Studies on the Function of Selenium Binding Protein 1 and its Relevance to Prostate Cancer Outcome

BY

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THESIS

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Contribution of Authors

My Introduction is a literature review that places my dissertation question in the context of the larger field and highlights the significance of my research question. My Results contains all unpublished data representing a series of collaborative work and my own experiments directed at determining the biological function of SBP1, if it's function affects prostate cancer recurrence, and if so identifying likely mechanisms of action. Qi Ying contributed the data found in **Figure** 2, Andy Hall assisted me in the experiment shown in **Figure 12** and Ryan Deaton assisted me in analyzing data shown in **Figure 13**, and **Figure** 14, and Tables II and III. I anticipate that this line of research will be continued in the laboratory after I leave and that this work will ultimately be published as part of a co-authored manuscript. My Discussion represents my synthesis of the research presented in this thesis/dissertation and my overarching conclusions. The future directions of this field and this research question are also discussed.

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List of Abbreviations

ATCC	American Type Culture Collection
5-FUra	5-Fluorouracil
ATP	Adenosine Triphosphate
BCR	Biochemical Recurrence
BSA	Bovine Serum Albumin
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxyribonucleotide triphosphates
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FU	Fluorescent Units
Gly	Glycine
GPx	Glutathione Peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
IFU	Infectious unit
MEM	Minimum Essential Media
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Phosphate buffered saline
PMSF	Phenylmethanesulfonylfluoride

pRX	pRetro-X
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
S.D.	Standard Deviation
SBP1	Selenium Binding Protein 1
Se	Selenium
Ser	Serine
TAE	Tris-acetic acid-EDTA
t-BOOH	Tert-Butyl Hydroperoxide
TBST	Tris-buffered saline with Tween
TrxRD	Thioredoxin reductase
UTR	Untranslated Region

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SUMMARY

SBP1 is a non-canonical selenoprotein which was identified due to its ability to bind selenium[75]. SBP1 levels are reported to be consistently lower in human tumors compared to normal tissue, suggesting that tumors gain an advantage by decreasing its expression. Furthermore, low tumor SBP1 levels are predictive of poor outcome of several cancer types, although its ability to predict prostate cancer outcome has never been examined. Therefore, it is hypothesized that low SBP1 is able to predict recurrence of prostate cancer and it may be useful in determining which patients will recur after radical prostatectomy.

In order to examine if SBP1 levels are predictive of prostate cancer recurrence, tissue from post-radical prostatectomy prostate cancer patients who experienced biochemical recurrence were compared to non-recurred controls. Patients in the lowest quartile of SBP1 expression were significantly more likely to recur compared with patients with higher expression, extending the association of low SBP1 and poor cancer prognosis to include prostate cancer.

Although it is unknown why low SBP1 is predictive of outcome in many cancer types, largely due to the fact that its function is unknown, SBP1 has been reported to associate physically with GPx-1 and VDU1, and its expression is increased in response to HIF-1α activity. In addition, cells expressing SBP1 have been reported to experience increased apoptosis in response to ROS compared with SBP1 null cells. In order to gain a better understanding of the function of SBP1, and why its low levels are associated with poor outcome, the response of cells with and without SBP1 to the DNA damaging agent 5-FUra was examined. Following of 5-FUra treatment, cells expressing SBP1 proliferated significantly less than SBP1 null cells.

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SUMMARY (continued)

Additionally, SBP1 expression was led to phosphorylation of serine-15 on p53, a post translational modification which facilitates p53 cell cycle arrest/apoptotic pathway. Taken together, the data collected from this study indicates that SBP1 affects the consequences of DNA damage in the cell, which may be the reason why its low levels lead to poor prognosis in cancer patients.

I. INTRODUCTION

A. Selenium and selenoproteins

1. History of selenium

The essential trace element selenium was discovered in by Jöns Jacob Berzelius in 1817. Berzelius was a Swedish chemist who noted the similarity of the new element to tellurium, (after *tellus*, Latin for "earth"), and coined his newly identified element selenium, after the Greek goddess of the moon, Selene. Years later, selenium was identified as a toxin in animals consuming food rich in the element [1]. The first instance of selenium's relevance to human health was the occupational hazard impacting industry workers who were exposed to high levels of selenium in factories. It wasn't until 1957 when selenium was understood as more than a dietary toxin, and was identified an essential element for mammalian life.

Since then, selenium has received considerable attention as its dietary intake has been associated with the reduced risk of a wide range of human diseases, including cardiovascular disease [2], diabetes [3], viral infections [4] and cancer [5]. These effects have been speculated to be due to the impact of selenium status on signaling pathways, immunity, cellular metabolism and reactive oxygen homeostasis. How selenium can affect these processes and ultimately human health continues to be an active research focus, and many of its properties have been attributed to selenium's role as a critical constituent of proteins in which selenium is specifically incorporated either during selenoprotein synthesis or post-translationally.

2. Synthesis of selenoproteins containing selenocysteine

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The most common and best studied class of selenium containing proteins includes those that contain the amino acid selenocysteine (Sec), which is incorporated co-translationally by its insertion in response to UGA codons that otherwise would serve as translational termination signals [6]. The recognition of UGA as Sec requires a regulatory sequence, called a Selenium Insertion Sequence (SECIS) element, in the 3'-untranslated region of the selenoprotein mRNA [7]. Selenoprotein mRNAs contain a SECIS element in the 3'-UTR which coordinates decoding of an in-frame UGA-codon. Selenocysteine is synthesized on its cognate tRNA from a serine pre-cursor in a series of reactions that require selenophosphate. The addition of Sec in response to the in-frame UGA during translation requires several translation factors, including the stem-loop structure formed in the 3' untranslated region named the Sec-insertion sequence (SECIS) element, to which the SECIS binding protein (SBP2) binds and stabilizes the translation complex required to add Sec to a growing amino acid sequence. [8, 9]. Based on *in silico* analyses, there are 25 selenoprotein genes in humans and 24 in mice [10]. Comprehensive reviews of the functions of the human selenocysteine-containing proteins and their possible role in disease risk and etiology have recently been published [5, 11].

B. Selenium associated proteins and Selenium Binding Protein 1

In contrast to selenoproteins, another class of selenium-containing proteins includes those in which selenium is tightly associated with the peptide although not as a component of selenocysteine. This is a much smaller family and the best studied of them is Selenium Binding Protein 1 (SBP1). The SBP1 gene is located on chromosome 1 at q21–22, and is the homologue of the mouse SP56 gene that was originally reported as a 56 kDa mouse protein that stably bound selenium^{75} [12]. The human cDNA contains a 472 amino acid encoding open reading frame [13]

and the protein was first reported to reside both in the nucleus and cytoplasm [14], although subsequent reports would indicate that its cellular location may be influenced by cell type, degree of differentiation and environmental signals [15, 16]. It is expressed in a variety of tissue types, including the heart, lung, kidney and intestine. The form of selenium in SBP1 is unknown as is the nature of its association: the selenium remains bound to the protein when electrophoresed in SDS acrylamide gels but dissociates at extremes of pH [12]. Additionally, data has recently been presented indicating that the selenium is bound to a cysteine residue (Cys57) [15]. While cys57 was not empirically shown to be the selenium binding site for SBP1, the study provided the most evidence for a potential binding site to date.

C. SBP1 and its interaction with GPx-1

1. SBP1 interacts with GPx-1 in a physical and antagonistic manner

Taking into account the tightly associated selenium atom in SBP1, studies were initiated to assess whether there was a physical or functional interaction between SBP1 and the first characterized and best studied Sec-containing protein, the ubiquitously expressed glutathione peroxidase-1 (GPx-1). This enzyme uses reducing equivalents from glutathione to detoxify lipid and hydrogen peroxides, and its levels are sensitive to intracellular selenium availability [17]. Changes in the levels of GPx-1 have been associated with a variety of human diseases, particularly where the lack of its antioxidant activity increases cellular sensitivity to oxidative stress. Accordingly, increasing its expression in cell culture models is protective against radiation and UV-induced DNA damage [18, 19].

Genetic analysis of several diseases have provided evidence that the expression of GPx-1 variants can affect disease risk and progression; either a single nucleotide polymorphism

resulting in a proline (Pro) vs. leucine (Leu) variation at codon 198, or a variable number of alanine (Ala) codon repeats that result in 5, 6, or 7 ala in the amino terminus of the protein (reviewed in [20]). These variations are functional and interactive, determining the levels of GPx-1 enzyme activity for a given concentration of available selenium [21, 22]. The *leu* allele has been associated with increased risk of cancers of several types while no clear pattern of association has emerged between the number of ala repeats and cancer risk [20]. In addition to cancer, allelic variants of GPx-1 have also been associated with the risk of cardiomyopathy [23], coronary heart disease [24, 25], hypertension [26], autism [27], intracerebral hemorrhage [28], asthma [29] and the metabolic syndrome [30].

An interaction between SBP1 and GPx-1 was first reported as a result of ectopically expressing each protein in human cell lines [31]. Ectopic expression of SBP1 in the HCT116 human colon cancer-derived cells that do not express SBP1 resulted in a decline in GPx-1 enzyme activity without any apparent effects on either GPx-1 mRNA or protein levels. Consistent with this observation, knock-down of SBP1 in the SMMC7721 non-transformed human liver cell line resulted in a 4-5 fold increase in GPx-1 activity, also without detectable changes in GPx-1 protein levels [16]. In contrast, ectopic expression of GPx-1 in human MCF-7 cells that don't express GPx-1 resulted in a reduction of SBP1 protein levels as well as reduced levels of the SBP1 mRNA [31].

The inverse association between GPx-1 activity and SBP1 expression was also demonstrated in mouse intestinal cells where GPx-1 levels were altered by feeding C57Bl/6 mice a selenium-deficient, -adequate, or -enriched diet and examining the levels of SBP1 by western blotting of extracts prepared from colonic and duodenal epithelial cells [31]. As GPx-1 levels increased in the tissues of mice fed the diets with elevated selenium content, there was a

corresponding decline in SBP1 levels [31]. In humans, an inverse association between SBP1 and GPx-1 levels has been reported for both prostatic [32] and liver tissues [16] obtained from patients with cancer.

2. Mechanisms of inter-regulation between SBP1 and GPx-1

The mechanism by which SBP1 and GPx-1 regulate each other remains to be determined. One possible mechanism of inverse regulation might involve competition for available stores of selenium in the cell. This possibility is unlikely as it was shown that increasing the amount of selenium in MCF-7 cells expressing both GPx-1 and SBP1 resulted in an increase in the levels of the GPx-1 protein and a decline in SBP1 levels, but selenium supplementation of MCF-7 cells that did not express GPx-1 had no effect on SBP1 levels [31]. This result indicates the necessity of GPx-1 for selenium to decrease SBP1 levels. Additionally, the decrease in GPx-1 activity following increased SBP1 expression was achieved without apparent changes in the GPx-1 mRNA or protein amounts [16, 31], suggesting that there might be a physical interaction between the two proteins. This notion is supported by co-immunoprecipitation of HA-tagged SBP1 and Green Fluorescent Protein (GFP) tagged GPx-1 [31]. These results indicate that the two proteins likely participate in a single complex. This result was also observed using modified GPx-1, where its selenocysteine was replaced with a cysteine, indicating that the selenium moiety of GPx-1 is not required for its inclusion in the complex with SBP1. Further evidence for the interaction of these proteins was obtained using confocal microscopy. SBP1 localized in both the cytoplasm and nucleus of liver cells, while GPx-1 was exclusively in the cytoplasm, but both proteins co-localized in the nucleus of these cells following challenge with H_2O_2 [16].

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GPx-1 regulates the levels of SBP1, likely involving control at the level of transcription. GPx-1 can decrease intracellular oxidative stress [33], which can have a profound impact on signal transduction pathways and transcriptional elements that respond to reactive oxygen species (ROS) [17]. Transcription of the SBP1 gene is a target of the oxygen-responsive Hypoxia-Inducible-Factor-1-alpha (HIF-1α) transcription factor, evidenced by the 80-fold increase in SBP1 mRNA observed in HIF-1α gain of function mice vs. non-transgenic controls [34]. While HIF-1α is considered a "master regulator of oxygen sensitive gene transcription" [35], it remains to be determined whether the effects of GPx-1 on SBP1 transcription is meditated through this pathway.

3. SBP1 –GPx-1 interaction is relevant to human disease

Based on the available information, it is likely that SBP1 and GPx-1 interact in such a way to modify the activity of one or both proteins. In the case of GPx-1, its ability to reduce hydroperoxides can have an impact on oxidative damage to biomolecules, DNA repair and a host of signaling pathways that are responsive to ROS, and this has been extensively reviewed [17]. To date, no particular enzyme activity has been associated with SBP1. However, increasing or decreasing its levels in cultured mammalian cells resulted in changes in several phenotypes associated with cancer, including proliferative capacity, apoptosis, cell migration, senescence, resistance to H_2O_2 and cisplatin, and growth of tumors in immunocompromised animals [16, 36-38]. What is noteworthy is that these observations have been made in cells obtained from different tissues and there has been consistency with regard to obtaining opposite results when SBP1 is over-expressed and knocked down.

Both GPx-1 and SBP1 are selenium-containing proteins relevant to several human diseases. They belong to classes of proteins distinguished by either the presence of selenocysteine or the ability to bind selenium. GPx-1 is an anti-oxidant enzyme implicated in disease etiology due to the association of specific alleles with risk of certain diseases. As described below, SBP1 levels are often reduced in tumors compared to corresponding normal tissue and lower levels are typically associated with a poorer clinical prognosis. The inverse association in the levels of these proteins in cells, animals and human tissues, as well as data indicating a direct interaction, reveal a need for the continued investigation of their biochemistry as well as determining how and why they are differentially expressed at progressing stages in the disease process.

D. SBP1 in human disease

1. SBP1 as a tumor biomarker

The earliest indication that reduced levels of SBP1 were associated with cancer was published in 1998 by Yang and Sytkowski [39]. SBP1 was detected as a gene that was differentially expressed in the LNCaP human prostate cancer cell line as compared to the slower growing DU145 and PC-3 lines, with the authors noting that only 4 of 11 tumor derived cell lines expressed detectable SBP1 mRNA levels [39].

Subsequent work examining the levels of proteins obtained from gastric adenocarcinomas and adjacent non-tumor tissues by 2-dimensional electrophoresis indicated that SBP-1 levels were reduced 2-fold in the tumor tissue [40] and using this same approach, similar results were reported for a different cohort of paired gastric tumors and normal mucosa [41]. While also reporting a reduction in SBP1 levels in gastric tumors compared to normal tissue from the same

individuals using tissue microarrays, Zhang et al. noted higher levels of SBP1 in normal and preneoplastic tissues as compared to that observed in tumors, providing evidence that the reduction in SBP1 seen in gastric carcinoma is likely to occur in the latter stages of tumor development [42]. This observation is consistent with data reported indicating a progressive loss of SBP1 levels from non-dysplastic Barrett's esophagus to Barrett's esophagus with high grade dysplasia and ultimately esophageal adenocarcinoma [38]. In the case of uterine leiomyomas, SBP1 levels were shown to be lower in tumors as compared to normal myometrium, with a trend towards a progressive loss with increasing tumor size [43].

By comparing the expression of SBP1 in tumor vs. normal tissues using 2D electrophoresis, expression arrays or tissue microarray analysis, the list of cancer types demonstrating reduced SBP1 levels now also includes adenocarcinoma of the lung [14], colorectal cancers [44, 45], esophageal adenocarcinoma [38], hepatocellular carcinoma [16, 46], liposarcoma [47] and ovarian cancer [48, 49]. In the case of ovarian cancer, reduced levels of SBP1 were also reported in ovarian tumors that developed in the only spontaneous animal model of human ovarian cancer, the laying hen [50]. A list of the types of human cancers in which carcinogenesis was accompanied by a reduction in SBP1 levels is presented in **Table I**.

The mechanism by which SBP1 is down-regulated in human cancers remains to be determined. One possible explanation would be the loss of one or two copies of the SBP1 gene during tumor progression. However, examination of DNA obtained from cells and tissues for either loss of heterozygosity or reduced gene copy number have not revealed any examples of *SBP1* loss [14, 38, 39, 44]. Less clear is whether epigenetic mechanisms of transcriptional control are involved with the changes in SBP1 levels observed in tumor cells, with gene methylation status having been investigated in several instances. The examination of whether

SBP1 is regulated by gene methylation using either bisulfite sequencing, MS-PCR or expression induction following treatment with the demethylating agent 5-azacytidine have yielded conflicting results, perhaps due to the different cell types being investigated [14, 37, 38, 44]. Data in support of the regulation of *SBP1* by promoter methylation was, however, obtained using human tissues by examining colon tumor cells and matched normal colonic epithelial cells from the same patients, revealing elevated *SBP1* promoter methylation in tumor DNA [37].

Table I. **Reduced levels of SBP1 in human cancers and as a predictor of poor clinical outcome**

Organ	SBP1 status	Patient groups	Ref
Lung	SBP1 acidic isoform & mRNA down regulated in adenocarcinoma and poorly differentiated tumors. Low levels of SBP1 correlated with poor survival.	Stage I cancer (n=64) Stage III cancer (n=29) Lung adenocarcinoma (n=80) Squamous cell carcinoma (n=45)	[14]
Ovary	SBP1 was reduced in 87% of invasive ovarian cancers & borderline tumors vs. normal & benign tissue.	Normal tissue & benign tumor (n=12) Borderline & invasive ovarian cancer (n=153)	[48]
	SBP1 expression decrease associated with increased epithelial proliferation and invasiveness.	Borderline carcinoma (n=73) Low grade carcinoma (n=7)	[49]
Colorectal	SBP1 markedly decreased in 12/14 carcinoma samples compared to matched non-tumor mucosa.	Colorectal cancer & paired normal tissue (n=14)	[44]
	3.5 fold less SBP1 in Stage III vs. normal. Low levels correlated with poor survival compared with patients with high SBP1 tumors.	Stage III colorectal cancer & paired normal tissue (n=80)	[45]
Uterus	SBP1 decreased in leiomyoma tissue vs. normal myometrium.	Non-tumor tissue (n=20) Uterine leiomyoma (n=20)	[43]

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Esophagus	SBP1 decreased during progression from Barrett's esophagus to adenocarcinoma.	Barrett's esophagus (n=31) Esophageal adenocarcinoma (n=37)	[38]
Stomach	Decreased SBP1 in gastric adenocarcinoma vs. normal tissues.	Gastric adenocarcinoma & paired normal (n=10)	[40]
	Decreased SBP1 in gastric adenocarcinoma vs. normal tissue.	Stage III gastric cancer & paired normal tissues (n=3)	[41]
	Lower SBP1 in carcinoma tissue, with 8/25 patients showing negative tissue SBP1 staining.	Precursor lesions (n=89) Gastric cancer & paired normal tissue (n=25)	[42]
	Negative tissue SBP1 staining correlated with advanced stage and poor prognosis	Stage II/III gastric cancer & paired nonneoplastic tissue (n=65)	[51]
Liver	SBP1 expression decreased as tumor grade increased.	Grade I (n=6) Grade II (n=12) Grade III (n=8), all with paired cirrhotic controls.	[15]
	Decreased SBP1 associated with vascular invasion. Low SBP1 expression is an independent risk factor for shorter overall survival and increased recurrence rates.	Hepatocellular carcinoma (n=342)	[16]
Prostate	SBP1 inversely correlated with GPx activity. GPx activity directly correlated with Gleason score.	Prostate cancer (n=24)	[32]
Breast	Low SBP1 in ER+ cancer patients associated with poor survival. SBP1 expression decreased with advanced clinical stage.	Breast cancer with paired normal tissue (n=95)	[52]
Fat	Nuclear & cytoplasmic staining decreased in well differentiated components. Lower SBP1 associated with decreased metastasis-free survival.	Atypical lipomatous tumor (ALT) (n=30) Dedifferentiated liposarcoma (DDL) (n=28)	[47]

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2. SBP1 is a marker of differentiation

Decreased expression of SBP1 with increasing tumor stage may be a result of SBP1 levels also being associated with cellular differentiation, as dedifferentiation is a hallmark of advanced tumor stage. SBP1 levels were examined in longitudinal sections of normal human colonic tissue by immunohistochemistry to assess SBP1 expression during different stages of colon cell differentiation [45]. As colon cells migrated along the crypt-luminal axis and became more differentiated, SBP1 expression also increased with maximal expression at the top of the intestinal villi. Evidence that SBP1 may play a functional role in this process was presented by showing that down regulation of SBP1 using siRNA in the Caco-2 colon cell line resulted in expression of the epithelial differentiation marker carcinoembryonic antigen (CEA) also being reduced [45].

3. SBP1 can function as a prognostic indicator

SBP1 was detected by gene expression profiling as one of 27 genes shown to comprise an expression profile that predicted both survival and progression among patients diagnosed with pleural mesothelioma [53]. Using a different approach, 2-dimensional electrophoretic separation of proteins from tumor vs. normal tissue, reduced levels of SBP1 protein was shown to be associated with poor survival among patients with colorectal cancer [44] and lung adenocarcinoma [14]. Specifically assessing the relationship between SBP1 levels and clinical outcome, reduced levels of SBP1 were shown to be an indicator of poor prognosis among patients with gastric cancer [51], hepatocellular carcinoma [16], breast cancer [52] and colorectal cancer [22]. The situation may be less clear for ovarian cancer. Although SBP1 levels

were reduced in 87% of the invasive ovarian carcinomas examined, high SBP1 levels were associated with poor overall survival, in contrast to what has been reported for other tumor types [48].

E. Prostate pathology

1. Biology of the prostate

The prostate is a small, walnut-sized gland located between the bladder and the penis. The urethra runs through the center of the prostate gland from the bladder to the penis, allowing urine to exit the body. The prostate functions in male reproductive function as an exocrine gland which secretes prostatic fluid, which increases the motility, survival time, and genetic stability of spermatozoa and the DNA they contain. Because of its location and structural anatomy, prostate disorders including swelling and dysplasia can interfere with both urination and reproductive function; surgical resection of the prostate may result in negative side effects in both of those areas especially in older men [54, 55].

The human prostate contains isolated glands composed of luminal epithelium surrounded by basal cells, which are in turn surrounded and supported by fibrous stroma with bundles of smooth muscle tissue. Most prostate neoplasms originate from luminal epithelium and cancerous glands are characterized by loss of their basal layer and smaller size than normal glands, with the highest grade of adenocarcinoma losing most functional architecture and often presenting as dispersed groups of few cells rather than glands [56].

2. Challenges of prostate cancer diagnosis

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Prostate cancer is the most commonly diagnosed cancer among men in the US, and is second only to lung cancer in lethality [57]. A patient whose prostate cancer is detected while it is at an early stage, and is given treatment for the disease, has a higher chance of survival than someone who is initially diagnosed with late stage cancer. In order to increase the chances of survival for men with prostate cancer, a major effort has been made to provide physicians with the tools to make earlier diagnoses of prostate cancer. Currently, a common way of detecting the existence of a prostate malignancy is through assaying blood levels of prostate specific antigen (PSA), a biomarker whose above normal presence in the blood is strongly associated with the existence of a malignancy in the prostate.

Since its introduction to the clinic in 1986, blood PSA level has served as a sensitive way for clinicians to detect prostate hyperplasia. Currently, a blood PSA level >4.0ng/mL usually results in a prostate biopsy to conclusively determine the presence of a tumor. As previously stated, early detection of prostate cancer increases chances of patient survival; therefore it was theorized that regular annual PSA screening of at-risk men (based on age) would significantly reduce mortality from prostate cancer. A cohort of men from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial were offered annual PSA testing for 6 years, and annual digital rectal examination (DRE) for 4 years. After 13 years of follow up, they were compared with men in a control group who were given usual care, which sometimes included screening (about half of the control group was screened at any point). Surprisingly, there was no difference in mortality from prostate cancer between the two groups after 13 years of follow up [58]. This result raises the concern that in order to be most effective in preventing prostate cancer mortality, additional methods of identifying lethal vs. nonlethal cancers are necessary. PSA has proven to be a sensitive way to identify the existence of prostate hyperplasia, but this makes it inherently

ineffective at revealing tumors which require more aggressive intervention, or those which are more likely to recur after treatment [59].

3. Conclusions

SBP1 expression has been repeatedly and consistently shown to be relevant to human cancers, and research involving the protein will likely continue in this vein. While its function is still largely unknown, SBP1 has been implicated in differentiation, hypoxia response, and antioxidant function. However, despite the evidence that SBP1 affects several capacities in the cell, its lack of any canonical amino acid motif makes its function difficult to study. Additionally, SBP1 does bind selenium but its protein level does not change in response to selenium titration in MCF7 cells. Because of these characteristics of SBP1, my thesis work has taken the approach of determining the way human cells with and without SBP1 respond differently in specific conditions. We also studied the potential of SBP1 as a marker of prostate cancer recurrence, despite our incomplete understanding of its mechanism of action. This work will contribute novel information about the molecular biology and biochemistry of SBP1, as well as expand its relevance to human cancer.

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II. MATERIALS AND METHODS

A. Tissue culture

Three cell lines were obtained from ATCC: HCT116 colon cancer cells, MDA-MB-231 breast cancer cells, and LNCaP prostate cancer cells. The authenticity of cell lines obtained from ATCC was verified by Genetica DNA Laboratories (Burlington, NC) using short tandem repeat proviling. Six ovarian cell lines were obtained from Dr. J. Luborsky at Rush University (Chicago, IL): ovarian cancer lines CaOV-3, OV-90, OVCar-3, SkOv3, and TOVII2D, and normal ovarian cells IOSE-3. HCT116 cells were maintained in McCoy's 5a medium, IOSE-386 and TOVII2D cells were maintained in a 1:1 mixture of Medium 199 and MCDB 105. All other cell lines were cultured in RPMI 1640 media (ATCC). All media was supplemented with 10% fetal bovine serum (FBS, Gemini Biosciences, West Sacramento, Ca), and 100 units/mL of both penicillin and streptomycin (Gibco, Grand Island, NY). For doxycycline treatments, adherent cells (HCT116, MDA-MB-231) were plated at minimally 30% confluency and treated with 0.5-1.0 ug/mL doxycycline (Sigma-Aldrich, St. Louis, MO).

B. Construct development and viral packaging

1. Tet-On SBP1 inducible expression construct synthesis

The pRetroX-Tight-Pur (pRX) vector was obtained from Bert Vogelstein through Addgene and used as a backbone for the construction of an inducible SBP1 expression construct. The pRX vector contains 5 tetracycline binding regions to which the Tet-On Advanced transactivator protein binds.

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Two oligonucleotides were synthesized containing NotI and EcoRI restriction sites (IDT, Coralville, IA, 5'-ATAGCGGCCGCTACAGCATGGCTAC-3'; 5'ACGAATTCGCTCAAATC CAGATGTCA-3') and used as primers to PCR amplify the SBP1 gene from the pCDNA3.1-Ha-SBP1 expression construct provided by Dr. Wancai Yang. The SBP1 amplicon and the pRX vector were both digested with NotI and EcoRI and isolated by excision from a 1% agarose gel; their sizes were 1-kb and 6-kb respectively. The DNA was then electroeluted from the agarose gel slices into Tris-acetic acid-EDTA (TAE) buffer in dialysis tubing. DNA was precipitated by alcohol precipitation adding 1/10 volume 3M sodium acetate and 3 volumes 100% ethanol. The solution was placed at -80°C for one hour, and then spun at 14,000 rpm in centrifuge for 10 minutes to pellet DNA. Supernatant was then removed and the pellet dried for 5 minutes. The pellet was then resuspended in water and DNA quantified by NanoDrop. Seventy nanograms of the SBP1 insert and 50ng of the pRX vector were ligated using T4 DNA ligase (Promega, Madison, WI), which proceeded overnight at 4°C. Following ligation , 5µL of ligation reaction was transformed into subcloning efficiency DH5α *E. coli* (Invitrogen). 200µL of the transformation was plated on LB agar (Fisher) made with 100µg/mL ampicillin (Sigma-Aldrich), and colonies were grown overnight at 37°C. Eight colonies were selected for expansion in LB broth containing 100µg/mL ampicillin and were shaken overnight at 225 rpm and 37°C. pRX-SBP1 DNA was isolated from the bacteria with the QIAPrep spin miniprep kit (Qiagen) and digested with NotI and EcoRI to verify insertion of the reporter fragment. Clones positive for fragment insertion were co-transfected with the pVSV-G retroviral envelope plasmid into GP293 cells for packaging.

Retroviral packaging of the pRetroX-Tet-On-Advanced plasmid was performed by Applied Stem Cell (Menlo Park, Ca). pRetroX-Tet-On-Advanced retrovirus was provided with

viral titers (8.65 ± 2.21 x 10^7 IFU/mL). GP293 retroviral packaging cells (Clontech, Mountain View, CA) were co-transfected overnight with the pRetroX-SBP1 tetracycline inducible SBP1 expression construct, and the pVSV-G pantropic viral envelope vector (Clontech) using Lipofectamine-2000 (Invitrogen). The following day, transfection media was replaced with fresh high-glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, 100 units/mL penicillin and 100ug/mL streptomycin. Transfected, viral producing GP293 cells were allowed to grow for 48 hours before virus containing media was removed and frozen to kill all cells remaining. Virus containing media was then thawed, and polybrene (Santa Cruz Biotechnologies, Santa Cruz, CA) added at 4 μg/mL. HCT116 cells were plated such that they were 50% confluent at the time of retroviral infection in 25cm^2 tissue culture flasks. HCT116 cells were infected overnight with 5mL of a 1:1 mixture of viral supernatant and McCoy's 5a media. The following day, the media was removed and fresh McCoy's 5a containing 1μg/mL puromycin (Santa Cruz Biotechnologies) was applied.

C. Proliferation and soft agar assays

For proliferation studies, HCT116 TetSBP1 and control HCT116 cells were treated with 1.0 ug/mL doxycycline for 48 hours with non-treated plates grown concurrently. Cells were trypsinized and resuspended in 10mL McCoy's 5a media and counted using a hemocytometer. Four 96 well plates were seeded with 500 cells per well and cells were incubated overnight to allow attachment. The following day, media was replaced with media containing specified treatments and allowed to grow for either 24, 48, 72, or 96 hours. When the determined growth duration was reached, media was removed from the relevant plate was stored at -80°C until all plates were ready for analysis. For treatment groups, half of the wells containing either inducible

or control cells received media containing 2.5% or 10% FBS. Cells receiving media containing either serum level were treated in the same fashion; with both groups receiving in triplicate media supplemented with 0nm, 22.5nm, 60nm, or 90nm sodium selenite ($NaSeO_3$) (Sigma-Aldrich). For proliferation studies involving growth in tert-butyl hydroperoxides (t-BOOH) (Sigma-Aldrich), cells were treated with 0uM, 50µM, 100µM, or 200µM t-BOOH.

The ability of cells to form colonies in soft agar was assayed to determine the effect of SBP1 on anchorage independent growth of HCT116 cells. A base layer of agar was prepared by adding 2mL of 0.6% agarose in DMEM media to each well of 6 well plates and allowed to solidify. The top layer was prepared by adding $1x10^4$ cells to 1mL of 0.4% agarose in DMEM, which was then placed over the base layer. Colony growth was monitored daily, and colonies were counted after 14 days of culture.

D. Cell lysis and protein isolation

Adherent cells were dislodged from the plate by scraping, and resuspended in Cell Lysis Buffer (Cell Signaling Technologies) with 200 µM phenylmethanesulfonyl fluoride (PMSF) protease inhibitor and lysed on ice for 15 minutes. Lysates were centrifuged at 14,000 rpm at 4°C for 15 minutes, and cleared lysate collected for each sample. Protein concentration was determined using the Bradford assay as previously described [60].

E. Protein analysis and enzyme activity

Cell lysates were analyzed by western blot analysis using antibodies targeted against proteins of interest. Thirty micrograms of total protein per sample was prepared for electrophoresis in NuPAGE LDS sample buffer (Invitrogen, Grand Island, NY) and NuPAGE

sample-reducing agent, then boiled at 100°C for 5 minutes. Ten micrograms of the prepared sample was loaded onto a 4-12% Bis-Tris denaturing polyacrylamide gel (Invitrogen) and electrophoresed for 45 minutes at 200 volts in the XCell electrophoresis chamber (Invitrongen). Spectra Broad Range Multicolor protein ladder (Thermo) was electrophoresed on each polyacrylamide gel for band size estimation after blotting. Protein was transferred from the polyacrylamide gel onto a methanol-activated polyvinylidene fluoride (PVDF) membrane for 70 minutes at 30 volts using the XCell Blot Module (Invotrogen). After transfer, membranes were blocked against non-specific antibody binding using 5% dry Carnation® milk in TBS containing 1% Tween-20 (TBST, Bio-Rad and Fisher) for 30 minutes at room temperature with gentle agitation. PVDF membranes were incubated overnight at 4°C with primary antibodies against proteins of interest. Antibodies against the following proteins were used: SBP1, GPx-1 (mouse, MBL International), β-actin, GPx4 (rabbit, Abcam), TrxR1 (rabbit, Proteintech, Inc., Chicago, IL), phospho-p53 (mouse, Cell Signaling), p53 (mouse, Santa Cruz), Akt, phospho-Akt-Ser473, and NF-κB (rabbit, Cell Signaling). β-actin was used as an endogenous control for protein concentration in each sample. All antibodies were diluted 1:1000 in 5% milk-TBST except β-actin which was used at a dilution of 1:10,000. After overnight incubation, membranes were washed twice for 5 minutes each in TBST, then incubated for 1 hour at room temperature in species-appropriate secondary antibody- anti rabbit (1:5000, Cell Signaling) or anti-mouse (1:1000 Cell Signaling) in 5% milk-TBST. Following secondary antibody incubation, the membrane was washed 3 times for 10 minutes each in TBST. The blot was imaged by enhanced chemiluminescence (ECL) with ECL plus (GE Life sciences, Pittsburgh, PA). The density of protein bands was quantified using ImageJ software (NIH, Washington, DC) and normalized to

β-actin band density. Total GPx enzyme activity was determined by a coupled spectrophotometric GPx assay, as previously described [61].

F. Phospho-p53 reporter construct and luciferase assay

In order to determine the ability of phosphorylated p53 to activate transcription, the pG13-Luc reporter construct was purchased from Addgene and used as previously published [62]. The construct was transfected into HCT116 Tet-SBP1 cells in triplicate, which had been induced with doxycycline to express SBP1 48 hours previously, using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, the cells were harvested, lysed, and luciferase activity quantified using the Dual Luciferase Assay kit (Promega).

G. Immunocytochemistry and confocal microscopy

HCT116-TetSBP1 and LNCaP cells were seeded on glass-bottom confocal microscopy culture plates and maintained as indicated above. After treatment, cells were fixed using 4% paraformaldahyde for 15 minutes, blocked with 5% BSA and subsequently stained overnight with anti-GPx-1 (rabbit, Cell Signalling) and anti-SBP1(mouse, MBL International) primary antibodies. Following incubation with primary antibodies, cells were stained with Alexa 488 (Rabbit, Invitrogen) and Alexa 568 (Mouse, Invitrogen) fluorescent tagged secondary antibodies, then nuclei were counterstained using DAPI. All cells were scanned and images obtained using the Zeiss LSM 510 Meta confocal microscope.

H. Immunohistochemistry

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All immunohistochemistry was performed by Andy Hall of the UIC Research Resource Center. Formalin fixed, paraffin embedded tissue samples were sectioned at 5 micron thickness and mounted on Starfrost/Plus slides. Tissue was hydrated through a xylene and alcohol gradient. Slides were rinsed in distilled water followed by heat induced antigen retrieval in citrate buffer for 20 minutes and rinsing in TBST for 5 minutes. For the demonstration of SBP1, tissue sections were blocked with 3% hydrogen peroxide for 30 minutes, and then treated with a protein blocking solution for 15 minutes at room temperature. Slides were rinsed in TBST for 5 minutes and incubated with monoclonal mouse anti-SBP-1 antibody (MBL International, Woburn, MA) at a titer of 1:100 and rabbit anti-pan-cytokeratin (pan-CK) antibody at a titer of 1:100 for 60 minutes at room temperature. Slides were rinsed in TBST for 5 minutes, and then treated with anti-mouse Alexa fluor 647, and anti-rabbit Alexa fluor 488 polymer for 20 minutes at room temperature. Slides were rinsed in distilled water, nuclei were counterstained with DAPI, dehydrated through an alcohol gradient and mounted with Micromount (Leica Microsystems). Anti-SBP1 and pan-CK was detected in stained cores by scanning using the Vectra quantitative imaging system.

I. Statistical analysis

All statistical analysis was performed by Ryan Deaton from Dr. Peter Gann's lab. The fluorescent intensities of nuclear and cytoplasmic SBP1 were assessed for normality and log-transformed for statistical analysis. Patients were subcategorized into three categories based on their Gleason grade (GleasonCat1 = Gleason $\leq$6, GleasonCat2= Gleason 7(3+4) GleasonCat3= Gleason 7(4+3) & $\geq$8). Association between SBP1 levels and GleasonCat was assessed using the

Wilcoxon Rank Sum test and the distribution of scores were represented using box-and-whisker plots.

Patient tissues were also assigned to quartiles based on SBP1 intensity. We fit conditional logistic regression models to estimate odds ratios and 95% confidence intervals for the risk of biochemical recurrence for each quartile of SBP1. The conditional models incorporated adjustment for case-control matching variables; additional models were fit with baseline PSA as an additional covariate, since PSA was not a matching factor.

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III. Results

A. GPx-1 extends the half life of SBP1 and the levels of the two proteins are inversely associated in ovarian cell lines

1. SBP1 is differently expressed in ovarian adenocarcinoma cell lines

SBP1 levels are decreased in multiple human tumor types compared to normal tissue, including ovarian tumors. When immunohistochemistry of invasive human ovarian tumors was performed using antibodies against SBP1, invasive tumors had 87% less SBP1 when compared with benign ovarian tissue [48]. In order to study the expression of SBP1 in ovarian cancer cells, we examined SBP1 expression in five established ovarian cancer cell lines: Caov3, OVCAR3, SK-OV3, TOV-112D, OV90, and in immortalized ovarian cell line: IOSE-386. All of the tumor cell lines were derived from adenocarcinoma, TOV-112D and OV90 cells were specified by ATCC as originating from grade 3 tumors. IOSE-386 was generated by immortalizing human ovarian surface epithelial (OSE) cells with genes from the simian virus 40 (SV40) virus [63]. Immunoblot of lysates from these six cell lines shows that there is no SBP1 expression in IOSE-386, SK-OV3, and TOV-112D cells, a small amount of expression in Caov-3, moderate expression in OV90, and very strong expression in OVCAR3 cells (**Figure 1**). Examination of GPx-1 levels in these cells showed moderate to strong expression in all cell lines except OV90 and OVCAR3, which were the two cell lines with moderate to strong SBP1 expression (**Figure 1**). Thioredoxin Reductase (TrxRD1) levels were not associated with SBP1 levels.

2. The half life of SBP1 varies based on the presence of GPx-1 protein.

When immunoprecipitation using anti-human SBP1 monoclonal antibody was performed on lysates from HCT116 cells following co-transfection of the cells with SBP1 and GPx-1

expression constructs, GPx-1 was co-precipitated with SBP1, indicating a physical interaction between the proteins [31]. To contribute evidence in support of this interaction, Qi Ying from Dr. Alan Diamond's lab determined the intracellular half life of SBP1 in the presence and absence of GPx-1. MCF-7 breast cancer cells which have high SBP1 levels but are null for GPx-1 were treated in culture with cyclohexamide, a protein synthesis inhibitor. Cells were harvested after 24, 48, and 72 hours of cyclohexamide treatment and immunoblotted to detect the rate of decay of the SBP1 protein. The same experiment was performed using previously generated MCF-7-GPx-1 cells which stably express GPx-1 [64]. The half life of SBP1 in MCF-7 cells was 43 hours, whereas in MCF-7-GPx-1 cells the half life was 64 hours, indicating that the presence of GPx-1, and the likely physical interaction it participates in with SBP1, extends the half life of SBP1 by 50% in MCF-7 cells (**Figure** 2).

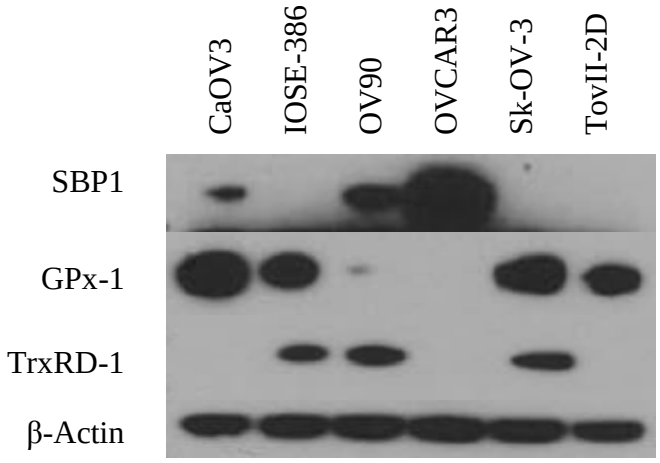

Figure 1. Inverse SBP1 and GPx-1 expression in ovarian normal and adenocarcinoma cell lines.

Total cell extracts from ovarian cell lines were analyzed for SBP1, GPx-1, and TxrRD-1 protein levels by western blot using antibodies for each protein. β-Actin is used as an endogenous protein loading control.

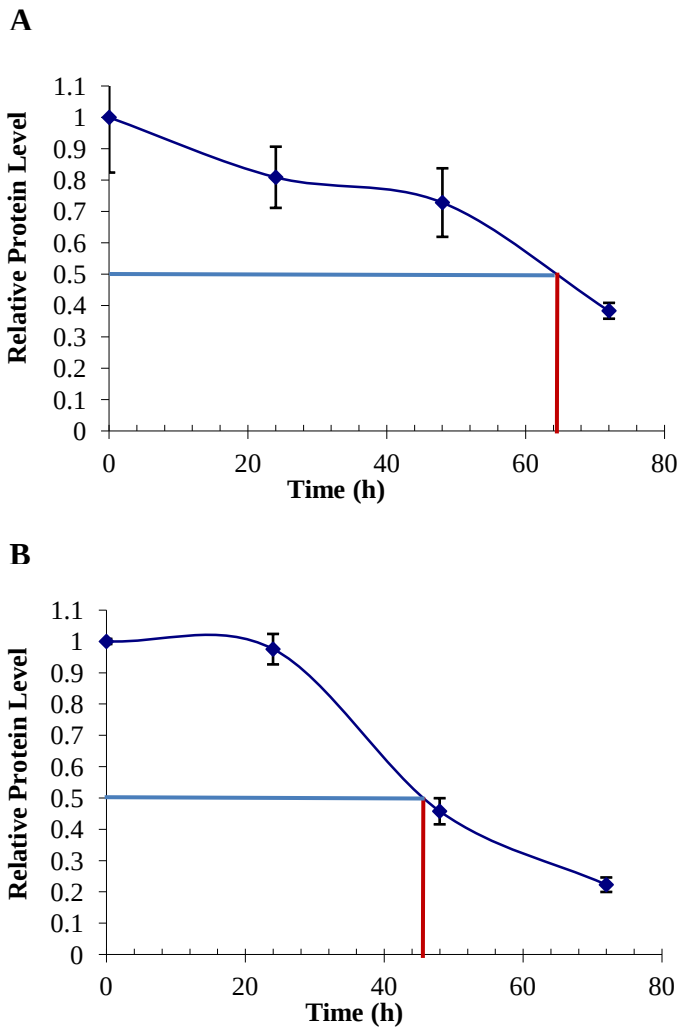

Figure 2. Half-life of SBP1 in MCF7 cells in the presence or absence of GPx-1.

SBP1 levels in MCF7 cells (A) and MCF7-GPx-1 cells (B) were determined at 4 time points after treatment with cyclohexamide by western blotting. Protein levels were normalized to actin at each time point. Error bars represent standard deviation (n=3).

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B. SBP1 in HCT116 cells results in decreased soft agar colony formation and decreased proliferation in response to chemotheraperutic 5-fluorouracil

1. HCT116 cells were engineered to express SBP1 in response to doxycycline treatment

Previous studies have indicated that ectopic expression of SBP1 in HCT116 cells reduces the growth of those cells in nude mouse xenografts [37]. In order to investigate SBP1's ability to inhibit cancer cell growth, we designed an inducible SBP1 expression construct, and subsequently generated a cell line in which SBP1 expression is induced in response to treatment with doxycycline. The HCT116 colon cancer cell line was chosen for these studies because of the lack of detectable SBP1 expression, and because those cells have been used previously in studying SBP1 function [37]. The inducible expression system includes the pRetroX-Tight-Pur-TetOn-Advanced plasmid containing the Tet-On transactivator gene. The Tet-On gene codes for a protein that binds to the promoter region of the pRetroX-Tight-Pur plasmid and induces transcription of the gene downstream of the promoter in response to doxycycline. The SBP1 gene was inserted downstream of the doxycycline responsive promoter region in the pRetroX-Tight-Pur plasmid yielding the pRetroX-Tight-Pur-SBP1 plasmid (**Figure 3**). Both the Tet-On transactivator and the SBP1 expression constructs were packaged into a retrovirus and used to infect HCT116 cells, followed by puromycin selection of infectants. From a single puromycin resistant colony, the HCT116-TetSBP1 cell line was expanded and treated with doxycycline to confirm the consistent induction of SBP1 these cells, with no expression of SBP1 seen in the non-treated cells (**Figure 4**).

2. SBP1 expression alone does not impact proliferation of HCT116 TetSBP1 cells

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The FluoReporter dsDNA proliferation assay is based on a Hoescht 33258 fluorescent stain to measure of the quantity of double stranded DNA present in each well of a 96 well plate as an indicator of the amount of live cells present at a given time point. The growth of HCT116 TetSBP1 cells with and without SBP1 induction was measured and no difference was seen under standard culture conditions (

Figure 5). HCT116 cells, which only contain the pRetroX-Tight-Pur-SBP1 plasmid without the TetOn transactivator plasmid, were used to control for the effect of doxycycline on proliferation, and were grown concurrently with the TetSBP1 cells. The difference in growth between control cells with and without doxycycline at each time point was used to adjust the TetSBP1 proliferation data. In order to do this, the fluorescent reported growth of doxycycline treated HCT116-TetSBP1 cells (fluorescent units, FU) was divided by the difference in FU seen between doxycycline treated and non-treated control cells, as per the following formula.

$$\text{Proliferation of +Dox TetSBP1 cells} = \left(\text{+Dox TetSBP1 FU} \Big/ \underbrace{\left(\text{+Dox Ctrl FU} / \text{-Dox Ctrl FU}\right)}_{\text{(Proliferation Change from Doxycycline)}}\right)$$

3. Selenium supplementation does not change proliferation of HCT116 cells; the proliferation of cells induced to express SBP1 is decreased by selenium supplementation.

Given the physical association of SBP1 with selenium, we studied the effect of selenium levels on the ability of SBP1 to affect cellular proliferation. To assess the impact of selenium on cells that don't express SBP1, 500 HCT116 TetSBP1 cells were seeded per well in a 96 well plate and grown in media supplemented with 25nM sodium selenite. The growth of cells between 48 and 96 hours was measured using the average signal produced from Hoescht stain of

dsDNA. The proliferation of selenium supplemented cells was not statistically different from that of cells grown in non-supplemented media (**Figure 6**, $p>0.05$), indicating that selenium supplementation of SBP1 null HCT116 cells does not result in an increase in cellular proliferation.

To investigate if SBP1 has an effect on the proliferation of selenium supplemented cells, selenium supplemented HCT116 TetSBP1 cells containing SBP1 were grown concurrently with selenium supplemented SBP1 null HCT116 cells (+25nM sodium selenite). Cell growth was measured by calculating the average increase in FU of cells between 48 and 96 hours after cells were treated. Cell growth was lower in the selenium supplemented cells compared with non-supplemented cells, indicating that selenium and SBP1 interact to decrease HCT116 cell proliferation.

4. SBP1 attenuates the growth of cells in soft agar

In order to determine if SBP1 affects the characteristics of malignant transformation in cancer cells, we examined the ability of cells to perform anchorage independent growth, which is a key characteristic of transformation. In order to measure how SBP1 affects anchorage independent growth, HCT116-TetSBP1 cells expressing SBP1 (+Dox) as well as SBP1-null (-Dox) cells were grown for two weeks in 0.4% agarose. The colony formation ability of SBP1 expressing cells was significantly less than cells SBP1-null cells, indicating that SBP1 decreased the ability of cells to perform anchorage independent growth (

Figure 7).

5. SBP1 does not change expression of Glutathione Peroxidases-1 & 4, Thioredoxin Reductase-1 & NF-κB

One potential explanation for the suppressive effect of SBP1 on cell growth in selenium supplemented cells could be that SBP1 decreases the levels of certain selenoproteins. In order to investigate this possibility, lysates from HCT116 TetSBP1 cells with and without SBP1 induction were immunoblotted using antibodies against TrxRD1, GPx-1, and GPx-4. These three selenoproteins are antioxidants which are involved in the etiology of several cancers [11]. Additionally, SBP1 has been repeatedly reported to posttranslationally decrease the activity of GPx-1 [16, 31] through an unknown mechanism that may involve a physical interaction between the two [31]. SBP1 did not change the levels of any of the selenoproteins examined, extending previous observations that SBP1 does not impact the protein levels of GPx-1 to two other selenoproteins (**Figure 8**).

6. SBP1 decreases the growth of cells treated with 5-fluorouracil, a drug used to treat colon cancer.

5-fluorouracil (5-FUra) is a drug which blocks the synthesis of thymidine and inhibits DNA replication [65], [66]. The affect SBP1 has on the proliferation of cells treated with 5uM of

5-FUra was measured using dsDNA to quantify cells. HCT116 TetSBP1 cells were treated with doxycycline to induce SBP1, then grown on 96-well plates for 6 days with 48 hour time points concurrently with SBP1-null control cells at the same concentration of 5-FUra (n=3). As a result of 5-FUra treatment, the proliferation of cells expressing SBP1 was an average of 33% slower than cells not expressing SBP1 after 144 hours, at the final time point (**Figure 9**). The significant increase in sensitivity to 5-FUra caused by SBP1 expression indicates that cells containing SBP1 have a stronger response to this treatment, and may provide insight into why colon cancer patients whose tumors express higher levels of SBP1 experience greater survival compared with patients whose tumors have low levels of the protein [45].

7. SBP1 induces phosphorylation of p53 at serine 15

Given the ability of SBP1 to sensitize cells to 5-FUra, as well as published data indicating that SBP1 increases apoptosis in HCT116 cells after exposure to hydrogen peroxide [37], we investigated whether SBP1 may affect the activation of the p53-dependent DNA damage response. 5-FUra causes DNA damage because of its inhibition thymidylate synthase (TS), which results in decreased DNA synthesis and repair [67]. Additionally, its antineoplastic activity is partially a result of incorporation of 5-FUra anabolites into DNA, which causes double strand breaks. [68]. Phosphorylated Serine 15 (Ser-15) on p53 is a post-translational modification that is required to activate the p53-dependent DNA damage response [69]. HCT116 cells normally do not have detectable phosphorylated p53-Ser-15, but when SBP1 was induced in these cells it resulted in robust phosphorylation Ser-15 on p53 (**Figure 10**). Although phosphorylation of Ser-15 results in a decrease of total p53 (**Figure 10**), this is consistent with previous reports indicating that there is a functional significance of changing p53 level; the

higher levels of p53 lead to apoptosis and lower levels, which are dependent on Ser-15 phosphorylation, result in cell cycle arrest.

To test if this phosphorylation increases the ability of p53 to activate the pGL13-Luc luciferase reporter construct which contains a luciferase gene under the control of a p53 responsive promoter region, the pGL13-Luc construct was transfected SBP1 expressing and SBP1-null cells. In cells containing the p53 reporter construct, SBP1 did not change luciferase levels, indicating that the SBP1-dependent phosphorylation of Ser-15 on p53 did not result in increased activation of the p53-dependent transcription of luciferase (**Figure 11**). In addition, supplementing culture media with selenium did not lead to an increase in p53-dependent luciferase transcription in SBP1 expressing cells (**Figure** 11).

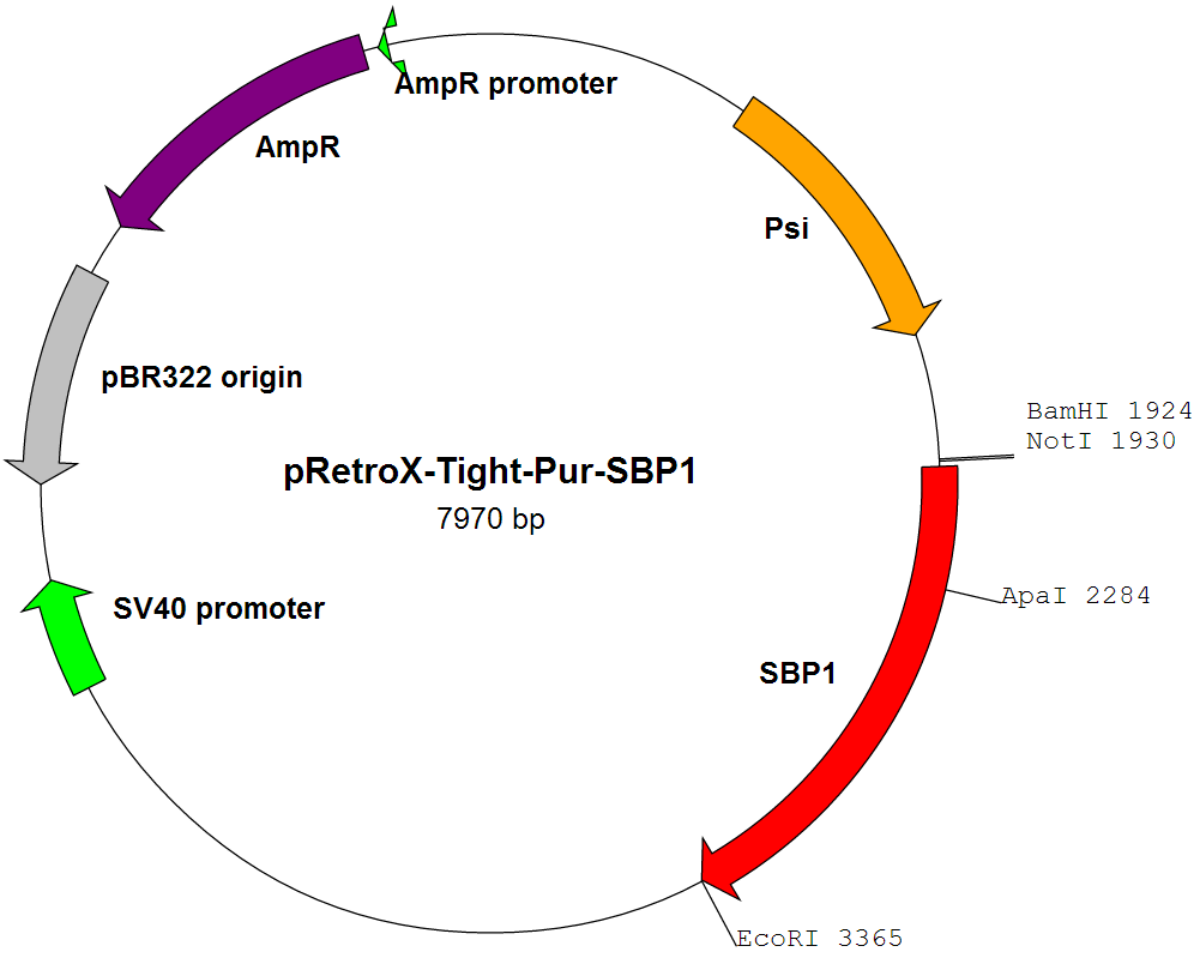


Figure 3. Plasmid map of pRetroX-Tight-Pur-SBP1 inducible expression construct.

pRetroX-Tight-Pur-SBP1 plasmid containing SBP1 gene under the control of the Tet-On Advanced responsive Tight promoter. The retroviral psi packaging element is present to facilitate the incorporation of the SBP1 gene into the retrovirus produced by packaging cell line GP293.

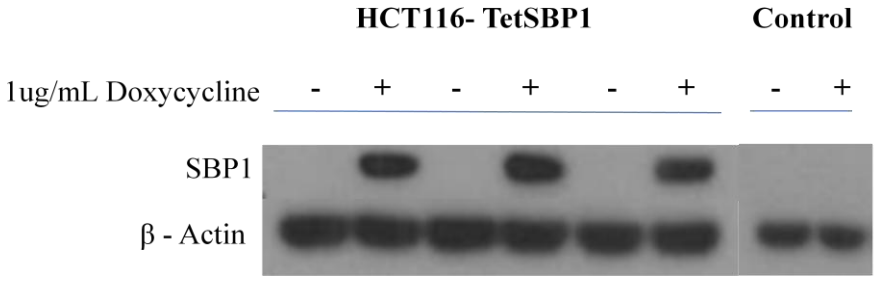
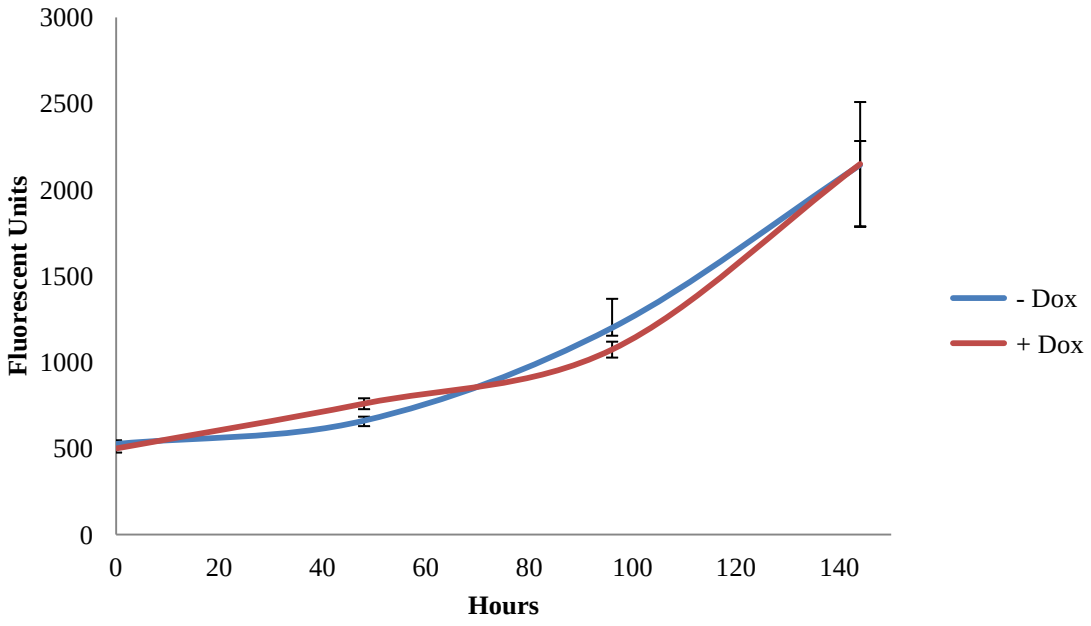


Figure 4. The induction of SBP1 by doxycycline in HCT116 TetSBP1 cell line.

The HCT116-TetSBP1 cell line was generated by infection with pRetroX-TetOn-Adv, a plasmid which expresses a gene coding for the doxycycline binding transactivator protein, and the pRetroX-SBP1 plasmid containing the SBP1 gene under a transactivator responsive promoter. Total cell extracts from doxycycline treated or non-treated HCT116-TetSBP1 cells were analyzed for SBP1 protein levels on western blot with anti-human SBP1 or β-Actin antibodies. Control cells only contain the pRetroX-SBP1 plasmid without the transactivator. β-Actin was used as an endogenous control.

A



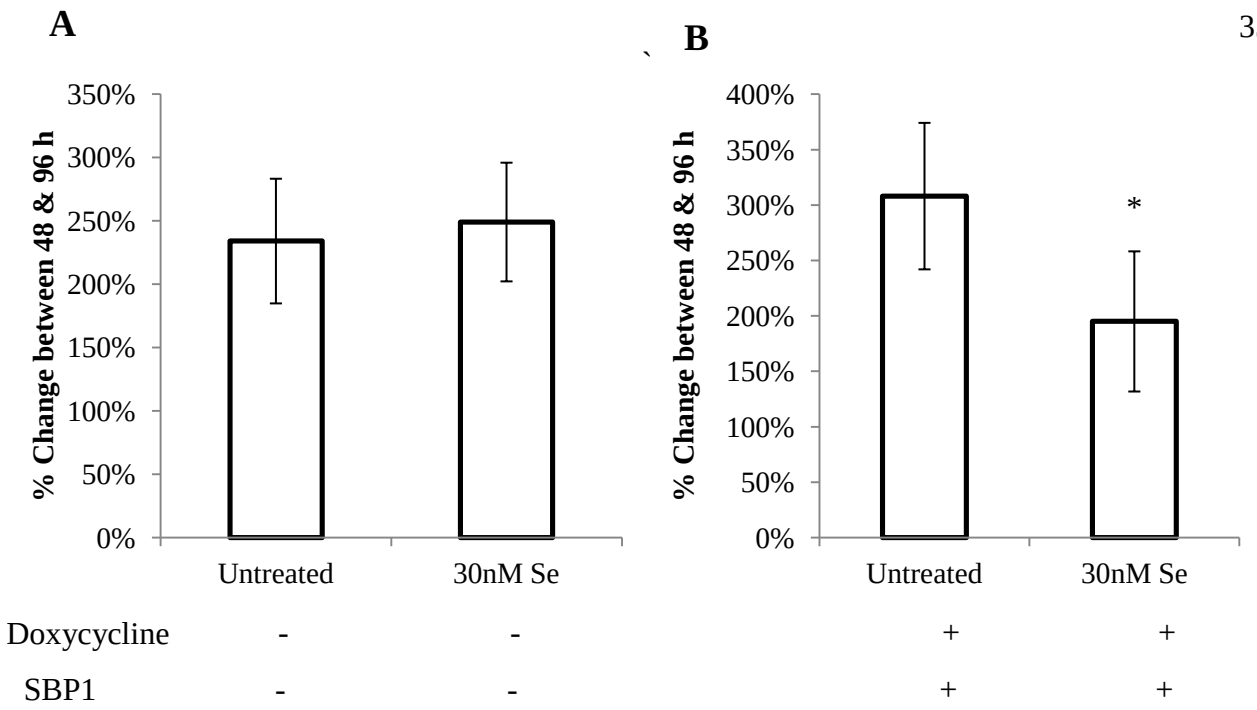
B

Proliferation Change from Dox treatment

Hours	Change in Proliferation
48	-12%
96	+11%
144	+12%

Figure 5. SBP1 does not change the proliferation of HCT116 cells.

HCT116-TetSBP1 cells with (+Dox) and without (-Dox) SBP1 were grown for 6 days on a 96 well plate. Cells were treated with 1ug/mL doxycycline 48 hours before the 0 hour time point. Five hundred cells were seeded in triplicate 24 hours before the 0 hour time point. Fluorescently labeled dsDNA from each well of a 96 well plate was quantified at four time points- 0, 48, 96, and 144 hours, and error bars represent standard deviations at each time point. (A) To eliminate the effect of doxycycline from these analyses, Fluorescent Units from SBP1 expressing cells were adjusted using the mean doxycycline dependent growth change observed in control HCT116 cells (B).



C

Growth change from Dox treatment		
Hours	Difference in growth	
48	+7%	Untreated
96	+21%	
48	+1%	+30 nM Se
96	+8%	

Figure 6. Selenium supplementation of SBP1-null cells does not change proliferation, whereas selenium decreases the proliferation of SBP1 expressing cells.

The growth of SBP1-null (A) and SBP1 expressing (B) HCT116-TetSBP1 cells was measured with and without supplementation with 27.5 nM selenium. Fluorescently labeled dsDNA from each well of a 96 well plate was quantified after cells were lysed while still attached to the plate. Five hundred cells were seeded in triplicate 24 hours before 0 hour time point. Double stranded DNA quantity was measured as an indicator of cell growth at three time points- 0, 48, and 96 hours. In order to account for the lag in growth observed between 0 and 48 hours of the assay, the percent change of quantified dsDNA was determined between 48 and 96 hours. Error bars represent the percent change standard deviations (*$p<0.05$). Each bar represents the average of 6 independent experiments. In order to eliminate the effect of doxycycline from these analyses, Fluorescent Units from SBP1 expressing cells were adjusted using the mean doxycycline dependent growth change observed in control HCT116 cells (C).

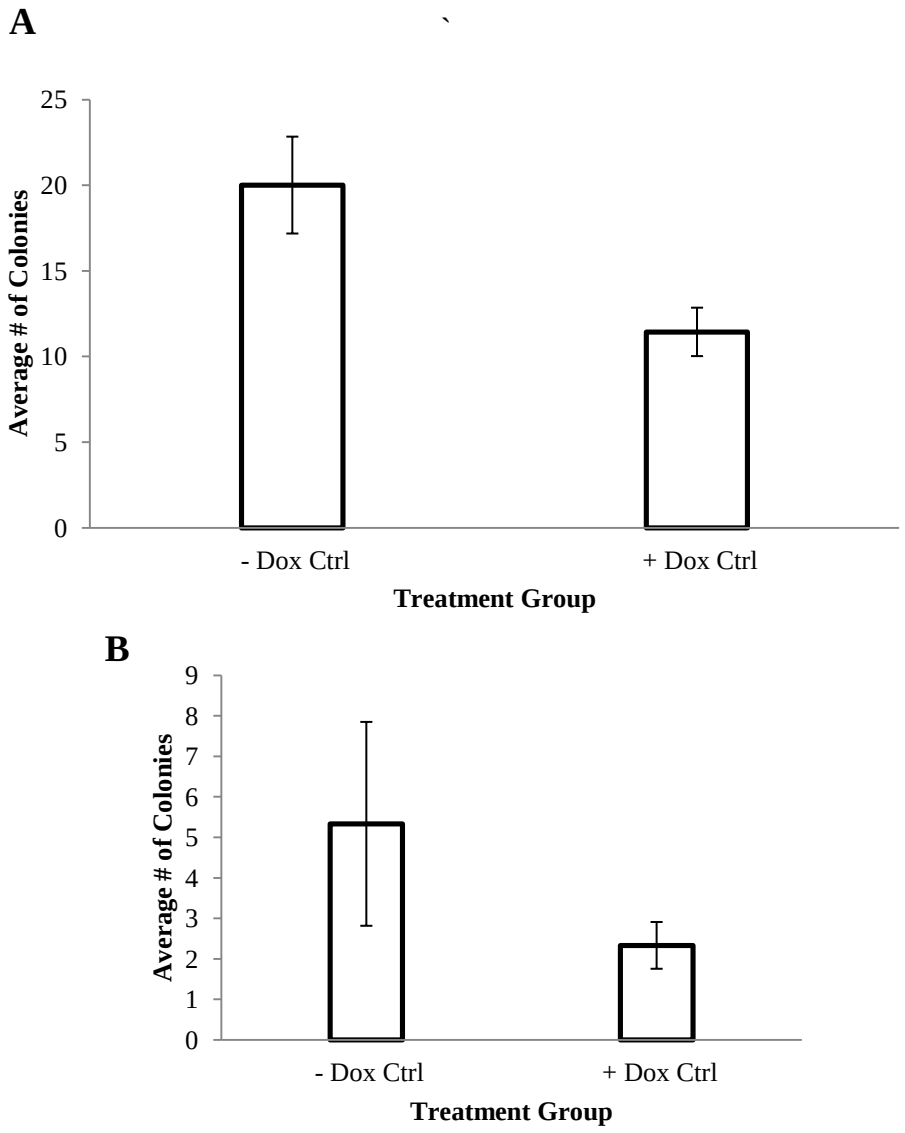


Figure 7. SBP1 decreases soft agar colony formation in HCT116 cells

The ability of HCT116 cells with and without SBP1 to form colonies in soft agar was measured. Cells induced to express SBP1 as well as SBP1-null cells were mixed with media containing 0.4% agarose, and each group was plated in triplicate on 6 well plates coated with 0.6% agarose in media. Each well contained $5x10^4$ cells, and colonies larger than 0.5mm were counted on day 15 of growth (A). Error bars represent S.D. (*$p<0.05$ n=3). To eliminate the effect of doxycycline from these analyses, average colony counts were adjusted using the effect of doxycycline on the colony formation of control cells (B).

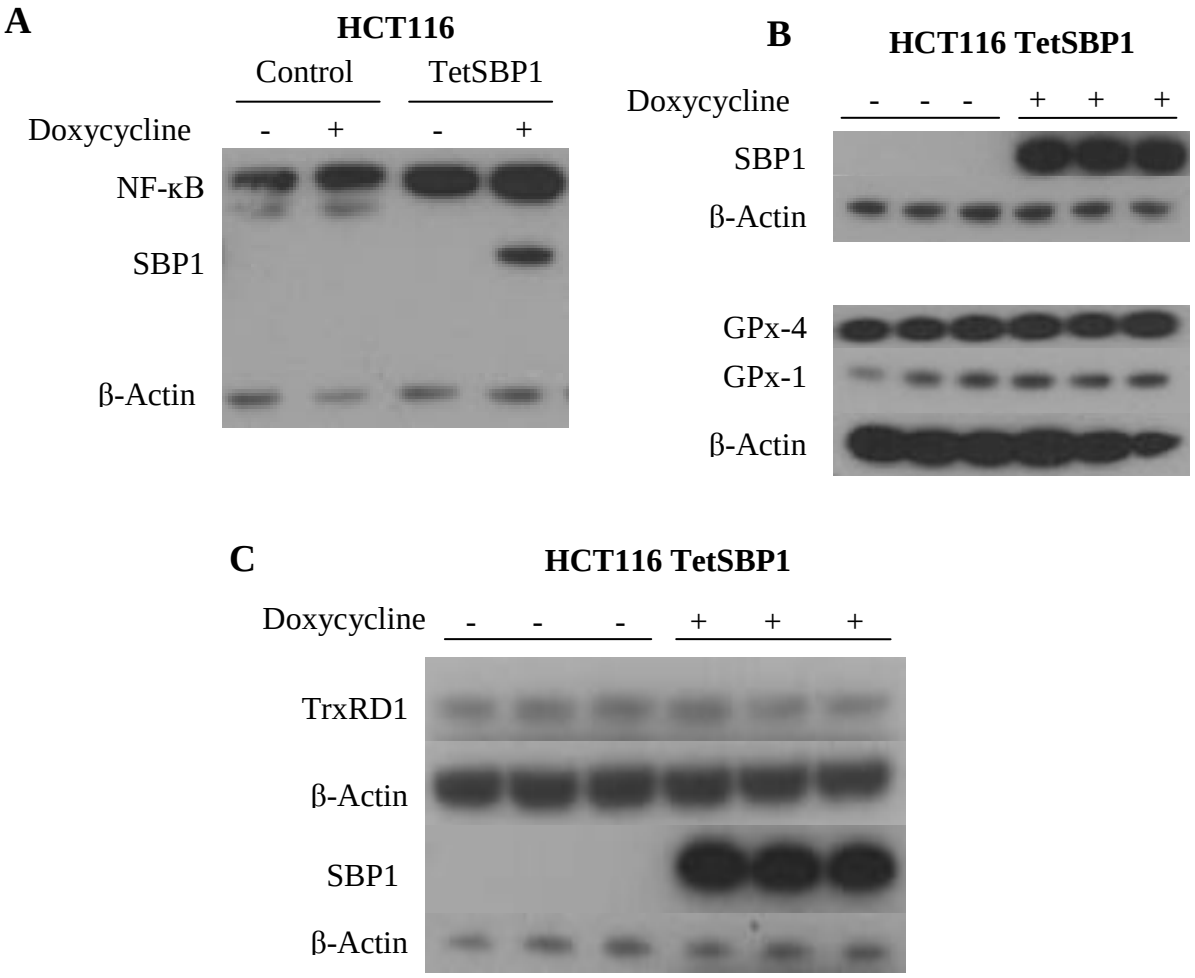
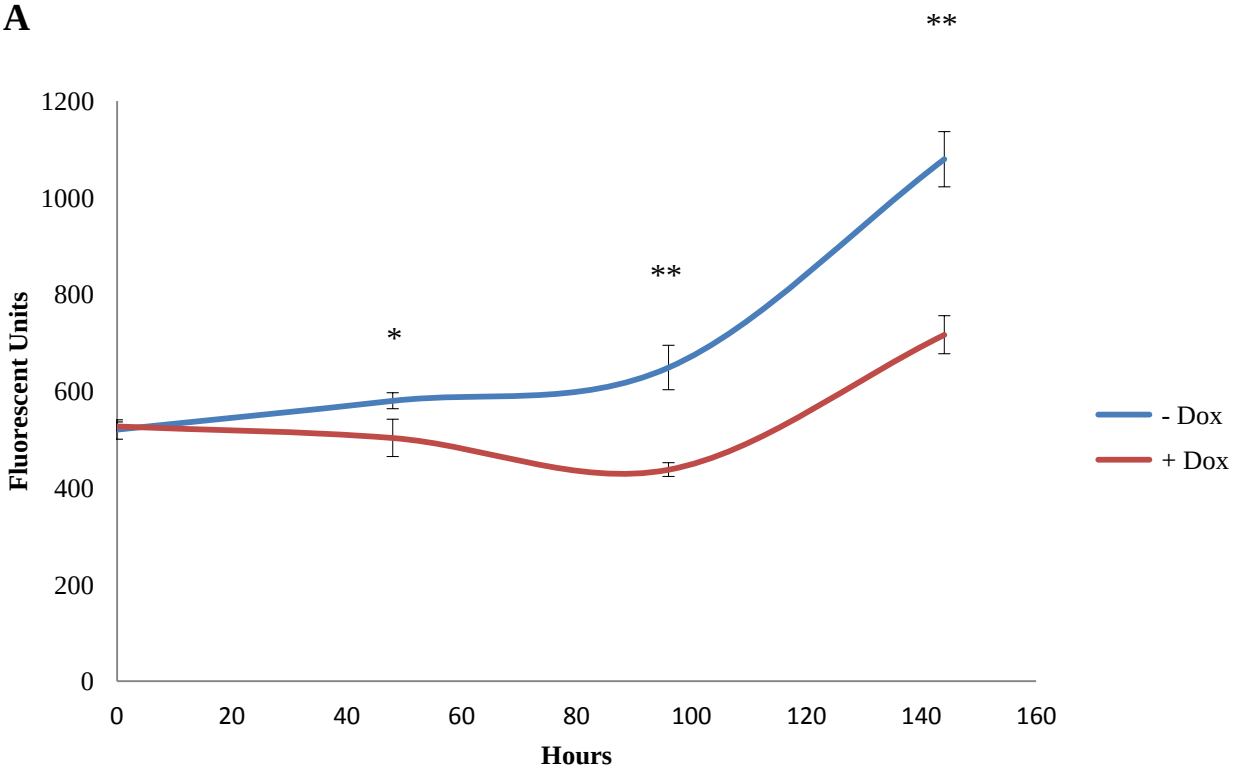


Figure 8. SBP1 expression does not change the levels of GPx-1, GPx-4, NF-κB or TrxRD1

Total cell extracts from doxycycline treated or non-treated HCT116-TetSBP1 cells were analyzed using immunoblot for changes in NF-κB (A), GPx-1, GPx-4 (B), and TrxRD1 (C) levels in response to doxycycline dependent induction of SBP1. Anti-human SBP1, TrxRD1, NF-κB and β-Actin antibodies were used to detect protein levels. Control cells only contain the pRetroX-SBP1 plasmid without transactivator. β-Actin was used as an endogenous control.

A



B

Growth Change from Dox Treatment

Hours	Change in growth
48	8%
96	14%
144	9%

Figure 9. SBP1 sensitizes cells to treatment with colon cancer chemotherapeutic 5-FUra.

The growth of HCT116-TetSBP1 cells was measured during a 6 day treatment with 5uM 5-FUra. Five hundred cells were seeded in triplicate 24 hours before 0 hour time point. Double stranded DNA quantity was measured as an indicator of cell growth at four time points- 0, 48, 96, and 144 hours, and error bars represent standard deviation at each time point (*$p<0.05$, **$p < 0.01$). Fluorescently labeled dsDNA from each well of a 96 well plate was quantified after cells were lysed while attached to the plate. In order to eliminate the effect of doxycycline from these analyses, Fluorescent Units from SBP1 expressing cells were adjusted using the mean doxycycline dependent growth change observed in control HCT116 cells (B).

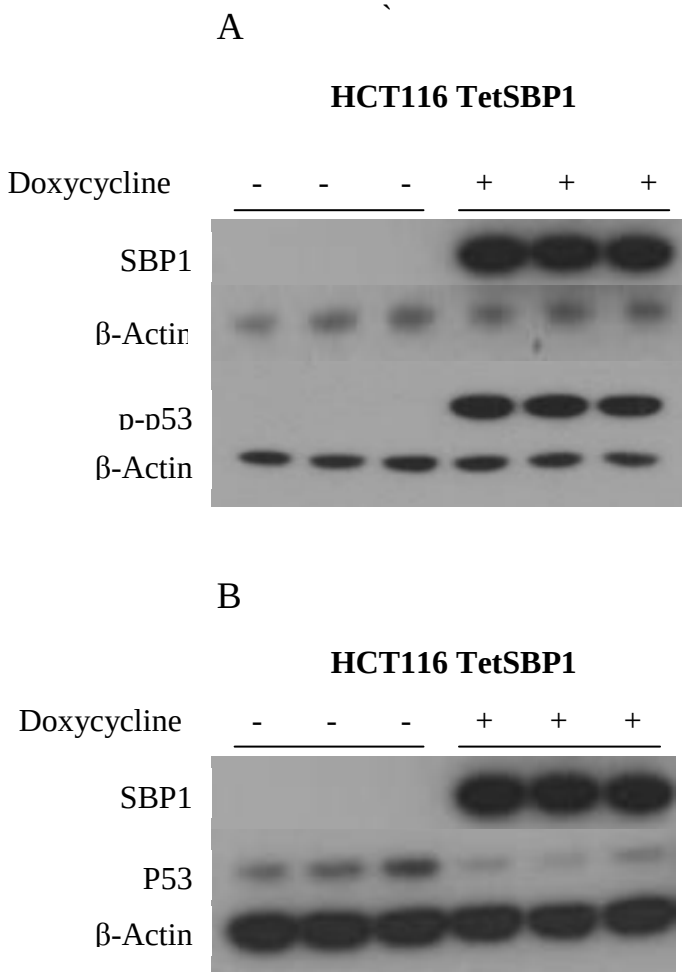


Figure 10. SBP1 induction results in an increase in phospho-p53 and a decrease in total p53 in HCT116 cells.

Total cell extracts from doxycycline treated or non-treated HCT116-TetSBP1 cells were analyzed using immunobloting for changes in phosphorylated Ser-15 on p53 (A) and total p53 (B) levels in response to induction of SBP1 using anti-human SBP1, phospho-Ser-15-p53, p53, and β-Actin antibodies. β-Actin was used as an endogenous control.

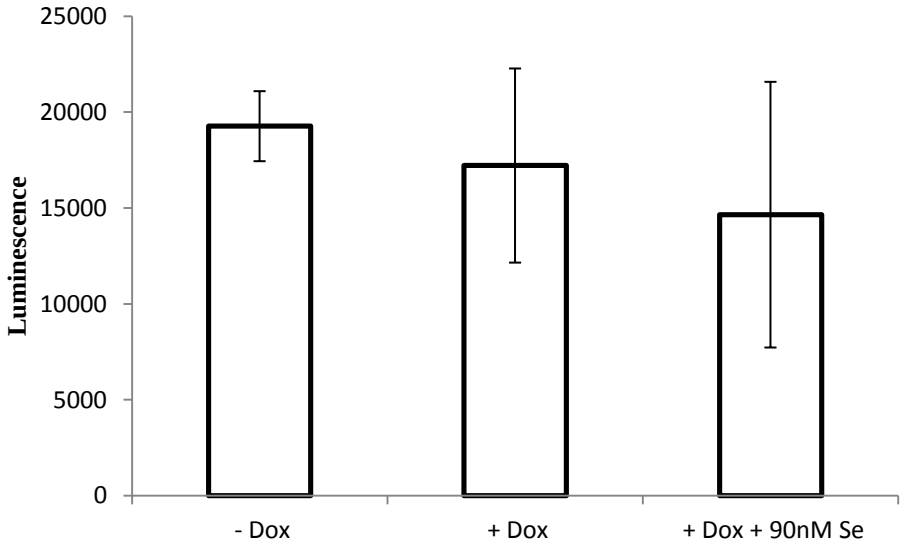


Figure 11. The induction of p53 phosphorylation by SBP1 does not activate p53 dependent expression of pG13-luc luciferase reporter construct.

HCT116-TetSBP1 cells were transiently transfected with pG13-luc luciferase reporter construct which contains a p53 response promoter upstream of the luciferase gene. Activation of the promoter results in expression of luciferase in the presence of transcriptionally active p53. Transfected cells were treated with 1ug/mL doxycycline for 48 hours to induce SBP1 expression and subsequent p53 phosphorylation. Following cell lysis, total cell extracts were assayed for luciferase activity. Error bars indicate the standard deviation (n=3).

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C. SBP1 expression in prostate cancer tissue

1. Fluorescent imaging of SBP1 in human prostate cancer indicates strong nuclear localization and sporadic intraglandular expression

SBP1 became relevant to human cancer in 1998 with the discovery of its androgen responsiveness in the LNCaP prostate cancer cell line, a model of androgen-sensitive prostate cancer [39]. Since then, it has been reported as decreased in many tumor types, and its low level is associated with increased risk of mortality in lung, liver, colon, and breast cancer [14, 16, 44, 52]. While the results of the aforementioned *in vitro* study presents the possibility that decreased SBP1 is relevant to the etiology of androgen driven carcinogenesis, a clinical relevance of SBP1 in human prostate cancer has yet to be identified. The only SBP1 study done using human prostate tissue indicated an inverse correlation between SBP1 expression and GPx activity. Although SBP1 levels didn't reach significance, its inverse association with GPx activity indicates a possible association between SBP1 and prostate cancer progression due to the direct correlation between GPx activity and Gleason score [32].

In order to determine if SBP1 levels are predictive of outcome of prostate cancer patients after radical prostatectomy, a tissue microarray from the Collaborative Prostate Cancer Tissue Resource (CPCTR) containing tissue from prostate cancer patients who underwent radical prostatectomy (RP) and subsequently experienced biochemical recurrence (n= 202) was used [70]. The tissue from cases who recurred were matched with non-recurred control patients based on age (+/- 5 years), ethnicity, cancer stage, and Gleason grade. Gleason grade is the sum of the primary and secondary Gleason scores, which indicate the degree of tumor dedifferentiation observed by the pathologist on a scale of 1 (most differentiated) to 5 (least differentiated). The Gleason grade is expressed using the (primary score)+(secondary score) nomenclature (ex. 4+5).

The primary score is determined by the characteristics of the most commonly observed cancer glands, and the secondary score refers to the characteristics of the second most common glands [71, 72]. Because of the increased lethality of a 4+3 grade tumor vs. a 3+4 tumor [73], cases with Gleason score 3+4 and 4+3 were matched with controls of the same primary and secondary grades. The microarray was stained using SBP1 mouse monoclonal antibody, and pan-cytokeratin (pan-CK) primary antibody as a marker of epithelial cells. The slides were subsequently stained with Alexa fluor secondary antibodies to report SBP1 at the 647 fluorescent wavelength (red) and pan-CK at the 488 wavelength (green); nuclei were counterstained with DAPI (blue).

Using the VECTRA, an automated quantitative imaging system, the stained slides was scanned. Each tumor epithelial cell was digitally segmented into nuclear and cytoplasmic compartments; DAPI was recognized by the software as the nucleus of each cell, and the cytoplasmic signal was obtained by sampling the peri-nuclear area. After segmentation, SBP1 expression was separately quantified in both subcellular compartments of all the cells scanned. Benign glands were manually excluded from analysis. After poor quality and benign-only cores were excluded, the cohort for analysis included tissue from 168 cases and controls. (**Figure 12**) is an example of a scanned core from the microarray showing the highly variable SBP1 levels among epithelial cells in the same gland, with adjacent cells often showing very different staining intensity from each other among the cells that were SBP1-positive. Strong nuclear localization was often observed.

2. Nuclear to cytoplasmic ratio of SBP1 is inversely correlated with prostate cancer Gleason score

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In order to determine if there is a relationship between SBP1 and more advanced prostate cancer, we distributed tissue from the patients included in the outcome tissue microarray into three groups based on their Gleason score- inclusion criteria for each group is detailed in (**Figure 13**). Patients with Gleason score 5 and 6 were in GleasonCat 1, GleasonCat 2 was comprised of Gleason score 7 (3+4), and GleasonCat 3 included Gleason score 7 (4+3), and 8+. We decided to include 4+3 samples in GleasonCat 3, the highest Gleason group, because the mortality rates of patients with prostate cancer of Gleason score 4+3 is three-fold higher than 3+4 cancers [73]. Analysis of SBP1 levels in these tissues indicated that the average nuclear SBP1 signal in each cell was significantly lower in GleasonCat 3 than in GleasonCat 2 (**Figure 13**). Cytoplasmic and total cell SBP1 levels were not significantly associated with Gleason score, nor was there a significant difference between GleasonCat 1 and 3. Additionally, the nuclear:cytoplasmic ratio of SBP1 was significantly lower in GleasonCat 3 vs GleasonCat 1.

3. Prostate cancer patients in the lowest quartile of SBP1 expression are significantly more likely to recur after radical prostatectomy.

SBP1 levels in the nucleus, cytoplasm, and total cell were quantified and analyzed using a paired t-test. There was not a significant difference between recurrent cases and non-recurred controls, indicating that there is not a relationship between SBP1 expression in prostate tumors and biochemical recurrence. However, because the SBP1 levels among patients were skewed due to a large amount of weakly stained tumors, we distributed patients into quartiles based on their tissue SBP1 levels. Linear regression analysis of the interquartile odds of prostate cancer recurrence indicated that patients in the lowest quartile of nuclear SBP1 expression are significantly more likely to recur than those in any other quartile (**Table II).** All estimates are adjusted for PSA, Gleason grade, stage, and age at diagnosis.

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In (**Figure 12)** an example of highly variable intraglandular SBP1 levels is shown- a frequent observation in this cohort which led us to investigate whether the lack of consistent staining among neighboring cells is related to likelihood of biochemical recurrence. Patients were assigned to quartiles based on the nuclear to cytoplasmic ratio of SBP1, and estimated odds ratios and 95% confidence intervals for the association between prostate cancer recurrence and each quartile. There was not a significant association between quartiles of nuclear to cytoplasmic ratio of SBP1 and prostate cancer recurrence **(Table III)**.

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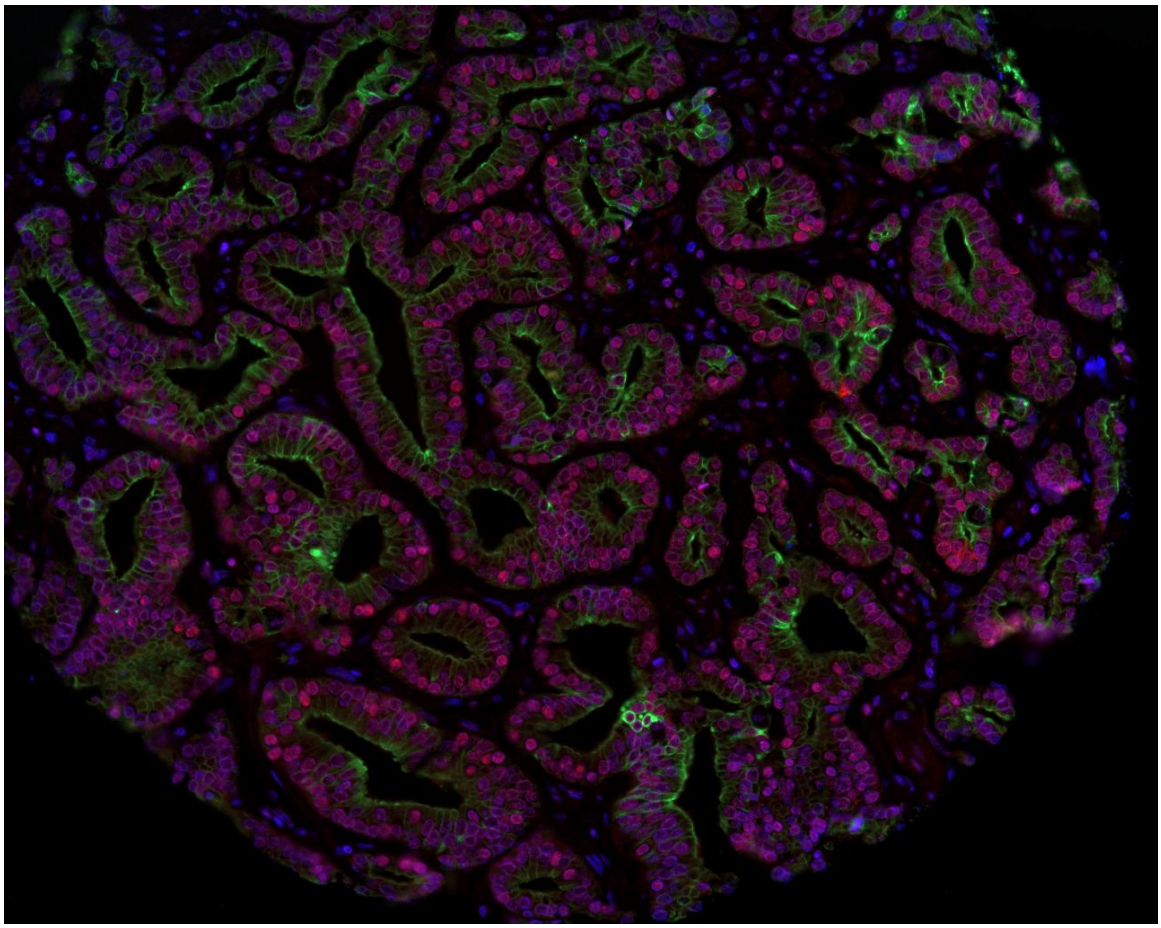


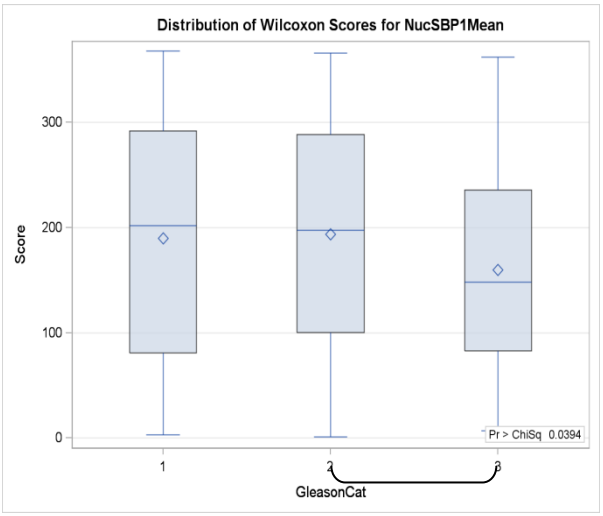
Figure 12. SBP1 is strongly nuclear in human prostate tissue.

An example of a core from the outcome CPCTR tissue microarray stained with anti-pan-cytokeratin (green) and anti-SBP1 (red) antibodies followed by Alexa488 goat anti-rabbit and Alexa647 goat anti-mouse respectively. Nuclei were counterstained with DAPI (blue).

A

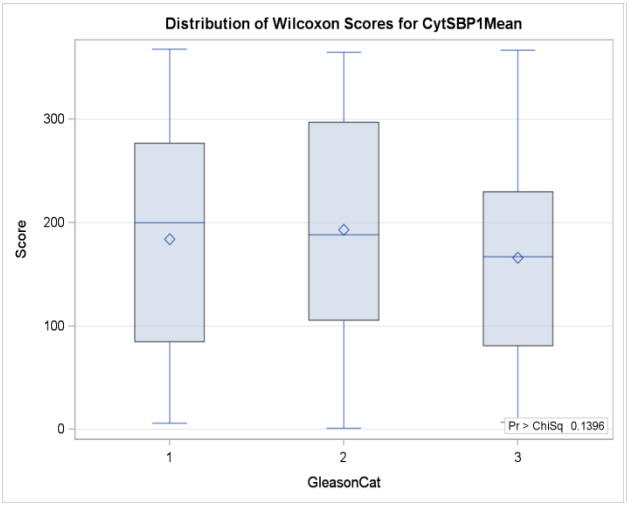
Category	Gleason Score	N	
1	≤ 6	87	
2	3+4	192	
3	4+3, ≥ 8	89	
		368	Total

B



P< 0.05

C



D

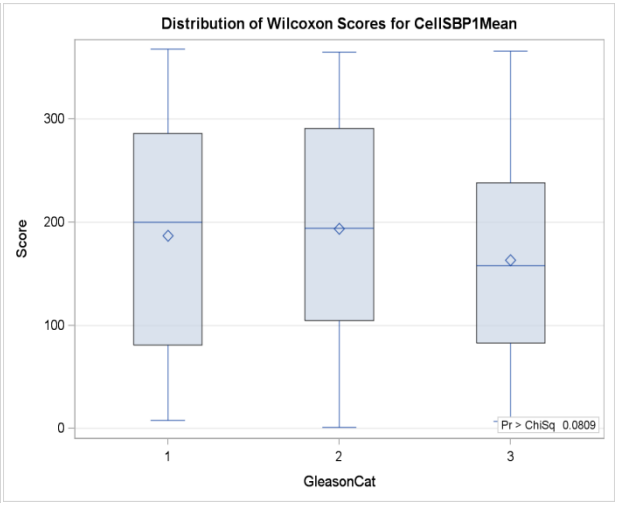
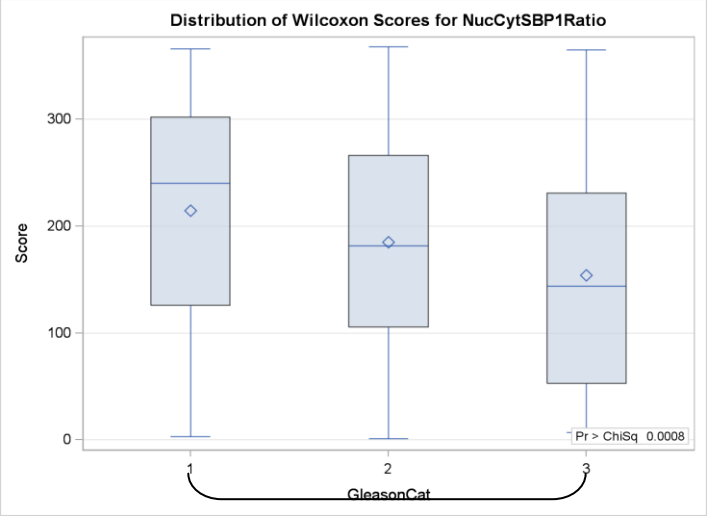


Figure 13. Lower SBP1 levels in the nucleus are significantly associated with advanced prostate cancer.

Patients in the prostate cancer outcome tissue microarray were distributed into three categories (GleasonCat) based on Gleason score (A). Mean quantified tissue SBP1 was ranked for all patients and assigned a score from 1-368 based on relative SBP1 levels where the patient with the lowest tissue SBP1 received a score of 1, and the patient with the highest SBP1 levels received a score of 368. Box-and-whisker plots indicate the range of scores from patients in each category (vertical lines) and the range of the first and third quartile in each category (boxes). The horizontal line inside the box indicates the median and diamonds are the means.



P < 0.05

Figure 14. Lower nuclear to cytoplasmic SBP1 ratio significantly associated with advanced prostate cancer.

Patients in the prostate cancer outcome tissue microarray were distributed into three categories (GleasonCat) based on Gleason score (A). Mean quantified tissue SBP1 was ranked for all patients and assigned a score from 1-368 based on relative SBP1 levels where the patient with the lowest tissue nuclear:cytoplasmic ratio of SBP1 received a score of 1, and the patient with the highest ratio received a score of 368. Box-and-whisker plots indicate the range of scores from patients in each category (vertical lines) and the range of the first and third quartile in each category (boxes). The horizontal line inside the box indicates the median and diamonds are the means.

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	Expression Quartile			
	1 (Low)	**2**	**3**	**4 (high)**
	OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
Nuclear SBP1	**1.00**	**0.355** (0.156, 0.81)	**0.441** (0.195, 0.994)	**0.337** (0.125, 0.911)
Cytoplasmic SBP1	**1.00**	**0.347** (0.164, 0.737)	**0.539** (0.244, 1.19)	**0.482** (0.19, 1.223)
Total SBP1	**1.00**	**0.24** (0.102, 0.564)	**0.586** (0.273, 1.259)	**0.283** (0.104, 0.768)

Table II. Patients in lowest quartile of nuclear SBP1 expression in their tumor glands are significantly more likely to recur after radical prostatectomy than patients with higher nuclear SBP1.

Odds ratios (OR) and 95% confidence intervals (CI) for prostate cancer recurrence by quartile of SBP1 expression. Expression level was measured by the VECTRA quantitative imaging system, and all OR estimates are adjusted for PSA, Gleason grade, tumor stage, and patient age at diagnosis.

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	Expression Quartile			
	1 (Low)	**2**	**3**	**4 (high)**
	OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
Nuc:Cyt SBP1	**1.00**	**1.610** (0.797, 3.255)	**0.929** (.443, 1.947)	**0.603** (0.266, 1.370)

Table III. There was not an association between quartiles of nuclear to cytoplasmic ratio of SBP1 and prostate cancer recurrence.

Odds ratios (OR) and 95% confidence intervals (CI) for prostate cancer recurrence by quartile of nuclear to cytoplasmic SBP1 expression. Expression level was measured by the VECTRA quantitative imaging system, and all OR estimates are adjusted for PSA, Gleason grade, tumor stage, and patient age at diagnosis.

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D. SBP1 tissue expression, subcellular localization, and physical interaction with GPx-1

1. SBP1 levels are sporadic in HCT116 and LNCaP cells, and it is found in both the nucleus and the cytoplasm.

In a previous report, SBP1 and GPx-1 were located in the cytoplasm, and after treatment with hydrogen peroxide both proteins appeared in punctuate patterns in the nucleus in liver cancer cells [16]. In order to determine the location of SBP1 and GPx-1 in LNCaP and HCT116-TetSBP1 cells, prostate and colon cancer cells respectively, both cell lines were stained with fluorescently labeled secondary antibodies following incubation with SBP1 and GPx-1 primary antibodies. GPx-1 is consistently cytoplasmic in both HCT116-TetSBP1 (**Figure 15**) and LNCaP (**Figure 16**) cells, regardless of the localization of SBP1. SBP1 however, was irregularly expressed among cells on the plate, evidenced by adjacent cells containing starkly different levels of the protein. In addition SBP1 was heavily nuclear localized, and this did not change following treatment with 50uM hydrogen peroxide.

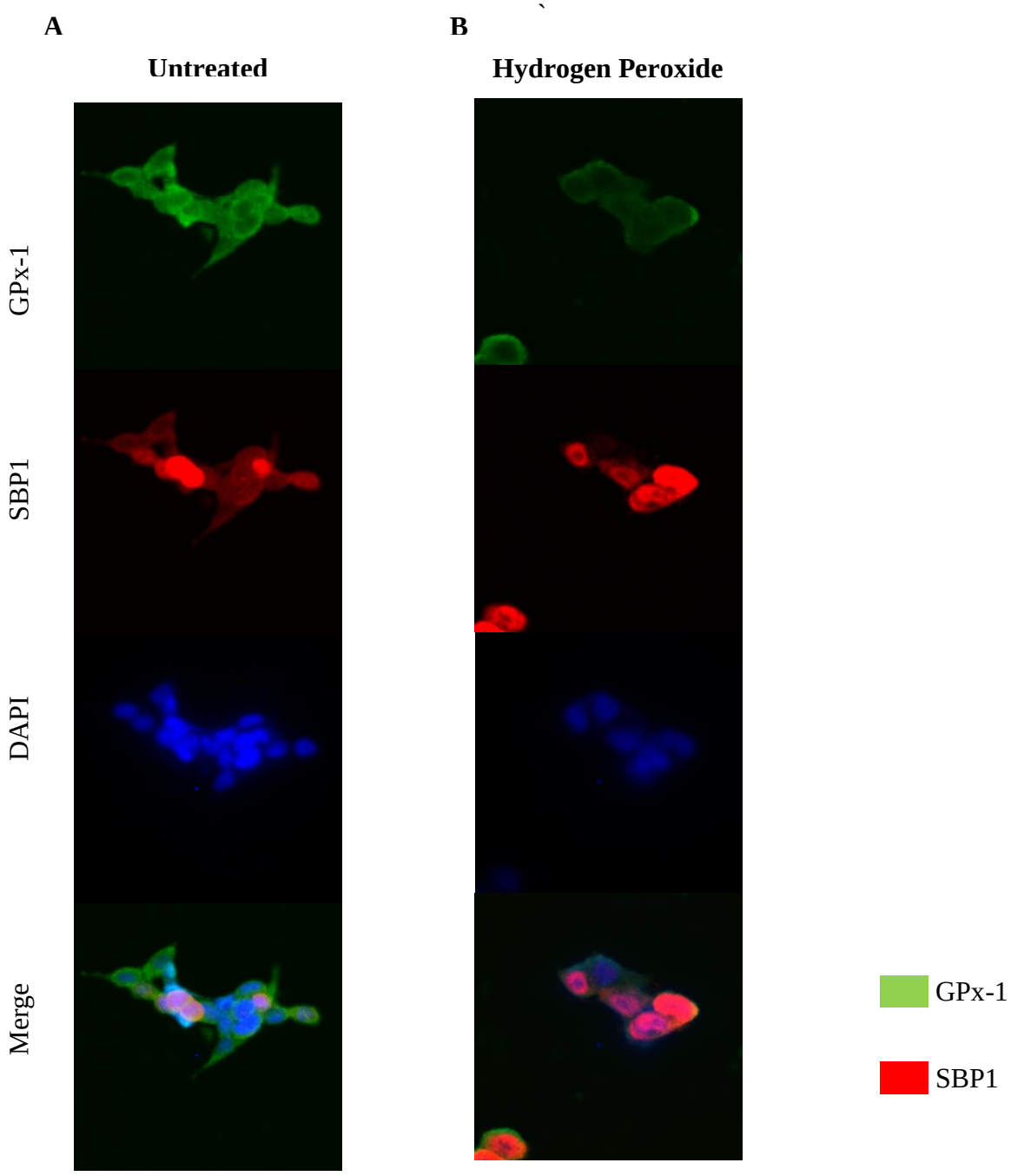

Figure 15. SBP1 is localized in the nucleus with or without hydrogen peroxide treatment in HCT116-TetSBP1 cells.

Immunofluorescence images of SBP1 and GPx-1 in HCT116 TetSBP1 cells. Cells were stained with anti-SBP1 monoclonal antibody (red) and anti-GPx-1 monoclonal antibody (green). Cells were cultured without hydrogen peroxide treatment (A), or treated with 50uM hydrogen peroxide for 10 minutes before fixation (B). DAPI is used as a nuclear marker.

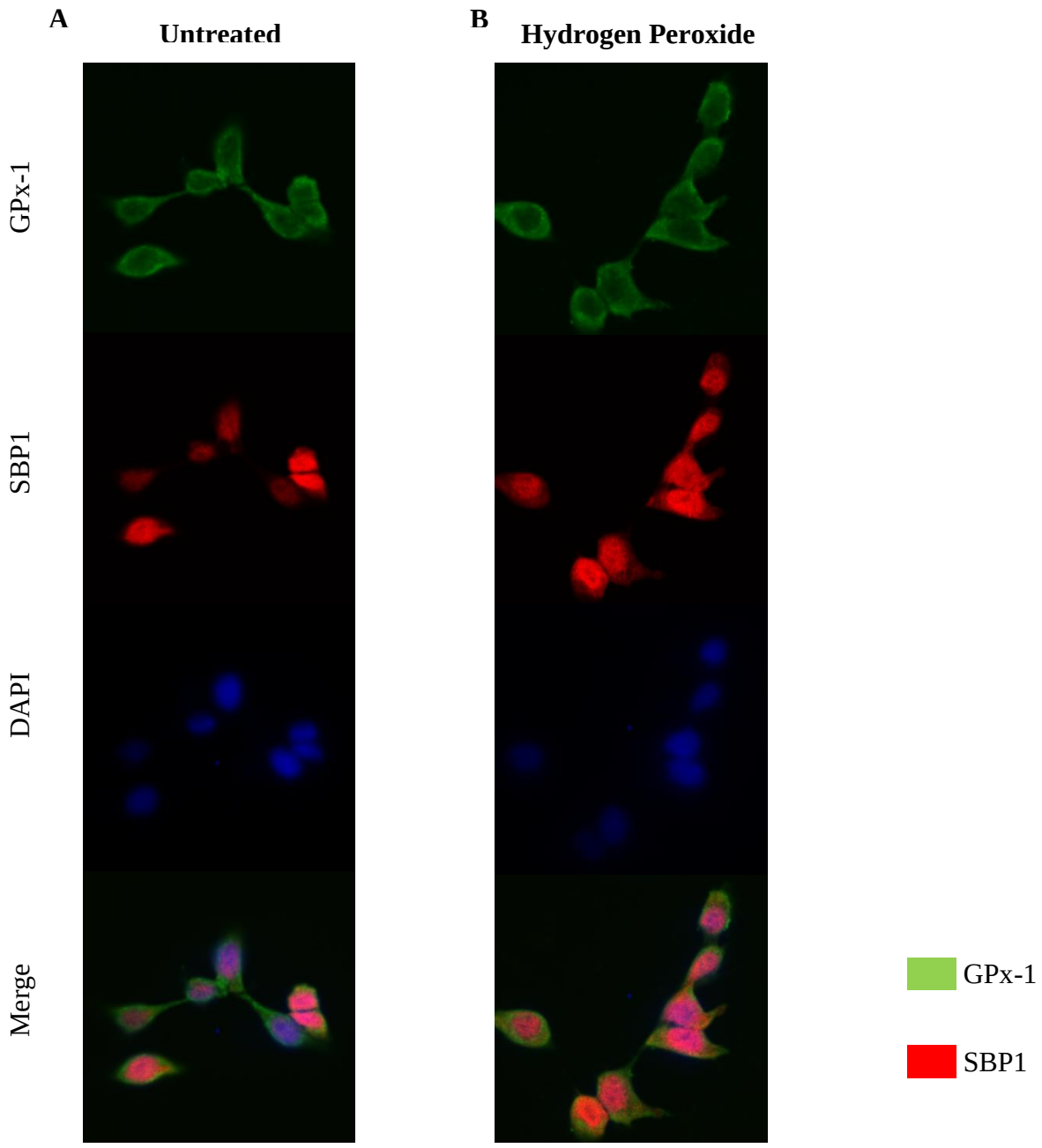

Figure 16. SBP1 is localized in the nucleus with or without hydrogen peroxide treatment in LNCaP cells.

Immunofluorescence images of SBP1 and GPx-1 in LNCaP cells. Cells were stained with anti-SBP1 monoclonal antibody (red) and anti-GPx-1 monoclonal antibody (green). Cells were cultured without hydrogen peroxide treatment (A), or treated with hydrogen peroxide for 10 minutes before fixation (B). DAPI is used as a nuclear marker.

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IV. Discussion

There were two major goals of this SBP1 study. The first was to gain an understanding of the function of SBP1 and the possible consequences of its frequent decreased expression in human tumors compared to normal tissue. We hypothesized that the loss of SBP1 allows tumors gain a growth or survival advantage. We explored the impact of SBP1 expression in cells either grown in soft agar, supplemented with selenium, or treated with 5-FUra. The second goal was to determine if the observation low SBP1 is a risk factor for poor outcome in lung, colon, breast, and gastric cancer, was an observation that could be extended to include prostate cancer. This was accomplished by determining a plausible association between SBP1 levels in prostate tissue and BCR using tissue samples from 338 patients whose cancer either did or did not recur after 15 years of post-radical prostatectomy follow up. The results of these studies have implications not only in the understanding of SBP1's function, but also in understanding of prostate cancer outcome.

A. The interaction between SBP1 & GPx-1 may be relevant to their function

1. Inverse relationship between the levels of SBP1 and GPx-1 is observed in ovarian cell lines

Using western blotting to determine SBP1 and GPx-1 levels in ovarian cell lines, we observed that they were inversely expressed in all six cell lines we examined (**Figure 1)**. Published studies have reported an inverse relationship between SBP1 and GPx-1 in HCT116, and SMMC7721 cell lines using ectopic expression or silencing of SBP1 and observing an inverse response in GPx-1 activity [16, 31]. Similarly, the levels of endogenous SBP1 in GPx-1 null MCF7 cells decrease in response to ectopic expression of GPx-1. The levels of SBP1 and activity of GPx-1 were also inversely associated in human prostate cancer tissue [32]. The

observation of this inverse relationship in colon, liver, and prostate [32] cells is now extended to include ovarian, showing that SBP1 and GPx-1's interaction is functional.

2. SBP1 half-life increases in the presence of GPx-1, indicating a likely physical interaction between the two proteins

The half life of SBP1 was measured to be 46 hours in MCF7 cells which are null for GPx-1, and 64 hours in MCF7-GPx-1 cells which express ectopic GPx-1 (**Fig. 2**). A previous report on the interaction between SBP1 and GPx-1 have shown that they colocalize in the nucleus of SMMC7721 cells after treatment with 50uM hydrogen peroxide using immunofluorescent antibodies [16]. Taken together with the data described above regarding their inverse relationship, it is likely that the extended half life of SBP1 in the presence of GPx-1 is due to the physical interaction between the two proteins which may decrease the exposure of SBP1 protein to degradation. Although we do not contribute evidence supporting a direct physical interaction between the two proteins, the increased half life of SBP1 in response to GPx-1 suggests that GPx-1 stabilizes SBP1 levels in the cell. This perhaps occurs because of SBP1 participating in a protein complex that requires GPx-1. Another possibility is that GPx-1 binding prevents SBP1 from interacting with other proteins, thus serving as a post-translational regulator for SBP1. Despite the lack of data regarding the nature of the physical interaction between SBP1 and GPx-1, these two selenium associated proteins are both likely relevant to cancer etiology, and their interaction may be an important aspect of their regulation.

3. The interaction between SBP1 and GPx-1 does not affect the nuclear localization of either protein

Using immunofluorescent confocal microscopy, we determined that SBP1 and GPx-1 do not colocalize in the nuclei of HCT116, or LNCaP cells (**Fig. 15, 16**). SBP1 is primarily nuclear in both cell lines, whereas GPx-1 was not detected in the nucleus in either cell type. As opposed

to the two cell lines we examined, a previous report indicated that in SMMC7721 cells nuclear SBP1 was not detected, indicating that SBP1 is not necessarily a nuclear protein, and that it may be located differently based on cell type. Additionally, absence of GPx-1 in the nuclei of HCT116 or LNCaP cells contradicts the observation made in SMMC7721 liver cancer cells where GPx-1 was nuclear following treatment of the cells with 50uM hydrogen peroxide [16]. In that study, GPx-1 did colocalize with SBP1 in the nucleus, but its localization did not require SBP1 as it was still found in the nucleus in H_2O_2 treated SMMC7221 cells whose SBP1 expression was silenced using siRNA [16] . The difference in localization of SBP1 and GPx-1 based on cell type and in response to hydrogen peroxide treatment indicates that the cellular location of SBP1 is likely important for its function. Nuclear proteins often function as gene regulators, whether interacting with DNA, RNA, or histones. Currently it is unknown if SBP1 affects gene expression, and the consequences of SBP1 nuclear localization also remain unknown.

The putative physical interaction between SBP1 and GPx-1 was not required to determine the location of either protein in SMMC7721 cells, in contrast to known regulatory interactions between other proteins, for example NF-κB and its regulator IkB. IkB binding to NF-κB sequesters the complex in the cytoplasm until IkB is phosphorylated and subsequently dissociates from NF-κB, allowing NF-κB to localize in the nucleus and bind its target DNA [74]. As shown above, SBP1 is heavily nuclear in HCT116 and LNCaP cells as well as in human prostate tissue, strongly suggesting that its presence in the nucleus is functional. Although the interaction between SBP1 and GPx-1 does not appear to determine either proteins location, the genotype of GPx-1 may participate in determining the location of SBP1. Recent work in our lab revealed that the *GPx-1* gene contains several polymorphisms which affect both its function and

location [22], (unpublished). Induction of GPx-1 levels in response to selenium supplementation differs based on its genotype, and given the likely ability of SBP1 to physically interact with GPx-1, it is possible that GPx-1 interacts with SBP1 differently based on genotype. Likewise, because the subcellular location of GPx-1 is partially affected by its genotype, the subcellular location of SBP1 may be affected similarly. Given the data shown above however, it is not likely that there is a direct physical interaction between SBP1 and GPx-1, and the way the two proteins interact is probably influenced by several factors.

A. Loss of SBP1 is likely to contribute to cancer progression and increases resistance to 5-fluorouracil

1. SBP1 decreases the ability of cells to form colonies in soft agar, but does not increase cell proliferation

Anchorage independent growth is the ability of adherent cancer cells to grow being attached to a culture dish, and is one of the major features of cell transformation *in vitro* characteristic functions as a model for the ability of tumor cells to eventually ability to invade and metastasize, which is a major hallmark of cancer [76]. In order to if SBP1 affects the ability of cancer cells to achieve anchorage independent growth, cells were grown with and without SBP1 in soft agar. Statistically fewer colonies were by cells with SBP1 than cells without (

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Figure 7). Although it is unknown how SBP1 decreases the efficiency of soft agar colony formation, SBP1 expression does not affect the proliferation of cells (

Figure 5), whereas the expression of other genes known to increase or decrease the soft agar colony growth of cancer cells typically affect the proliferation of those cells. For example, Phospholipase C-Epsilon 1 (PLC-E1) decreases the ability of SW620 colon cancer cells to form colonies in soft agar, but also decreases their proliferation. PLC-E1 is similar to SBP1 in that they both are frequently downregulated in colon tumors compared with normal tissue, however SBP1 differs from PLC-E1 due to its ability to decrease anchorage independent growth without affecting cell proliferation.

The proliferation of immortalized cancer cells *in vitro* can be increased or decreased by several mechanisms, and changes in cell proliferation often result from the perturbation of molecular pathways which affect proliferative signaling, cell differentiation, or apoptosis [77]. The ability of SBP1 to decrease anchorage independent growth without a corresponding decrease in proliferation is likely mediated through its ability to specifically attenuate the resistance of cells to apoptosis. Due to the nature of the soft agar colony formation assay, decreasing cell proliferation, whether through decreasing proliferative signaling, or by promoting apoptotic pathways, would result in an apparent attenuation of the colony forming ability of those cells. PLC-E1 expression increases apoptotic signaling in colon cancer cells, whereas SBP1 does not. While it is unknown how SBP1 attenuates anchorage independent growth without decreasing cell proliferation, its affect on that aspect of cell transformation likely has implications for cancer progression and metastasis of the many tumor types in which SBP1 expression is low compared to normal tissue.

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2. Loss of SBP1 confers resistance to 5-FUra through its effect on p53

SBP1 can alter anchorage independent growth of colon cancer cells, therefore impacting the transformed phenotype of those cells. In order to elucidate how SBP1 does this, we investigated SBP1's affect on p53, a major regulator of apoptosis, cell cycle, and DNA repair signaling [78]. Using an antibody against phospho-serine 15 on p53, a post-translational modification which increases p53 binding to its negative regulator MDM2, we observed increased SBP1 leading to increased phosphorylation a Ser-15 of p53 (**Figure 10**). This increase in phosphorylation was accompanied by the decrease in total p53, a result that sheds light on the likely purpose of the SBP1-dependent Ser-15 phosphorylation. Due to the two-phase dynamics of p53 in the cell cycle arrest/apoptosis mechanism, a moderate decrease in total p53 likely indicates that p53 levels have switched to that which is required for cell cycle arrest as opposed to apoptosis. It is of note that HCT116 cells do contain wild type p53 [79]. In addition, when the ability of p53 to activate a transcriptional reporter construct was examined in these cells, the increase in phosphorylation of p53 at Ser-15 did not increase the expression of the reporter plasmid, indicating the post translational modification does not increase the ability of p53 to bind to and activate the promoter region of the reporter plasmid. The impact of SBP1's ability to increase levels of p-Ser-15-p53 is unknown due to the lack of difference in proliferation or gene expression observed between cells induced to express SBP1 and SBP1-null controls. Likewise, the broad range of functions associated with p53 and its associated pathways make it difficult to speculate on the consequences of p53's interaction with SBP1 might be.

It is known, however, that the levels of p53 are not only dichotomously functional as stated above [80], but that the temporal fluctuation of its levels determine cell fate [69]. p53

levels increase and are suppressed repeatedly in response to DNA damage, a phenomenon which is partially mediated through phosphorylation at Ser-15. The oscillation of p53 level leads to cell cycle arrest, allowing the cell to repair the DNA damage. The amount of p53 oscillations are directly related to the extent of DNA damage, effectively giving the cell an appropriate amount of time in the arrest phase based on how much DNA repair needs to occur. When DNA damage is excessive, p53 oscillations characteristic of cell cycle arrest occur, but are followed by an increase of p53 levels several times above any previous cycle arrest spike, and the apoptosis pathway is activated. Total p53 levels in SBP1-null HCT116 cells are relatively high compared with levels seen in SBP1 expressing cells, suggesting that SBP1 may facilitate the switching of p53 from one phase to another, which may be why tumor cells gain an advantage by suppressing SBP1, particularly if those cells have previously obtained a mutation which inactivates one phase of the two-phase p53 cell cycle arrest/apoptosis mechanism.

One inherent limitation to our study is that we did not examine how SBP1 might affect the DNA damage repair mechanism described above. Given previous reports of SBP1's ability to increase apoptosis after ROS [37], SBP1 may function as a co-factor for the p53 oscillation mechanism, allowing it to function more effectively. The ability of SBP1 to attenuate resistance of cells to 5-FUra, a drug which causes DNA damage, strengthens the idea that SBP1 may play a role in the p53 DNA damage response pathway. It is important to note that it is unlikely that SBP1 "activates" or "deactivates" apoptosis or cell cycle arrest in response to DNA damage, but that its expression supports the function of the p53 DNA damage response through its ability to facilitate Ser-15 phosphorylation, which is a necessary post-translational modification for p53's functional oscillation.

B. SBP1 is predominantly nuclear localized, and its levels predict outcome of prostate cancer patients

1. Patients with prostate tumors that have low nuclear SBP1 are twice as likely to experience chemical recurrence

The association between low tumor SBP1 levels and poor patient outcome has been well documented, and a correlation exists for tumors of the lung, colon, and stomach. The need for more effective prognostic tools for prostate cancer has been described above, as well as in publications examining how the sensitivity of PSA testing often results in overtreatment of patients whose cancer is indolent or unlikely to be lethal [59]. Given the consistent observations made in numerous tissue types of SBP1 being lower in tumors than in normal tissue, as well as its association with outcome, we predicted that SBP1 levels are likely associated with recurrence in prostate cancer. Indeed, when prostate cancer patients in the CPCTR outcome TMA were separated into quartiles based on tissue SBP1 levels, patients in the lowest quartile of SBP1 were significantly more likely to recur than patients in the other three quartiles (**Table II**). This association was independent of PSA, Gleason score, or age at surgery.

Fifteen percent of prostate cancer patients who undergo radical prostatectomy (RP) experience biochemical recurrence (BCR) after 5 years [81]. Biochemical recurrence after RP is defined by detectable serum PSA, which if left untreated is most often followed by metastasis and death [82]. The fact that patients with low tumor SBP1 are at higher risk of BCR likely means that cells with low SBP1 are more capable of metastasis than cells containing higher SBP1 levels. Because the association between low tumor SBP1 and recurrence was independent of Gleason score, it is unlikely that SBP1 levels are predictive because of an inverse correlation between SBP1 and tumor progression. Alternatively, given SBP1-dependent phosphorylation of Ser-15 on p53, which may potentiate the DNA-damage response, it is possible that tumor cells

with low SBP1 are more likely to accumulate genetic mutations, increasing the chances that the acquire the ability to invade and metastasize. If loss of SBP1 occurs early in the natural history of an individual's tumor, the subsequent the loss of potency for p53 dependent DNA damage response may be the reason for the frequent observation that tumors with low SBP1 are likely more advanced and have worse outcome.

2. Sporadic SBP1 levels in prostate tissue and cell culture may be due to SBP1's post-translational regulation by the p53 cell cycle arrest/apoptosis oscillatory mechanism, or a change in the epitope recognized by the monoclonal antibodies used.

Repeated observations of sporadic SBP1 expression among cells *in vitro* and in human tissue have been shown above. Using fluorescent immunocytochemistry with antibodies against SBP1, we observed highly varied intensity of SBP1 among LNCaP and HCT116 cells grown in the same culture dish (**Figure 15**, **Figure 16**). Unexpectedly, even HCT116-TetSBP1 cells which were expanded from a single colony contained highly differing levels of SBP1 following doxycycline induction of the SBP1 gene under the control of a tet-responsive promoter. The phenomenon of SBP1's sporadic levels is not only interesting physiology; it occurs in human prostate glands as well, demonstrating that it is not merely an artifact of cultured cell lines. Considering the fact that SBP1 levels were sporadic regardless of whether the gene expressed was under an exogenous (HCT116-TetSBP1) or endogenous (LNCaP, human prostate) promoter, it is likely that SBP1 is regulated by a post-transcriptional mechanism.

Of the possible mechanisms responsible for regulating SBP1 levels, the previously discussed p53 cell cycle arrest/apoptosis dynamic is the most likely. SBP1 expression results in phosphorylation of Ser-15 on p53, therefore it is possible that the oscillation of SBP1 levels is

necessary for the oscillation of p53 levels that results in cell cycle arrest [69]. If this occurs, the sporadic SBP1 levels we observed in cell lines and human tissue indicate the current status of SBP1 oscillation when the cells were stained. Alternatively, the apparent difference in SBP1 levels may be a result of a change in the epitope recognized by the primary antibody we used for immunohistochemistry. SBP1 is known to be phosphorylated at an unknown location, and if the modification occurs in the region of the protein that our primary antibody recognizes, that may result in the antibody not being able to efficiently bind SBP1, subsequently causing the cells to appear as if their SBP1 levels are widely different, when the actual difference between the cells is the amount of modified SBP1. Even if this explanation is correct, however, it still remains possible that SBP1 is post-transcriptionally modified in an unknown way, and this modification is likely relevant to its function, as well as its importance to human cancers.

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V. Conclusion and future directions

SBP1 belongs to an unusual subcategory of selenoproteins which do not contain a selenocysteine, but tightly bind selenium. Given this characteristic of SBP1, a reasonable prediction is that it interacts with one or more other selenoproteins, whether physically or by changing the availability of selenium for incorporation into selenocysteine. The data provided by our studies do not establish a physical interaction between SBP1 and GPx-1, but the observation that SBP1 half-life is longer in cells containing GPx-1 compared to its half-life in GPx-1 null cells strengthens support for the occurrence of a physical interaction between the two. Although there are publications which have provided evidence for a likely physical interaction between GPx-1 and SBP1, there has not been any conclusive proof that such a relationship exists between the two proteins. SBP1 and GPx-1 co-localize in the nuclei of liver cells, [16] and co-immunoprecipitate from the lysates of colon cells [31], but neither of those experiments excludes the possibility of both proteins belonging to the same protein complex rather than interacting with each other directly.

Studying SBP1 is made difficult by the fact that *in silico* analysis of its amino acid sequence indicates that it does not contain any known conserved functional domains. Typically such domains would provide direction in studying the function of a protein and give insight into its likely binding partners. The lack of characterized domains presents not only an obstacle to learning the function SBP1, but also an opportunity to discover previously unknown functional domains through studying SBP1. For example, a recent study suggests that SBP1 binds von-Hippel Landau protein-interacting deubiquitinizing enzyme 1 (VDU1) [83] with greater affinity when SBP1 is also binding selenium [84]. Future studies in this vein will continue to shed light into the biochemistry of SBP1, which until now has been obscure.

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The fact that SBP1 expression leads to phosphorylation of Ser-15 on p53 is a finding that merits extensive further study. SBP1 being associated with VDU1, and GPx-1 does not provide much clarity into the mechanism behind SBP1's involvement with p53 function. The p53 oscillations that require Ser-15 phosphorylation involve periodic degradation of p53, which may involve the activity of VDU1. VDU1, however, has not been extensively studied which makes it difficult to speculate on the likelihood of an interaction between VDU1, SBP1, and p53. SBP1's likely supportive role in the p53 DNA damage response pathway supports the large body of publications indicating that SBP1 expression decreases the degree of transformation in cells. While the details of the interaction are unknown, SBP1s interaction with p53 may contribute to the reason why prostate cancer patients with tumors expressing SBP1 are over 50% less likely to recur after radical prostatectomy. Although SBP1 may not be developed as a clinical biomarker of prostate cancer recurrence, investigating why low levels of SBP1 is predictive of recurrence may lead to further understanding of patient response to therapy, as well as important molecular changes occurring in tumors during progressing to more malignant stages.

The data presented in this dissertation represent a part of our ongoing work in understanding the biology of selenium and selenoproteins, as well as elucidating how this subset of the human proteome affects the pathobiology of cancer. SBP1, as a member of the family of selenium containing proteins, will be increasingly integrated into the historical narrative of selenoproteins as its function is further understood. It's unique physiology in human cells and its involvement in the response of cells to DNA damage, as well as the implications its expression has on human cancers, will continue to make it an attractive subject of study, leading to greater understanding of not only SBP1, but all the proteins and pathways it interacts with.

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