization was used to examine *HNF4α* mRNA levels in prostate cancers.[34]

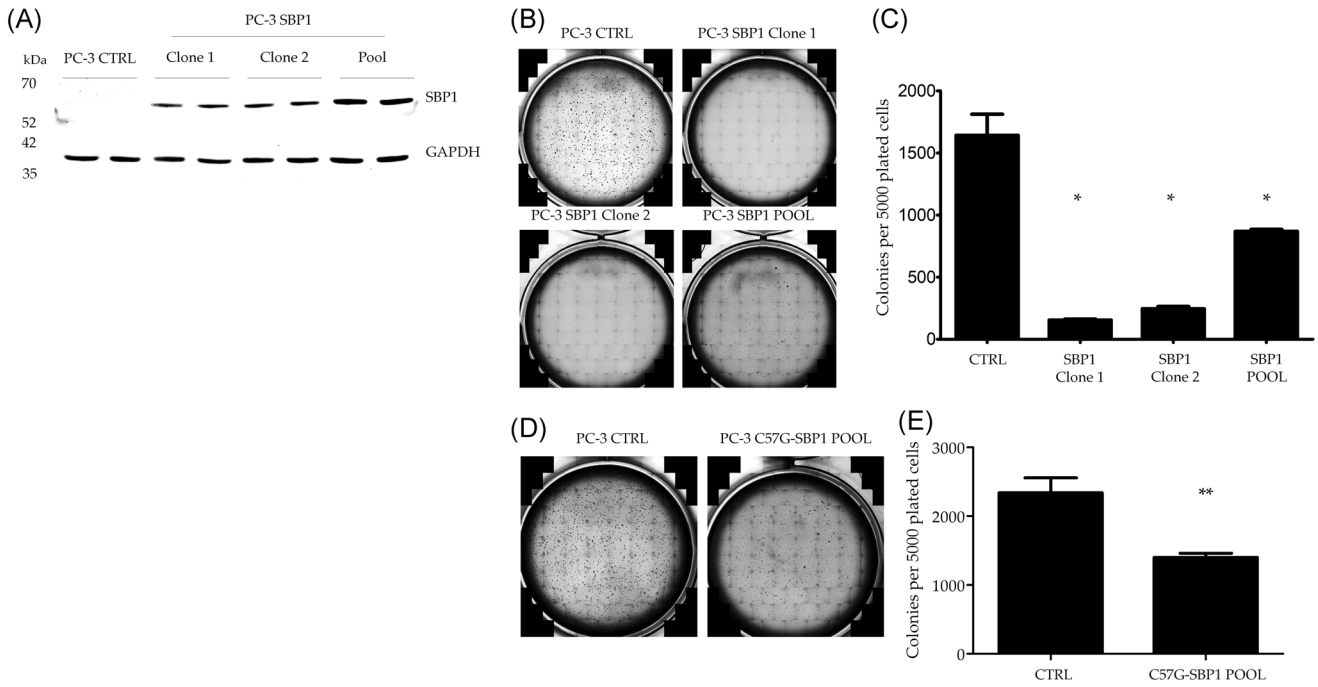

FIGURE 5 Ectopic expression of SBP1 attenuates anchorage-independent growth of PC-3 cells. A, A Western blot analysis showing SBP1 levels in clones and a pool of PC-3 cells transfected with a constitutively active SBP1 expression construct, compared to control vector-only transfected cells. GAPDH levels are presented as a loading control. B, Representative images of cell culture plates of transfected and control cells (refer to (C) for quantification of colonies). C, Quantification of an average number of colonies formed per 5000 plated cells shown in B. D, Representative images of cell culture plates of SBP1-overexpressing and control cells (refer to (E) for quantification of colonies). E, Quantification of an average number of colonies formed per 5000 plated cells shown in D. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SBP1, selenium-binding protein 1; $^{*}P < .05$; $^{**}P < .01$. Data are represented in averages ± standard error of mean. n = 3

The analysis of 14 prostate cancer studies[35-48] indicated that *HNF4α* was significantly elevated in prostate carcinoma compared to benign tissues ($P = .03$).

4 | DISCUSSION

SBP1 loss has been implicated in the progression of cancers of many different tissue types,[9] based on observations of lower levels of SBP1 in cancers compared to benign tissues, or the association of lower SBP1 levels with poor clinical outcome.[9] 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.[10] Overexpressing SBP1 has been shown to inhibit phenotypes related to cellular transformation or tumorigenicity,[26,49,50] but none of these studies used prostate-derived cancer cells. The data presented here show that overexpressing SBP1 in human PC-3 prostate carcinoma cells attenuated their anchorage-independent growth and migration in vitro, two conventional assays of transformation, supporting the evidence that SBP1 is a tumor suppressor in prostate cancer.

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.[51] 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.[52,53] 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.[52,54] 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 of 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, overexpressing SBP1 inhibited OXPHOS in prostate cancer cells, therefore mimicking the metabolic phenotype of the healthy prostate, where OXPHOS is also inhibited. Although our previous data indicated that the SBP1 nuclear-to-cytoplasmic ratio

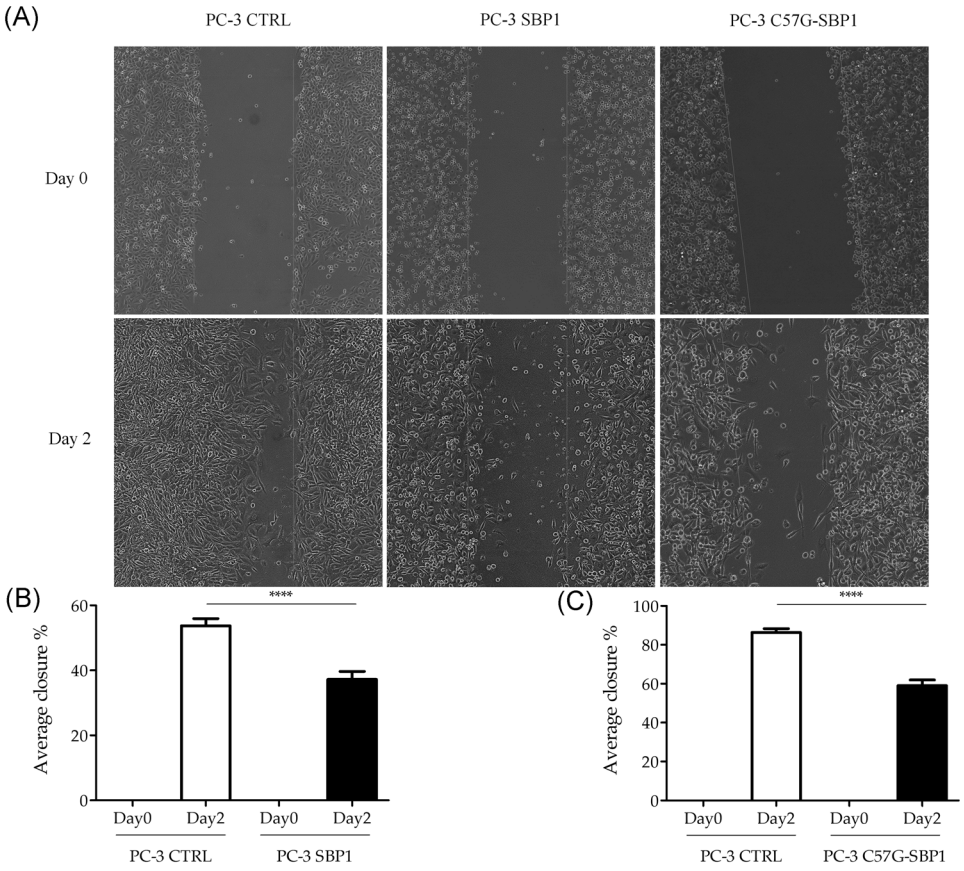

FIGURE 6 Ectopic expression of SBP1 attenuates the migration of PC-3 cells in a scratch assay. A, Representative images captured at day 0 and day 2 for control cells transfected with just vector, SBP1-expressing, and cells expressing a derivative version of SBP1 in which cysteine[57] is converted to glycine by in vitro mutagenesis. B, C, 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 (ThermoFisherScientific). Data are represented in averages ± standard error of mean. SBP1, selenium-binding protein 1; *****P* < .0001, n = 3

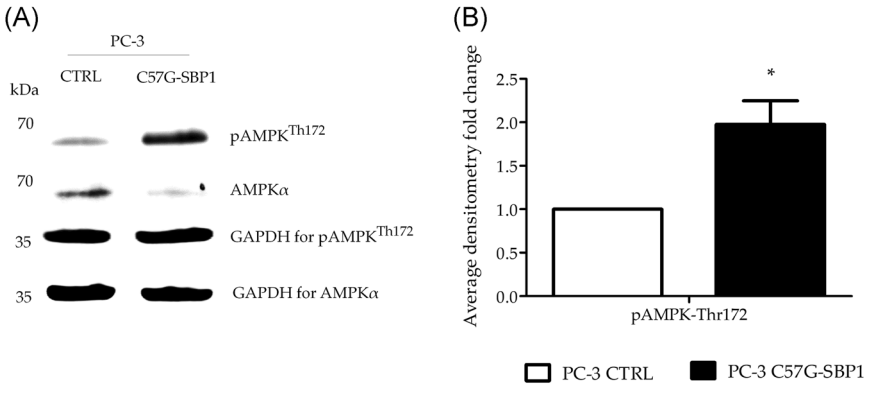

FIGURE 7 C57G-SBP1 activates AMPK PC-3 cells. A, Representative Western blot analysis showing the phosphorylation of AMPK by ectopic expression of C57G-SBP1 in PC-3 cells. B, Quantification of densitometries obtained from three independent experiments is shown. AMPK, AMP-activated protein kinase; *ns*, nonsignificant; SBP1, selenium-binding protein 1; **P* < .05. Data are represented in averages ± standard error of mean. n = 3

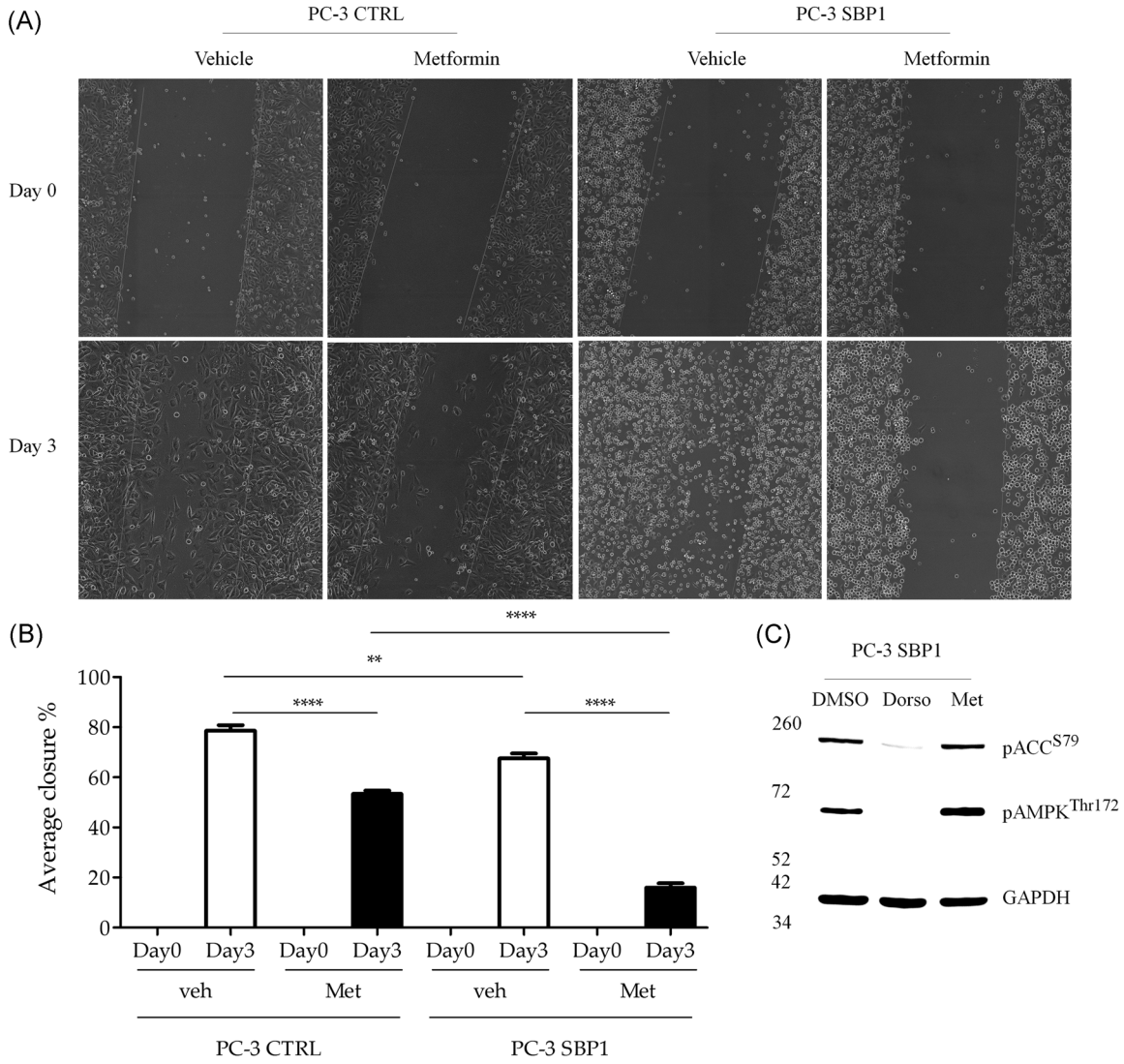

FIGURE 8 Pharmacological activation of AMPK attenuates migration of PC-3 cells. A, Representative images of PC-3 cells incubated with aphidicolin and metformin (Met, 1 mM) showing the attenuation of migration in cells treated with Met, compared to control vehicle-only (veh) treated cells. B, Quantification of the data obtained from three independent experiments with the bars representing the width of the scratch. C, Western blot analysis showing successful induction, and inhibition of AMPK by metformin 1 mM, and dorsomorphin 10 μM (Dorso), respectively. Data are represented in averages ± standard error of mean. AMPK, AMP-activated protein kinase; DMSO, dimethyl sulfoxide; ****$P < .0001$, n = 3

was inversely associated with tumor grade,[10] both nuclear-targeted and nuclear-excluded SBP1 were capable of suppressing OXPHOS to a similar extent (Figure 3). It is possible that H_2S generated by SBP1 MTO enzymatic activity can cross the nuclear membrane and affect mitochondrial function. In support of this possibility, elevated levels of H_2S can suppress mitochondrial respiratory complex IV.[13-15]

In addition, AMPK 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 noncatalytic β and γ subunits that exist in several isoforms.[55-59] The α2 subunit isoform has been shown to be preferentially found in the nucleus,[60] and both α and β subunits shuttle between the nucleus and the cytoplasm.[61] SBP1 may, therefore, activate AMPK located in either the nucleus and the cytoplasm. It is also likely that the loss of SBP1 that occurs in prostate cancer has pleiotropic effects in addition to those involving energy metabolism.

A high AMP/ATP ratio activates AMPK to restore intracellular energy balance. Several studies have found a beneficial effect of metformin, an AMPK activator, and commonly used therapy in the treatment of diabetes mellitus, in reducing prostate cancer incidence and improving overall survival.[62-67] Metformin also inhibits the proliferation of prostate cancer cells.[68-71] In this study, SBP1 activated AMPK, and metformin-induced AMPK activation reduced the migration of prostate cancer cells, therefore indicating that the impact of SBP1 overexpression may be mediated by activation of AMPK. Furthermore, both H_2O_2 and H_2S, products of the MTO activity of

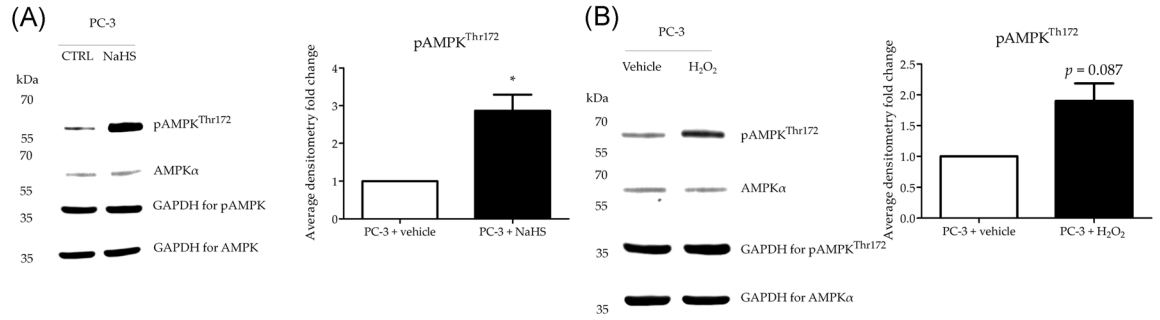

FIGURE 9 NaHS and H_2O_2 activate AMPK. A, Representative Western blot analyses showing the effects of the exposure of PC-3 cells to NaHS (1 mg/mL for 15 minutes), or B, H_2O_2 (250 μM for 1 hour) on the phosphorylation of AMPK. GAPDH is included as the loading control. Quantification of densitometries obtained from three independent experiments is shown next to the corresponding blot. Data are represented on average fold changes ± standard error of mean. AMPK, AMP-activated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $^*P < .05$, $^{**}P < .01$. n = 3

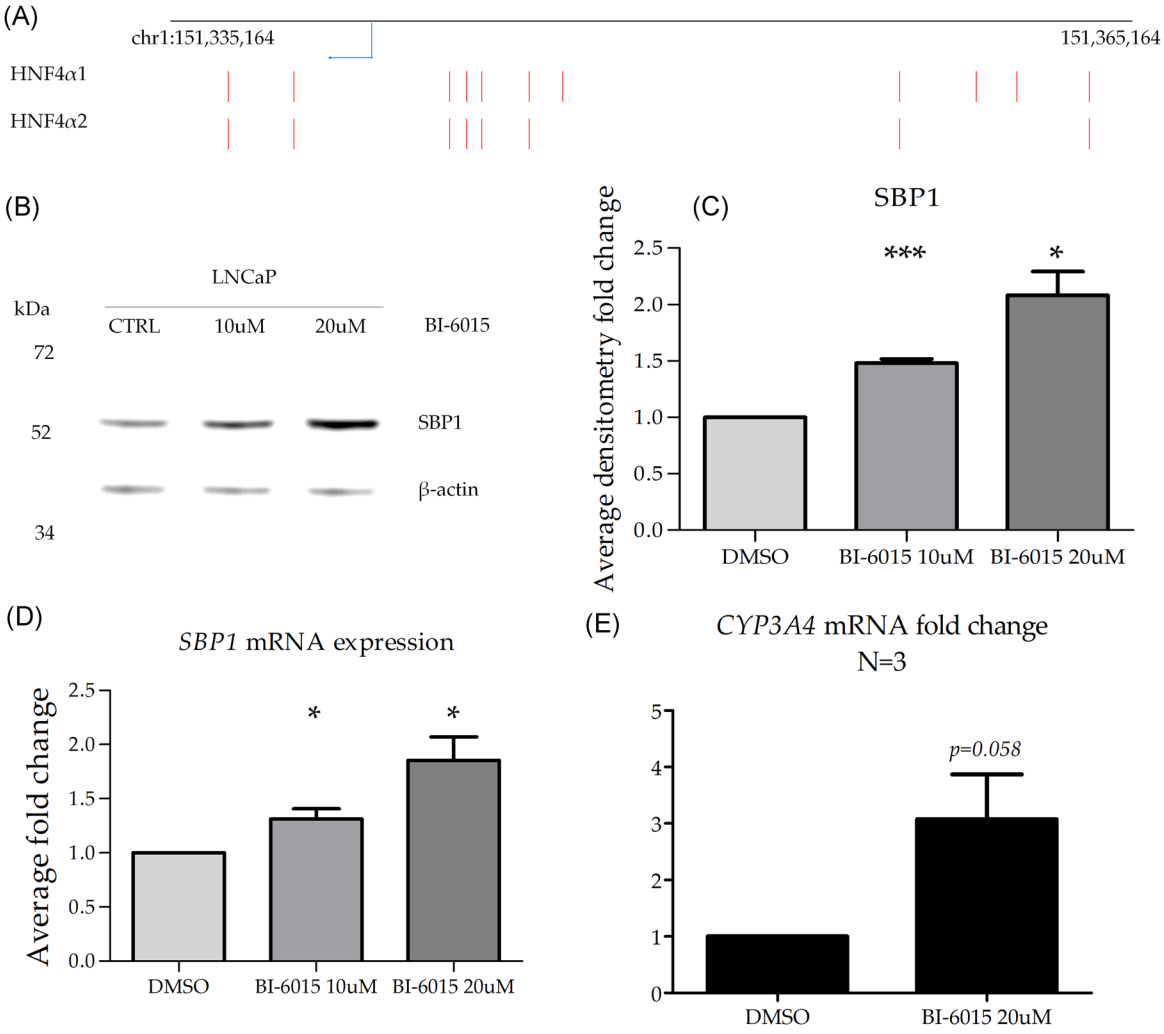

FIGURE 10 HNF4α suppresses SBP1 expression in LNCaP prostate cancer cells. (A) An illustration showing the putative HNF4α transcription factor binding sites within the *SBP1* promoter. (B, C) Representative Western blot analysis (B) and its densitometric analysis (C) showing a dose-dependent increase in SBP1 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 ± standard error of mean. AMPK, AMP-activated protein kinase; DMSO, dimethyl sulfoxide; mRNA, messenger RNA; RT-qPCR, real-time quantitative polymerase chain reaction; SBP1, selenium-binding protein 1; $^*P < .05$, $^{***}P < .001$, n = 4 [Color figure can be viewed at wileyonlinelibrary.com]

SBP1,[12] were also capable of activating AMPK. The contribution of H_2S in the pathobiology of the prostate cancer cells has been recognized,[72,73] and the results of several studies indicated impaired sulfide metabolism in prostate cancer.[74-76] H_2S and/or sulfide-containing compounds have been demonstrated to inhibit the survival of prostate cancer cells in vitro and in vivo,[77,78] as well as to repress androgen receptor (AR) transactivation, which is post-translationally modified by H_2S through S-sulfhydration.[79]

Prostate cancer is driven by AR signaling dysregulation, which involves a complex interplay of a network of signaling molecules.[80,81] AR regulates many genes involved in the metabolism of prostate cancer cells.[82-86] Additionally, AR suppresses *SBP1* expression in LNCaP cells,[29] and we also previously observed a dihydrotestosterone-induced suppression of *SBP1* in LAPC-4 cells (data not shown), indicating a potential AR-mediated mechanism of SBP1 suppression in prostate cancer.[10]

We have also identified HNF4α as a novel negative transcriptional regulator of *SBP1* expression, and the elevated expression of *HNF4α* in prostate cancer compared to benign tissues by an in silico analysis using the Oncomine platform. HNF4α is a transcription factor essential for liver development and differentiation,[30] as well as a regulator of several enzymes involved in glucose and lipid metabolism.[31,32] Here we provide evidence that elevated HNF4α may contribute to the reduction in SBP1 levels in prostate cancer. AMPK can also repress the transcriptional activity of HNF4α by directly phosphorylating it on serine 304,[87] indicating the possibility of a regulatory feedback in the *SBP1* transcriptional regulation by HNF4α.

Low levels of dietary selenium have been associated with prostate cancer risk in several studies,[5] and reduced SBP1 stability may be one of several possible mechanisms by which reduced levels of SBP1 occurs in prostate cancer. 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 sodium dodecyl sulfate polyacrylamide.[8] Based on structural considerations, selenium was predicted to bind SBP1 at its cys57 residue,[24] and mutagenesis of that cysteine did not measurably alter its MTO activity.[12] 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. 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.[88]

In summary, 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 observation that HNF4α inhibition resulted in a dose-response increase in the levels of SBP1 mRNA and protein. Additionally, the elevated expression of *HNF4α* in prostate cancer compared to benign tissues may identify HNF4α as an oncogene in this disease. SBP1 overexpression in PC-3 cells attenuated their anchorage-independent growth and the migration in culture, both properties associated with transformation. One mechanism by which SBP1 may impact 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 overexpressed. Data were also 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.

Based on the obtained data, a model is proposed for the role of SBP1 in prostate cancer etiology (Figure 11). 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 the production of the building blocks of biomolecules needed for tumor growth and progression.

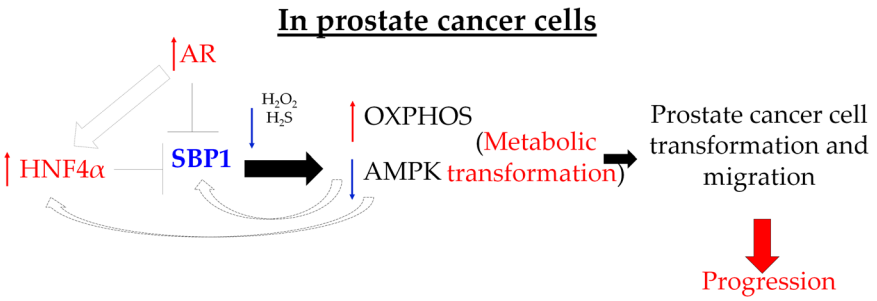

FIGURE 11 The hypothesized role of SBP1 and its regulation in prostate cancer. The figure depicts the inhibition of SBP1 expression by HNF4α and the androgen receptor (AR) and the reduction of the SBP1 reaction products, H_2O_2 and H_2S which contribute to the metabolic reprograming of prostate cancer cells that promote progression. AMPK, AMP-activated protein kinase; OXPHOS, oxidative phosphorylation; SBP1, selenium-binding protein 1 [Color figure can be viewed at wileyonlinelibrary.com]

ACKNOWLEDGMENT

This work was supported by grants from the National Institute of Health (Grant # R21CA182103 and R01CA193497).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

ME conceived, designed, and conducted the study, analyzed data, and prepared the manuscript. AMD was involved in the conceptual design and manuscript preparation. LKH participated in the conceptual design of some experiments. SK was involved in performing the site-directed mutagenesis to generate the C57G-SBP1 construct.

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How to cite this article: Elhodaky M, Hong LK, Kadkol S, Diamond AM. Selenium-binding protein 1 alters energy metabolism in prostate cancer cells. *The Prostate*. 2020;80:962–976. https://doi.org/10.1002/pros.24028