The Bca2 And Ampk Paradigm: Unraveling The Cancer Connection

Daniela (buac) Ventro
Wayne State University,

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DEDICATION

The work, tears and laughter that went into this dissertation are dedicated to my original mentor, Dr. Angelika M. Burger, who lost her own personal battle to a very rare form of cancer in May of 2011. As the discoverer of BCA2, her love for science and meticulous dedication to her work has instilled in me the motivation to also continue along the academic route. In addition to being my mentor, she was also a friend and touched my life in many ways, both personally and professionally.

All of my experimental success and failure throughout this process is dedicated to my now husband, George Ventro Jr., who has been by my side since I began this journey and very tolerantly listened to my daily frustrations and accomplishments. Your patience, words of encouragement and hugs of comfort were very much appreciated and there when I needed them most. Your intelligence and ambition to follow your dreams, I have always admired, and it has inspired me more than you’ll ever know.

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PREFACE

The purpose of this dissertation is to define the relationship between the newly discovered ubiquitin E3 ligase, Breast Cancer Associated Gene 2 (BCA2), and the master regulator of cellular energy metabolism, AMP-activated protein kinase (AMPK), in a breast cancer context. Negative regulation of AMPK by BCA2, including the proposed limitation of metformin use in the treatment of cancer and all that follows in the results section, has never before been reported. It is my hope that the discoveries made within this body of work provide new grounds for the basis of developing specific inhibitors of BCA2 for the treatment of breast cancer that may one day translate into the clinic.

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CHAPTER 1
INTRODUCTION

Breast Cancer

Breast cancer is the most commonly diagnosed cancer type in women, with an estimated 232,340 new invasive cases in 2013 (American Cancer Society, 2013). Following the 2002 study from the Women’s Health Initiative that linked the use of combined estrogen and progestin for menopausal hormone therapy (MHT) to an increased risk of developing breast cancer, a 7% decrease in incidence rates was seen and can be attributed to cutbacks in this treatment (Rossouw et al., 2002). However, the most recent five year data available, from 2005 to 2009, suggests incidence rates to be fairly steady (American Cancer Society, 2013). As the second most commonly diagnosed cancer type in women, breast cancer comes in second to lung cancer for cause of cancer death taking roughly 39,620 fated lives in 2013. Although death rates have steadily decreased in women over the last 25 years or so, presumably due to a decrease in MHT, earlier detection, and better treatment options, breast cancer still remains at the top of the list for both incidence and mortality, indicating a continued need for novel detection methods, prognostic biomarkers and treatment options. In the clinic it is currently treated locally with surgery plus/minus radiation and systemically with cytotoxic chemotherapy, hormonal therapy, immunotherapy and targeted therapy (National Cancer Institute, 2013; Rakha, 2013). Surely these treatment options provide many survival benefits to patients, but they also include a large category of adverse side effects related to toxicity that significantly affect the patient’s quality of life. The clinical decision of which treatment course to pursue is based on several tumor descriptive factors: histopathology (ductal or lobular), grade (tumor cell appearance vs. normal
breast), stage (TNM system), and receptor status (estrogen receptor (ER), progesterone receptor (PR) and HER2) (National Cancer Institute, 2013; Rakha, 2013). Although cytotoxic chemotherapy is still the most commonly chosen route, targeted therapy has become a major component of many treatment regimens. Drugs like tamoxifen and Herceptin for the treatment of ER and HER2 positive cases, respectively, has not only revolutionized the management of this group of cancers, but also the outlook for future application of novel targeted therapies in the treatment of breast cancer.

**Ubiquitin-Proteasome System**

The ubiquitin-proteasome system (UP-S) is responsible for about 80% of all protein degradation in the cell, with the lysosome being accountable for the other 20% (Ciechanover et al., 2000; de Duve, 2005). Proteasome degradation is essential for the progression of all normal cell processes as it removes misfolded, damaged, and toxic proteins that may otherwise be harmful to the cell. However, a look back through history tells us that the proteasome was not always accepted in this role. In the early 1950s the lysosome was discovered as a proteolytic organelle and the proteases within this compartment were thought to be primarily responsible for all intracellular proteolysis (De Duve et al., 1955). Then, in the 1980s, reports of an entirely new and non-lysosomal protein degradation system surfaced. The accumulated evidence suggested this system to be very specific and dependent on a novel post-translational phenomenon termed polyubiquitination. The discovery of the UP-S (figure 1) is accredited to Aaron Ciechanover, Avram Hershko and Irwin Rose; their work was acknowledged with the 2004 Nobel Prize in Chemistry.
Figure 1. The Ubiquitin Proteasome System

Ubiquitin is activated by the ubiquitin activating enzyme (E1) and requires ATP. It is then transferred to the ubiquitin conjugating enzyme (E2). In the presence of an E3, ubiquitin is transferred to a specific protein substrate and targeted for degradation by the proteasome. The protein is chopped into small peptide fragments and the ubiquitin recycled.
As a large multi-subunit protease, the 26S proteasome was described to contain a ring-shaped 19S regulatory lid and a 20S catalytic core component, further made up of polypeptide subunits (Arrigo et al., 1988; Gerards et al., 1998; Hoeller and Dikic, 2009). The 20S core is composed of two identical outer and inner rings each having seven α and seven β subunits, respectively; together they are arranged in a stacked cylindrical structure with a narrow pore. It is through this pore that specific protein substrates pass before they are ultimately degraded (Gerards et al., 1998). The proteasome is ubiquitously expressed and can account for up to 1% of total protein content in the cell (Arrigo et al., 1988; Ciechanover et al., 2000; Gerards et al., 1998). Furthermore, it is essential for many key cell regulatory mechanisms such as, cell cycle, transcription, DNA repair and protein transport. It is no surprise that modulating its function seemed to at first be an intangible strategy when considering novel molecular targets in the treatment of cancer (Glickman, 2000; Goldberg et al., 1995; Groll et al., 2009). However, proteasome inhibitors have been developed, have proven to be relatively cancer cell-specific and as a result, in the last decade, have translated into the clinic. Bortezomib, the first FDA approved proteasome inhibiting anticancer agent used for the treatment of relapsed multiple myeloma and mantle cell lymphoma, is the field’s prototype. Carfilzomib, a second generation product, was FDA approved (2003) for patients with multiple myeloma progression while on, or after treatment with, bortezomib and an immunomodulatory agent. Furthermore, proteasome inhibition has been shown to be a valuable strategy in the sensitization of cancer cells to effects of traditional chemotherapies and radiation (Buac et al., 2012). While bortezomib has achieved some success in the treatment of hematological malignancies, it has produced only disappointment in solid tumor cases. But if we continue to look within the confines of the UP-S,
we find a rich supply of other promising molecular drug targets just upstream of the proteasome. The remarkable level of specificity achieved by this system after all, is accredited to the series of events that actually precede the proteasome’s activity, the process of polyubiquitination (Burger and Seth, 2004; Glickman and Ciechanover, 2002; Nalepa and Wade Harper, 2003; Weissman, 2001).

Ubiquitination is a sequence of ATP-driven events that involves the activation and transfer of ubiquitin to particular protein substrates (figure 1); it is dependent on three different groups of enzymes: the ubiquitin-activating enzyme(s) E1, ubiquitin-conjugating enzymes E2, and the ubiquitin E3 ligases, which are present in a hierarchal nature. For a while only one E1 enzyme was known to exist, however there are now reports of at least one other E1 in the human genome and an estimated 55 E2 conjugating enzymes and 500 E3 ligases. E1 mediates the ATP-dependent transfer of ubiquitin to an E2 and the latter either directly transfers it to the substrate, in the presence of an E3, or to an E3 that then ubiquitinates the specific protein substrate, which may be itself (autoubiquitination) (Handley-Gearhart et al., 1994; Jin et al., 2007; Nandi et al., 2006). Generally speaking, polyubiquitinated proteins at the lysine 48 residue are degraded by the 26S proteasome. Ubiquitin chains can also form on alternative lysine residues, like lysine 63 and other non-traditional sites, in which case regulatory functions are carried out such as, protein transport, regulation of DNA repair and translation (Kimura and Tanaka, 2010; Komander and Rape, 2012; Mukhopadhay and Riezman, 2007; Pickart and Eddins, 2004). Efficient and targeted ubiquitination of the UP-S therefore depends on the E3 ligases and an imbalance in their activity and expression has been extensively reported to play a role in the pathogenesis of breast cancer (Burger et al., 2006; Chen et al., 2006a; Ohta and Fukuda, 2004; Sun, 2006).
are typically classified according to structure domain homology, of which the HECT (Homologous to E6-associated protein C Terminus)-type and adaptor type are possible (figure 2). The adaptor type E3s can further be broken down into those that contain a RING (Really Interesting New Gene)-finger, a U-box or PHD domain (Ardley and Robinson, 2005; Dikic and Robertson, 2012; Hatakeyama and Nakayama, 2003; Metzger et al., 2012). While the PHD and U-box E3s are similar in structure to the RING-finger E3 ligases, in a breast cancer context, the RING-finger E3s are the most well characterized of the group.
In total, about 500 E3 ligase enzymes exist in the human genome. They are broken down into either the HECT type or Adaptor type. The latter is further classified by domain homology into the RING, U-Box or PHD groups. Also shown is a cross-section of the RING CH or H2 domain, complexing two zinc ions critical for E3 ligase activity.
RING-finger containing E3s make up about 230 of the known 500 genes and are classified as either RING-HC-type (C3HC4) or RING-H2-type (C3H2C3), with the cysteine (C) and histidines (H) representing zinc binding residues that form a sort of cross-brace structure in which two zinc ions are complexed within (figure 2). The RING domain can either be a part of a multisubunit complex, such as the SCF (Skp1/Cullin-1/F-box) protein, or be simply monomeric, like Mdm2 (Mouse double minute 2 homolog) (Deshaies and Joazeiro, 2009). The RING domain is critical for E3 ligase activity and first became the target for inhibition by a series of zinc ejecting compounds first developed by the National Cancer Institute (NCI) in an effort to uncover new drugs for the treatment of human immunodeficiency virus 1 (HIV-1). The nucleocapsid protein, p7, is essential to the development of the virus and encompasses a highly conserved cysteine/histidine-rich retroviral Zn-finger domain, from which zinc could be ejected and prevent its function (Rice et al., 1993; Rice et al., 1997b).

RING-finger E3 ligases are typically tightly controlled, but tend to have a deviant nature in a breast cancer setting as they regulate the turnover of key tumor suppressor proteins and oncogenic receptor tyrosine kinases. For example, the E3 ligases that degrade tumor suppressor proteins p53, p27, and 14-3-3σ (Mdm2/Hdm2, SCF and Efp respectively), are often overexpressed; and those that degrade oncogenic receptor tyrosine kinases, like c-Cbl, are deregulated (Burger et al., 2006; Dong et al., 2003; Fang et al., 2000; Honda et al., 1997; Jia and Sun, 2011; Kubbutat et al., 1997; Miura-Shimura et al., 2003; Talis et al., 1998). That RING-finger E3 ligases are a class of “druggable” anti-cancer targets has also been reported. Most notably, trastuzumab (Herceptin) has been reported to induce c-Cbl mediated degradation of HER2. Efforts placed on inhibiting Mdm2 activity have culminated in the development of some
small molecule inhibitors that either directly inhibit its activity or interaction (Nutlin) with p53 (Klapper et al., 2000; Secchiero et al., 2011). These examples provide evidence of the fact that the door is open to E3 ligase therapeutic intervention in cancer. Along these lines, the optimal cancer drug target should: i.) be minimally expressed in normal cells, but overexpressed in cancer cells and associated with poor prognosis, ii.) play an essential role in maintaining and/or promoting the cancer cell phenotype, iii) be an enzyme or cell surface molecule that can be easily targeted, iv.) result in either inhibition of cell growth or promotion of cell death upon inhibition of its activity and/or expression (Gashaw et al., 2011). The overall goal corresponding to the above criteria is to achieve a maximal therapeutic effect to toxicity ratio. One such target that meets most of these precedents is also a RING-finger ubiquitin E3 ligase, termed BCA2 (Breast Cancer Associated Gene 2).

BCA2

The partial BCA2 cDNA sequence was initially called Di12 and cloned from a subtractive hybridization library, derived from Hs578T and Hs578Bst matched invasive and normal breast cell line mRNAs in the laboratory of Dr. Arun Seth by Dr. Angelika Burger at the University of Toronto, ON (Burger et al., 2003). The full length BCA2 gene was later cloned from the human breast cancer cell line MDA MB 468 and after the release of the complete human genome sequence in the year 2000, was identified to be an exact match of the hypothetical zinc finger protein 364 (Genebank accession no. NM_014455). BCA2 maps to chromosome one (1q21.2), has 9 exons, mRNA is 1927 base pairs long, and is synonymous to T3A12/ZNF364/Rabring7(Burger et al., 2005). The BCA2 protein is a RING-H2-type ubiquitin
E3 ligase, contains an AKT/14-3-3 binding site, as well as a zinc-finger and RING-finger domain near its C-terminus (figure 3).

![Figure 3. Schematic of BCA2 (T3A12/ZNF364/Rabring7)](image)

Shown are the 9 exons of BCA2 and its three respective domains. An N-terminal zinc finger domain and a C-terminal RING-finger domain are shown as well as the AKT phosphorylation site.
Both the Zn-finger (BZF domain) and RING finger domains bind zinc ions and are capable of binding ubiquitin, however the RING domain is the protein-interacting domain, which is also required for its autoubiquitination activity (Amemiya et al., 2008; Burger et al., 2005). The unique ability of E3 ubiquitin ligases to autoubiquitinate made it possible to identify BCA2 without the knowledge of its substrates. An initial yeast two-hybrid screen performed in the Seth lab identified several potential candidates, among which several have been confirmed as binding partners of BCA2 and include: ubiquitin, Rab7, UBC9, 14-3-3σ, tethrin, and hHR23a. However, none of which have been confirmed as BCA2 substrates (Bacopulos et al., 2012; Miyakawa et al., 2009). Only recently has the cell cycle progression regulator, p21, been identified as its first substrate (Wang et al., 2013). This may in part explain how BCA2 contributes to breast cancer cell proliferation, at least in the cell lines studied by Wang et al. In addition, members of the UbcH5 group of E2 conjugating enzymes were found to be active, and be required for, BCA2 ubiquitination, with UbcH5b being most significant (Amemiya et al., 2008). Other E2 enzyme contributors are also likely. Ubiquitination of proteins occurs on lysine residues and BCA2 contains six, present in twos. One set is located near the N-terminus, another near the COOH terminus and the final in the middle portions of the protein. The lysine residues determined to be responsible for its ubiquitination are K26 and K32, located in its BZF domain at the N-terminus, as mutations in both of these residues was found to be required for a complete loss of BCA2 ubiquitination (Amemiya et al., 2008).

Evidence continues to accumulate that ubiquitin ligases influence the development of cancer, particularly due to their regulation of the majority of proteins in the cell including, growth factors, hormones and those controlling cell death or survival decisions. A breast tissue
microarray taken from the Henrietta-Banting Breast Cancer Collection (HBBCC) (Sunnybrook Health Sciences Centre, Toronto) revealed overexpression of BCA2 in more than half of the 945 invasive samples, compared to normal tissues. This demonstrated that this novel E3 ligase must have a distinct role in breast cancer (Burger et al., 2005). Furthermore, BCA2 has been linked to breast cancer cell proliferation in vitro, and correlates with patient regional reoccurrence history, making it a good target for therapeutic intervention (Burger et al., 2005). The BCA2 protein is localized to both the nucleus and cytoplasm of breast tumor cell lines, and the BCA2 promoter was recently shown to be estrogen responsive (Kona et al., 2012). The function of cytoplasmic BCA2 remains to be determined, although one can postulate that the action of this form may correspond to its endemic role, as any BCA2 found in normal tissue primarily localizes to the cytoplasm. Much of what is known and published on BCA2 contributes greatly to our understanding of how this protein is regulated. However its downstream targets, of which only p21 has thus far been confirmed a substrate, would provide the substantial evidence required to directly link its expression and activity to overall cancer cell survival. This work provides new evidence of the role BCA2 plays in the regulation of cellular metabolism; by negatively affecting AMP-kinase activity, thereby promoting breast cancer cell growth and compromising the effect of the now popularly studied AMP-kinase activator, metformin (Buac et al., 2013). Although AMP-kinase is not the direct target of BCA2 E3 ligase activity, TiO₂ phosphopeptide enrichment followed by mass spectrometry, before and after BCA2 knockdown in breast cancer cells, has revealed several promising targets in this regard. Furthermore, the characterization of BCA2 protein expression patterns, localization and cell cycle dependence is further explored within this dissertation and has even been extended to prostate cancer cells. All of this work amounts to one
supporting conclusion: BCA2 is not only a novel target for therapeutic intervention, but may be a valid biomarker to better predict which patients will respond to particular cancer prevention and/or treatment modalities.

As previously mentioned, the use of zinc-ejecting compounds for the inactivation of zinc-finger/RING-finger containing proteins has widely been reported and such agents comprise three major classes: disulfides,azoics, and nitroso aromatics (Beerheide et al., 1999; Burger and Seth, 2004; Nash and Rice, 1998). Chemical inhibition of BCA2 enzymatic activity has been reported and exploring such use would not only minimize the dependence on siRNA transfections to study its cellular functions, but also potentially provide new therapeutic options (Brahemi et al., 2010). After an initial screen of compounds from the NCI Developmental Therapeutics Program Central Drug Repository, disulfiram (Antabuse), a known zinc-complexing compound, was found to inhibit BCA2 autoubiquitination, comparable to that of the genetically induced cysteine mutations in the Zn$^{2+}$ complexing RING-H2 domain (Rice et al., 1997a).

**Disulfiram and Cancer**

Disulfiram (DSF, Antabuse®) (figure 4) has been an FDA approved agent for the treatment of alcoholism since 1951, but has a coincidental origin of discovery. It was initially developed by Medicinalco, a Danish drug company, in the later part of the 1940s for the treatment of parasitic infections.
Figure 4. Chemical structure of disulfiram (DSF)

The chemical structure of the zinc-ejecting compound, DSF, is depicted.
However, several employees testing it out on themselves expressed unpleasant hangover-like side effects, including nausea, vomiting, fatigue, and stomach irritation if combined with alcohol intake. As an anti-parasitic agent, the thiuram structure of DSF was found to act against the zinc ions contained within the RING-finger like surface proteins of the *Giardia lamblia* protozoa (Nash and Rice, 1998). Another unrelated protein enzyme with zinc binding ability is the hepatic aldehyde dehydrogenase (ALDH). ALDH1 is critical for the last step of ethanol metabolism that results in the conversion of toxic acetaldehyde to acetic acid (Agarwal and Goedde, 1987; Vallari and Pietruszko, 1982). It is the build-up of acetaldehyde in the blood after alcohol consumption that is responsible for the undesirable hangover symptoms those Danish scientists reported. Those DSF associated side effects can now be attributed to the drug’s ability to irreversibly inhibit ALDH1 activity in the liver, via zinc ejection. Therefore, it certainly serves its purpose in treating patients with chronic alcohol addictions as its effect is almost immediate, causing a 5-10 fold increase in acetaldehyde concentration, should you even have just one drink. DSF is otherwise well tolerated and usually prescribed at an initial dose of 500 mg/day for 1-2 weeks, followed by a maintenance dose of 125-500 mg/day (Johansson, 1992).

That DSF has anticancer activity was reported as early as the 1960s and to date there are over 200 publications showing DSF activity in various cancer types (Cen et al., 2004; Chen et al., 2006b; Cvek, 2011; Kona et al., 2011; Lovborg et al., 2006; Wickstrom et al., 2007). Most notably, in the 1970s Wattenberg published several studies that led to further investigation of DSF use as a chemopreventative agent, noting that if added to the diet of mice, DSF inhibited large bowel and forestomach cancers, induced by dimethylhydrazine and benzo[a]pyrene carcinogens, respectively (Borchert and Wattenberg, 1976; Wattenberg, 1975). During the next
decade, reports surfaced that DSF, amongst other ALDH inhibitors, could restore sensitivity of leukemia cell lines to oxazaphosphorines. (Sladek and Landkamer, 1985). In addition, in an in vivo leukemia mouse model, pretreatment with DSF significantly potentiated the cytotoxic effects of the alkylating agent, nitrogen mustard (Valeriote and Grates, 1989). Our past in vitro and in vivo experiments using a MCF7 breast cancer model studying the effect of DSF in combination with cisplatin indicated a synergistic effect between the two drugs, significantly enhancing the antitumor activity of cisplatin with combination indices (CI) below 1μM at the inhibitory concentration 50% (IC50) (Kona et al., 2011). Also impressive are the phase I and II clinical trial results studying this combination in 10 patients, in which no toxicity to the kidneys was reported and the antitumor response of cisplatin (at 50-120mg/m²) was not compromised (Qazi et al., 1988). Patients actually found relief of nausea and vomiting symptoms commonly associated with cisplatin use, suggesting a protective effect of DSF. These results are also echoed in another larger clinical trial of 53 patients with cisplatin sensitive malignancies, however the overall survival benefit of DSF inclusion to standard chemotherapy regimens remains unclear (Verma et al., 1990).

A plethora of molecular mechanisms exists in the literature to explain DSF’s antitumor activity in a spectrum of cancer types such as breast, prostate, hepatic and blood, including: inhibition of NFκB, AP-1, Bcl-2, P-glycoprotein efflux pump (P-gp), and key DNA methylating enzymes; G1/S phase arrest and upregulation of the tumor suppressor protein, p53, have also been reported (Lin et al., 2011; Liu et al., 1998; Loo and Clarke, 2000). Furthermore, DSF also has proteasome inhibition ability, including associated TNFα induced NFκB translocation inhibition and cytotoxic effects (Lovborg et al., 2006). The desirable antitumor consequences
found with its use could be achieved at concentrations comparable to that of the clinically approved proteasome inhibitor, bortezomib, and its cytotoxic effects were attributable to the initiation of apoptosis as measured by caspase -3/7 activation, nuclear fragmentation and cell membrane permeability (Lovborg et al., 2006; Wickstrom et al., 2007). The means by which DSF can inhibit the proteasome is at least in part due to its ability to inhibit the chymotrypsin (CT)-like activity of the proteasome (Chen et al., 2006b). In this study they show that a DSF-copper complex can inhibit proteasomal activity in cultured breast cancer MDA MB 231 and MCF10DCIS.com cells, but not in the normal MCF10A. Furthermore, cadmium (Cd)-DSF complexes were also explored (Li et al., 2008). The results reported also indicate inhibition of the proteasome and induction of apoptosis via inhibition of CT-like activity in breast cancer cells. Similar to the DSF-copper complexes, this proteasome inhibition was associated with an accumulation of ubiquinated proteins and the proteasome substrate p27, leading to apoptosis. DSF’s metal complexing and proteasome inhibitory qualities were also tested in melanoma cells where it was found to more potently reduce cyclin A expression and cell proliferation in combination with copper or zinc than DSF alone (Brar et al., 2004). It also inhibited growth and angiogenesis in melanomas transplanted in severe combined immunodeficient (SCID) mice, which was potentiated by zinc supplementation (Brar et al., 2004). Encouragingly, a patient with stage IV metastatic ocular melanoma achieved clinical remission with its use and remained on oral zinc gluconate and DSF maintenance for 53 continuous months with few side effects (Brar et al., 2004). Such promising data have culminated in phase I II and III clinical trials assessing DSFs antitumor activity in metastatic melanoma and solid tumors of the lung and liver. The phase I/II trial in metastatic melanoma (NCT00256230) and phase II/III studies in non-small cell
lung cancer (NCT00312819) have recently been completed and a report of the results is pending. Furthermore, the phase I study at the Huntsman Cancer Institute in Utah (HCI 26679) is still ongoing.

**Disulfiram and E3 Ligases**

As briefly previously mentioned, the critical role of zinc fingers and their development as potential therapeutic targets was first recognized by the NCI, specifically in the advancement of novel drugs for the treatment of HIV-1, via targeting the zinc ions in RING-finger E3 ligases (Rice et al., 1993; Rice et al., 1997b). These efforts produced a series of compounds that specifically modified the highly conserved cysteine/histidine-rich retroviral Zn-finger domains of the HIV-1 nucleocapsid protein, p7, by ejection of zinc and therefore inactivation of its essential activity for virus replication. One such agent, azodicarbonamide, even entered clinical trials against acquired immunodeficiency syndrome (AIDS), pointing to the translational potential of drugs with such protein modifying mechanisms (Rice et al., 1997b). Another representative of this type of drug class with similar capabilities in a cancer setting is 4,4-dithiodimorpholine (C16). C16 has been shown to remove zinc from zinc-finger domains critical for E6 function, the HECT-type E3 ligase responsible for the degradation of p53 in cervical cancer. It selectively reduced cell viability, associated with higher levels of the p53 protein (Beerheide et al., 1999). Several hundred zinc-fingers and RING-fingers exist in the human genome and thus the concern for pleiotropic effects is valid, however, the literature suggests that unique primary sequences and secondary protein structures of RING-finger E3 ligases that depend on zinc for catalytic activity could be specifically inhibited by such agents discussed herein (Burger, 2009; Deshaies and Joazeiro, 2009).
It has been shown that DSF could inhibit recombinant BCA2 and BCA2 expressing cells specifically in the sub-micromolar range, while the p7 HIV-1 nucleocapsid zinc-ejecting agent, NSC667089, could not (Beerheide et al., 1999; Brahemì et al., 2010; Burger et al., 2005; Kona et al., 2011). Furthermore, the TSQ (N-(6-methoxy-8-quinoyl)-p-toulenesulfonamide) zinc release fluorescence assay using recombinant BCA2 confirmed DSF’s capability of removing zinc ions from BCA2 (Kona et al., 2011). However, because of the multiple modes of action of DSF, analogs were designed based on the structural requirements for inhibition of BCA2, with the hope of increasing selectivity for BCA2 zinc-ejection and have since been tested (Brahemi et al., 2010). From the structure activity relationship (SAR) analysis, we found that a N(C=S)S-S motif was required for specific inhibition of BCA2 and notably two DSF analogs, ANFD24 and DPT001, were found to potently (submicromolar range) inhibit BCA2 autoubiquitination (figure 5) (Brahemi et al., 2010). Based on our SAR analysis, 77 compounds containing the N(C=S)S-S were received from the NCI and have since been screened. These data are discussed in Chapter 4. Therefore, although DSF can inhibit specific E3 ligases such as BCA2, its chemical structure elements can also be used as a basis for SAR studies in the identification of more selective E3 ligase inhibitors.
Figure 5. Chemical structures of DSF analogs

ANFD24 and DPT001 contain the N(C=S)S-S motif (circled) found to be required for inhibition of BCA2 autoubiquitination and breast cancer cell growth, compared to the inactive D23 compound.
AMPK and cancer cell metabolism

That cancer cells exhibit an altered metabolism was most notably recognized by Otto Warburg almost a century ago. He noted their preference of glucose breakdown via glycolysis over oxidative phosphorylation, even in the presence of oxygen, and in 1956 suggested it to be a cause of cancer development (Warburg, 1956). However, whether or not this phenomenon is a cause or symptom has been debatable and only recently has altered cancer cell metabolism been acknowledged as one of the hallmarks of cancer (Hanahan and Weinberg, 2011). Why would cancer cells prefer such an inefficient energy producing process when oxidative phosphorylation may be an available alternative? The tumor cell mitochondria must be defective. But they are not. In fact recent evidence suggests them to be intact and glucose oxidative phosphorylation to be functioning normally (Cantor and Sabatini, 2012; Pollak, 2012; Ward and Thompson, 2012). Yet still, neoplastic cells preferentially choose to undergo vigorous amounts of anaerobic metabolism. The answer is deeper than the ATP energy produced by either of these processes. Anaerobic metabolism also provides the necessary biomaterials required for proliferation, and therefore tumor growth, such as, nucleic acids, lipids and proteins (Cairns et al., 2011; Vander Heiden et al., 2009). In this way the Warburg effect is complicated far beyond what he initially described, in that tumor cells are not only capable of reprogramming glucose metabolism, but also amino acid, lipid and protein breakdown. Also required for the successful reprogramming of glucose metabolism is the suppression, or re-activation, of key metabolism related genes and/or proteins. Many oncogenes have been reported to take part in this movement such as, MYC, KRAS, AKT1, SRC, and BCR-ABL (Cairns et al., 2011; Dang et al., 2009; Munoz-Pinedo et al., 2012; Ying et al., 2012; Yuan and Cantley, 2008). But it is a key tumor
suppressive protein, and master regulator of cellular energy homeostasis, that has taken up much of the limelight in recent years and has become an attractive anti-cancer drug target. It is adenosine monophosphate-activated protein kinase (AMPK), whose function in normal cells is to respond, much like the flip of a switch, to drops in the ATP:AMP ratio and thereby restore cellular energy balance. AMPK is a heterotrimeric enzyme consisting of an α catalytic subunit and βγ regulatory subunits (figure 6); it was purified and sequenced in 1994 (Woods et al., 1996).
Figure 6. Schematic of the heterotrimeric AMPK enzyme.

AMP levels in the cell rise when ATP is low. As a result, AMP binds to the \( \gamma \) subunit leading to the phosphorylation of the \( \alpha \) subunit by an upstream kinase, triggering a conformational change to activate the enzyme.
Adding to its structural complexity, various isoforms of each subunit also exist; two α subunits, two β subunits and three γ subunits with twelve possible combinations that are differentially expressed and activated across specific tissue types (Carling, 2004). The AMPK α1 and α2 subunits contain approximately 550 amino acid residues and are highly conserved, the β subunits differ in the first 65 of about 250 residues, but are otherwise also similar (Steinberg and Kemp, 2009). However, the γ subunits vary in length at their NH$_2$ terminal: 331 residues in γ1, 569 in γ2, and 489 in γ3, but share a conserved approximate 300 residues at the COOH-terminus (Steinberg and Kemp, 2009). α1β1γ1 is the most widely distributed isoform of AMPK across various rat tissues, and is the most commonly studied in terms of cancer, while α2β2γ2 is more abundant in rat skeletal muscle.

Cellular ATP levels are required to be at high concentrations in order to ensure cell survival (Nelson et al., 2008). A drop in ATP, even by just 10%, is sensed by AMPK due to a subsequent rise in AMP and direct binding to the cystathionine-β-synthase (CBS) repeat sequences, positioned within the Bateman domains of AMPK’s γ subunit. Binding of AMP results in a conformational change in the enzyme, followed by phosphorylation of the α subunit at Thr$^{172}$ by upstream kinases, LKB1, CaMKK or Tak1 (Figure 6) (Carling et al., 2008; Hawley et al., 2003; Woods et al., 2003). Consequently, AMPK activation shuts down processes that would further consume ATP energy, like the synthesis of fatty acids and cholesterol, and conversely stimulates energy producing pathways like glucose uptake and fatty acid oxidation. In this way energy homeostasis is restored before proliferation can occur and consume even more energy. Therefore, AMPK function can be said to be required for both cell survival and inhibition of cell growth. Such metabolic regulation of anabolic and catabolic pathways by the
ratio of adenine nucleotides was first reported by Daniel Atkinson, which he called the “adenylate charge hypothesis” (Atkinson, 1968). AMPK is perhaps the best example of this theory in motion, and although the name AMPK is now widely accepted, it was previously referred to as Acetyl-CoA (ACC) kinase-3, due to its association, direct phosphorylation and therefore inhibition of ACC, the rate limiting step in fatty acid synthesis (Steinberg and Kemp, 2009). Labeling it as “AMP-activated protein kinase” was first suggested by Munday et al. in 1988 and was then adopted in 1989, when 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), required for cholesterol synthesis, was recognized to be phosphorylated in parallel with ACC (Munday et al., 1988). In addition to its allosteric activation by rising AMP levels, it is now known that AMPK can be activated completely independent of this by Ca\(^{2+}\) pathways mediated by calmodulin-dependent kinase kinase β (CaMKK) (Hawley et al., 2005; Woods et al., 2005). In addition, AMPK function in the brain also affects whole body metabolism by increasing one’s food appetite (Andersson et al., 2004; Minokoshi et al., 2004).

Neoplastic cells have a dysregulated metabolism and AMPK protein levels are generally kept at a minimum in order to ensure their continued anabolic needs and survival. The molecular mechanisms responsible for the activation of AMPK are becoming increasingly more clear as in the past 10 years it has received a lot of attention in the cancer research field as a potential drug target, perhaps owing to the now widely accepted AMPK-activating ability of metformin, an FDA approved agent and first line treatment for type II diabetes. The idea of sustained activation of AMPK, and therefore limited ATP production, is expected to produce a state of energy homeostasis that would halt energy consuming processes and create an unfavorable environment for neoplastic cells. While many laboratories report these results, which have been
recently reviewed (Shackelford and Shaw, 2009), several other groups have also shown that AMPK activation in certain tumor types may contribute to cancer cell survival via different mechanisms (Jeon et al., 2012; Park et al., 2009; Phoenix et al., 2009; Rios et al., 2013; Vazquez-Martin et al., 2009) of which the downstream survival effectors have not yet been defined.

In an effort to better understand its regulation, the crystal structure of the C-terminal regions of AMPK’s α1, β2 and full-length γ1 subunits together in a regulatory core have led to the revelation that the γ1 subunit has four potential AMP CBS binding sites, yet only three of the four sites were capable of binding nucleotide (Xiao et al., 2007). After some confusion with conflicting previous reports describing two exchangeable nucleotides bound in phosphorylated full-length AMPK (Scott et al., 2004), it was resolved that one of the three is bound so tightly that it does not engage in exchange and therefore would not be picked up in the earlier binding assays (Kemp et al., 2007). The question of whether the specific binding to each CBS site has differential effects on the allosteric and regulation of Thr\(^{172}\) phosphorylation, and therefore activity of AMPK, has the potential to clarify some of this disagreement seen in the literature. However, although attempts have been made to mutate the aspartic residues within each respective nucleotide binding site, they have been unsuccessful and therefore the answer to this question remains to be addressed (Mayer et al., 2011). Along these lines, the structure of phosphorylated AMPK, whereby its Thr\(^{172}\) containing activation loop makes plenty of contact with the C-terminal regions of the α and β subunits described above, thereby blocking access to inactivating phosphatases, is also well characterized (Carling et al., 2011; Xiao et al., 2011). While these structural studies provide much insight into the key points of AMPK regulation, how
AMP aids in Thr^{172} phosphorylation is unknown. From a genetic standpoint, AMPK knockdown is embryonically lethal; however there is no evidence of germline mutations in any of the AMPK subunits that would result in a predisposition to cancer (cancergenome.nih.gov/). Furthermore, somatic mutations are quite rare, less than 3% for any subunit, and they are actually more frequently amplified in human cancers (cancergenome.nih.gov/). In contrast, germline mutations in one of its upstream activating kinases, liver kinase B1 (LKB1) are known to be the cause of Peutz-Jegher Syndrome (PJS), a rare autosomal dominant predisposition to cancer described by benign intestinal hamartomatous polyps and mucocutaneous pigmentation that put the patient at an increased risk for developing varying malignant tumor types (Bardeesy et al., 2002; Jishage et al., 2002; Nakau et al., 2002; Ollila and Makela, 2011). Somatic mutations of LKB1 exist primarily in lung and cervical cancers, with some epigenetic inactivation reported to occur in papillary breast cancer (Ollila and Makela, 2011). Therefore, LKB1 is classified as a tumor suppressor gene. Taken together, AMPK activity would therefore be predicted to decrease in tumor cells short of functional LKB1, however it is important to note that its activation can occur independent of LKB1 in a wide array of cancer lines, with high levels seen even in lung cancer where LKB1 mutations are common (William et al., 2012). A plausible explanation for this discrepancy is the existence of other upstream kinases, such as CaMKK and Tak1, or unknown phosphatases, which may have differing roles in different tumor lineages where metabolic statuses also differ. Also unclear in this regard is to what extent the energy balance restoring function of AMPK really adds to the tumor suppressive activity of LKB1. Worth noting though, is that AMPK activation in LKB1-deficient cells may trigger differing downstream consequences and therefore be insufficient for cancer cell growth inhibition, but rather sufficient for cell
survival. Conflicting reports adding to the apparent two-fold function of AMPK have indeed divided the literature in terms of its credibility as an effective anti-tumor drug target (Carling et al., 2012; Jeon et al., 2012; Li et al., 2012; Liang and Mills, 2013; Zhong et al., 2008).

**Controversies surrounding AMPK activation in cancer**

The downstream repercussions of AMPK activation in a cancer setting include a multitude of molecular mechanisms that are both in favor of and in opposition to its use as a molecular drug target in the treatment of various cancer types. In its favor are reports outlining AMPK’s ability to inhibit cancer cell growth via direct and/or indirect inhibition of the notorious mammalian target of rapamycin (mTOR) (Figure 7) (Hahn-Windgassen et al., 2005; Steinberg and Kemp, 2009). mTOR exists as two complexes, namely mTORC1 and mTORC2; mTORC1 predominantly functions to activate pathways that promote cell growth when nutrient supply is adequate and not surprisingly, its abrogated activity has been connected to many cancers (Dazert and Hall, 2011; Zoncu et al., 2011). AMPK can directly phosphorylate the Thr^{2446} residue of mTORC1 and/or Ser^{792} of RAPTOR, resulting in overall inhibition of its activity (Cheng et al., 2004; Gwinn et al., 2008). Upstream of mTOR is the tuber sclerosis 1/hamartin/tuber sclerosis 2/tuberin (TSC1/TSC2) complex of which TSC2 Ser^{1345} can also be phosphorylated by AMPK, thereby indirectly lowering mTORC1 activity (Inoki et al., 2003).
Receptor tyrosine kinases signal through Akt and mTORC1 to promote cell growth. AMPK has an opposing function on mTOR in that it inhibits cell growth. The activation of AMPK by various kinases may have differing overall cellular effects in cancer cells. mTOR feedback signaling to Akt is also possible. Importantly, receptor tyrosine kinase signaling is often in abundance in many cancer types and furthermore, many of the proteins shown converged on common targets in this pathway.

**Figure 7. Simplified schematic of AMPK on the tumor cell signaling map**
In this way AMPK is at the center of the merge between cellular energy metabolism and cell growth signaling pathways. Many cancers also exhibit high levels of fatty acid synthase (FAS) activity for de novo fatty acid production by and unknown mechanism that is independent of lipid and hormone levels already present in circulation (Alo et al., 1999; Swinnen et al., 2000). AMPK has been shown to indirectly inhibit FAS gene expression in the liver by suppression of sterol regulatory element binding protein 1 (SREBP1) and these results have been reproduced in LNCap prostate cancer cells treated with the AMPK activator 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) where a reduction of FAS protein was observed (Xiang et al., 2004). AMPK can also inhibit breast cancer cell growth via inhibition of fatty acid synthesis by phosphorylation and inhibition of ACC (Chajes et al., 2006). Other reports describe yet other molecular anti-tumor effects in breast, ovarian, lung, liver and colon cancers such as, but not limited to, cell cycle arrest at the G1 phase, promotion of an anti-inflammatory phenotype, phosphorylation and activation of tumor p53 and p27 tumor suppressor proteins, as well as inhibition of HIF-1α (Isoda et al., 2006; Jones et al., 2005; Motoshima et al., 2006; Sag et al., 2008; Treins et al., 2006). Interest in the activation of AMPK as a plausible anti-cancer strategy really took off after reports surfaced (2005) attributing an overall reduction in cancer risk (~30%) in diabetic patients taking metformin daily to the indirect activation of AMPK, in addition to its known ability to inhibit gluconeogenesis in the liver (Evans et al., 2005). Since then, thousands of papers assessing metformin’s anti-cancer properties both in vitro and in vivo have been published. Phase II and III clinical trials are also underway (Dowling et al., 2012; Pollak, 2012). The use of metformin has been experimented with in a wide variety of cancer types, but it has also exposed some not-so-desirable consequences of AMPK activation in a cancer. This has led
to much controversy in the literature and it is now highly debatable whether or not AMPK is a friend or foe in the treatment of cancer, with a large amount of work done particularly in breast cancer. Generally speaking, it is agreeable that metformin works via indirect activation of AMPK. The mechanism(s) leading up to this event are unclear, but it is believed to be due to metformin’s ability to inhibit the mitochondrial respiratory chain complex I, thereby disrupting the ATP: AMP ratio and activating AMPK (Logie et al., 2012; Owen et al., 2000; Turner et al., 2008). While most of the data to date are in support of AMPK activation by metformin, those that are not suggest that the benefits of AMPK activation, and metformin protection, may vary by patient and tumor type. Indeed, a number of studies have reported AMPK activation to actually promote tumor growth in prostate cancer and that its inhibition, by siRNA, leads to reduced cell proliferation (Park et al., 2009). Interestingly, these effects could be prevented with an inhibitor of CaMKKβ (Jung et al., 2009). Others implicate AMPK in increasing glucose uptake rate and glycolysis, and even in mitosis during times of low nutrient supply by the control of myosin regulatory light chain (MLC) (de)phosphorylation (Banko et al., 2011; Massie et al., 2011). Autophagy, the cell’s means of protein and whole organelle disposal and recycling by way of the lysosome, occurs in times when the cell is desperate for energy and nutrients to meet its demands. Therefore, one can immediately deduce that AMPK must participate in activating this mechanism if need be, and the activation of AMPK in various cancer types resulting in autophagy and cancer cell protection has certainly been published (Hoyer-Hansen et al., 2007; Kim et al., 2011; Liang et al., 2007; Lum et al., 2005). Furthermore, since AMPK and Akt signaling converge on mTOR with conflicting effects (Figure 7), and AMPK phosphorylation being dominant over Akt, that AMPK antagonizes Akt signaling is certainly an enticing
proposition. While this may be true in the case of TSC2 and FOXO3, which are phosphorylated by both and at different sites, it may not translate to cellular outcome. AMPK and Akt have numerous targets outside of the classical TSC2 and FOXO3 that even overlap such as, IRS1, TBC1D1, TBC1D4, PFKFB2, PFKB2, TSC2, p27Kip1 and often work together in the regulation of other physio-regulatory mechanisms, particularly bioenergetics and cell viability (Hardie et al., 2012; Neufeld, 2012; Steinberg and Kemp, 2009; White, 2012). What’s more is that in glucose deprived conditions, AMPK and Akt are active and actually function to ensure cell survival, regardless of mTOR cascade shutdown (Inoki et al., 2003; Zhong et al., 2008). Taken together then, one should proceed with caution when making conclusions in regards to the complex and intertwined relationship between AMPK and Akt, as also shown in this body of work.

The function of AMPK in normal cells is indisputable; it is active only to increase energy demands in times of need, but also hinders cell growth and proliferation by way of mTOR inhibition. How cancer cells adapt to weaken this pathway and alter the possible downstream effects thereof is still not well understood. What is becoming clearer is that the effects of its activation seem to be tumor and cell type specific. It is possible that nucleotide binding to one site over the other in the CBS domain, altered upstream kinase expression from LKB1 to CaMKKβ, or the existence of some other post-translational mechanism could be important in understanding the apparent switch in signaling and dichotomy in overall effect.
Motivation and Approach

Post-translational regulation of AMPK is just beginning to be understood. As the master regulator of cellular metabolism, this field of study has large implications in our understanding of cancer development, progression, and even occurrence and prevention, as recently nicely summarized in two review articles (Carling et al., 2012; Zungu et al., 2011). Evidence for ubiquitination of the catalytic α and regulatory β subunits of AMPK does exist. The E3 ligase, cidea, is the only identified E3 in this regard to be responsible for the ubiquitination of the AMPK β subunit in brown fat tissue, resulting in decreased AMPK activity due to proteasomal degradation (Qi et al., 2008), whereas the α subunit-specific E3 still remains to be discovered. Furthermore, ubiquitination of the known upstream AMPK activating kinases has been reported to also decrease AMPK activity (Al-Hakim et al., 2008; Witczak et al., 2008). As the cell’s primary means of maintaining protein homeostasis, it is not surprising that AMPK activity is also regulated by the UP-S, which may represent an alternative avenue in the identification of novel molecular drug targets controlling AMPK activity in cancer.

As a previously identified E3 ligase with tumor promoting characteristics, BCA2 is an attractive and novel anti-tumor drug target. This dissertation work identifies BCA2 as a negative regulator of AMPK activity by regulating an event(s) that controls the (de)phosphorylation of the Thr172 site within the α1 subunit (see Chapter 3). While this is not a direct interaction, mass spectrometry data following phosphopeptide enrichment before and after knockdown of BCA2 in breast cancer cells has identified potential BCA2 substrate-targets that may play a role in AMPK activation in breast cancer (see Chapter 4). Therefore, the overexpression of BCA2 in breast cancer cells may be one of the previously discussed mechanisms neoplastic cells utilize to
modify and/or weaken the AMPK pathway to ensure their survival. Furthermore, this work provides innovative rationale for the use of metformin in select tumor types, or in combination with a BCA2 inhibitor, as the overexpression of BCA2 may weaken its anti-tumor effect. Previous DSF SAR related work has led to a collection of drugs from the NCI that possess the N(C=S)S-S described earlier, which have been assessed for their ability to inhibit cancer cell proliferation (see chapter 4). As potential inhibitors of BCA2, their impending use in combination with metformin may have valuable future therapeutic implications in breast cancer treatment. Finally, similar results were reproduced in prostate cancer and the characterization of BCA2 protein expression/localization assessed in a cell-cycle dependent manner in both tumor types (see Chapter 5).
CHAPTER 2  
MATERIALS AND METHODS

**Reagents** Anti-BCA2 antibody was purchased from LifeSpan BioSciences, Inc (Seattle, WA). FLAG (M2) and β-actin antibodies were obtained from Sigma-Aldrich Corp (St. Louis, MO). Antibodies to phosphorylated AMPK (Thr 172), ACC (Ser 79) and AKT (S473), as well as to total AMPK, ACC and AKT, were all purchased from Cell Signaling Technologies (Beverly, MA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All primary antibodies were stored at -20°C and secondary at 4°C. The AMPK inhibitor Compound C and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Corp (St. Louis, MO). AMPK activators, metformin and AICAR, were obtained from Toronto Research Chemicals Inc (North York, ON) and Tocris Bioscience (Minneapolis, MN), respectively. The PI3 Kinase inhibitor LY294002 was purchased from Cell Signaling Technologies (Beverly, MA). The stocks of these chemical reagents were made up as follows: 20 mM Compound C dissolved in DMSO, 1 M metformin in sterile H$_2$O, 75 mM AICAR in sterile H$_2$O and 10 mM LY294002 in DMSO. All drug stocks were stored at -20°C.

**Expression Vectors and Constructs** The BCA2 amplicon was subcloned into the FLAG-tagged pCMV-tag2B vector, as previously described (Amemiya et al., 2008; Burger et al., 2005). Mutations in the BCA2 RING domain and predicted AKT phosphorylation site were engineered in the wild type construct as also previously described (Amemiya et al., 2008). The AMPK alpha 1 catalytic subunit (PRKAA1) pCMV6-XL5 vector construct was purchased from the TrueClone® Human Collection, OriGene Technologies Inc (Rockville, MD). Bacterial stocks of
all vector constructs were generated in our laboratory. Once amplified and DNA isolated, plasmid maxiprep stocks were stored at -20°C.

Cell Culture Human breast cancer MDA MB 231, MDA MB 468, MCF7 and Hs578t cells, human prostate cancer LNCap, C42B cell lines, and the human embryonic kidney fibroblast HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA). MDA MB 231 and MDA MB 468 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), and MCF7, Hs578t, LNCap and C4-2B cells in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% FBS (Hyclone from Fisher Scientific, Pittsburgh, PA), 100 µg/ml streptomycin and 100 units/ml penicillin (Invitrogen, Carlsbad, CA). HEK293T cells were also grown in DMEM containing 10% Fetal Bovine Serum (FBS), but no antibiotics. Cells were passaged routinely and maintained at 37°C and 5% CO₂.

Serum starvation Exponentially growing MDA MB 231, MCF7, LNCaP or C42B cells were plated at 80% confluency in 100 mm dishes and allowed to adhere overnight. The next day, media containing 1% FBS was added in place of the 10% and cells were serum starved for 72 to 96 hours. Changing the media back to 10% for 24 to 48 hours synchronized the cells into S phase of the cell cycle. Drug treatment(s) were performed for the additional time and concentration indicated in each figure during the respective phase of the cycle.

Transfection (i) Transfection using AMPKα and BCA2 wt and mt constructs (created by site-directed mutagenesis) (Amemiya et al., 2008). Breast cancer or fibroblast cells were plated in 100-mm dishes at 80% confluency and allowed to settle overnight. A fresh complete medium change, devoid of antibiotics, was given 2 hours prior to transfection. For transfection
experiments using MDA MB 231 cells, 1-3 µg of FLAG-BCA2, wild-type (wt), S132/S133 mutant (mt) (serine 132 and 133 to alanine) or RING (cysteine 228 and 231 to alanine) (Amemiya et al., 2008; Bacopulos et al., 2012) mt vector DNA was transfected using the Lipofectamine® LTX reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA), with pCMV-tag2B empty vector used as a control. Following a 48 hour incubation period, cells were treated as indicated, harvested and lysed for Western blot analysis. For co-transfection experiments, 2.5 µg of FLAG-BCA2 wt, S132/S133 or RING mt and 2.5 µg of AMPKα vector DNA were co-transfected into HEK293T cells using the FuGENE® HD transfection reagent and protocol (Promega, Madison, WI), with pCMV-tag2B empty vector co-transfection with AMPKα as a control. For co-transfection experiments studying the concentration-dependent effects of wt or mt BCA2 on AMPKα, the AMPKα DNA concentration was held constant at 1 µg, while that of BCA2 ranged from 1-5 µg. After 48 hours, cells were treated as indicated, harvested and lysed for analysis by Western blotting. (ii) Transfection using BCA2 specific small interfering RNA. The custom designed small interfering RNA (siRNA) duplexes were purchased from Qiagen (Valencia, CA) [BCA2 siRNA duplex 2: sense = r(CGUCUGAAUAGAAUUAAUU)dTdT, antisense = r(AAUUAAUUCUAUUCAGACG)dGdG] and dissolved in siRNA suspension buffer to yield a stock of 20 µmol/L, stored at -20°C. Non-silencing control siRNA was used as a negative control and RNAiFect (Qiagen) as the transfection reagent. Experiments were carried out in 6-well plates following the manufacturer’s protocol. The siRNA transfection incubation period lasted 72 hours, after which cells were treated accordingly, harvested, lysed and analyzed by Western blotting.
**Western Blotting** Cells were transfected, drug treated, or both, and then harvested. Cell lysates (30-40 µg) were mixed with 3X sodium dodecyl sulfate (SDS) buffer, boiled for 5 minutes and analyzed by SDS-polyacrylamide gel electrophoresis using 4-20% tris-glycine gradient gels (Invitrogen, Carlsbad, CA), and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) and blocked in 5% milk powder in TBST for 1 hour at room temperature. The primary antibodies were diluted in blocking buffer (phosphorylated antibodies at 1:500 and all remaining at 1:1000) and incubated overnight at 4°C. Membranes were washed in TBS-Tween 20 (0.2%) and incubated with species-specific secondary antibodies conjugated to HRP (1:5000) for 1 hour at room temperature. Signals were developed using the mobilon western chemiluminescent HRP substrate (Millipore, Billerica, MA) and the FOTO/Analyst® Luminary/FX® Systems Flexible chemiluminescent and fluorescent imagining workstation (Fotodyne, Hartland, WI). Densitometry analysis was done using ImageJ software and relative intensity calculated as a percentage of the control.

**MTT Assay** *(i)* siRNA: breast cancer MDA MB 231, MDA MB 468 and Hs578t cells were grown under standard conditions as described above and seeded in 96 well plates (20 000 cells per well) and allowed to adhere for 24 hrs. BCA2 siRNA (0.75 µg) or non-silencing scrambled control (0.75 µg) was added for a period of 72 hrs after which metformin was added in concentrations ranging from 5-30 mM for 24 hrs. *(ii)* DSF analogs: breast cancer MDA MB 231 and MCF7 cells were seeded in 96 well plates (1 000 cells per well) and allowed to adhere for 24 hrs. DSF analog was added in increasing concentrations (0.01 to 10 µM) for a period of 5 days. Cell proliferation in either case was determined by addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Invitrogen, Carlsbad, CA). The conversion of MTT to
purple formazan by viable cells was measured using a Wallac Victor\textsuperscript{3} 1420 multilabel plate counter (550 nm) and analyzed in Microsoft Excel. Growth curves were generated as percent of the control and statistical analysis as described below.

**Colony Formation Assay** An agar concentration of 0.75\% in DMEM medium, containing 10\% FBS and 100 µg/ml streptomycin and 100 units/ml penicillin, was dispensed at 200 µl/well of a 24 well plate to create a base layer and allowed to solidify. 200 µl of MDA MB 231 cells in 0.4\% agar were added in the next layer (1000/well) and placed at 4°C for 5 minutes to solidify. Cells are incubated at 37°C and 5\% CO\textsubscript{2} overnight after which the respective drugs are added in a total volume of 200 µl/well and colonies are allowed to form over a period of 5-7 days. Once the colonies formed iodonitro tetrazolium blue (INT) (2 mg/ml) was added at 100 µl/well and incubated overnight at 37°C and 5\% CO\textsubscript{2}. Colonies of at least 30 µm in diameter were counted using the Oxford Optronix GelCount\textsuperscript{TM} automated mammalian cell colony counter and CHARM algorithm software. Colony-forming ability was determined as a percent of those in the control.

**RNA analysis by Real-Time PCR** Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) and was reverse transcribed to complementary DNA (Two Step DNA kit, Invitrogen). The cDNA was amplified using primer pairs for BCA2 forward, 5′-GGGGTCACCAGACTCACACT-3′ and reverse 3′-CAGGAAAAAGGGGTGTTGGAGA-5′. The loading control was β-actin, primers: forward 5′-GAGCGCGGCTACAGCTT-3′ and reverse 5′-TCCTTAATGTCACGCACGATTT-3′

**BCA2 Promoter Luciferase Assay** Cells were plated in 24-well plates and co-transfected with BCA2 promoter–luciferase vector (Switch Gear Genomics) and Renilla vector (Promega) as
previously described (Kona et al., 2012). After 24 h, cells were treated with 20 mM metformin or 1 mM AICAR for 6 hours. Luciferase activity was measured using the Dual Luciferase Reporter Assay Kit (Promega) and promoter activity was calculated as relative luciferase units (RLU).

**Immunoprecipitation** HEK293T cells were co-transfected with AMPKα and FLAG-tagged BCA2 (as above) and co-immunoprecipitation was performed using 700 µg of protein, FLAG-antibody, and the Pierce Classic IP Kit (Thermo Scientific, Rockford IL) as per the manufacturer’s protocol.

**Nuclear/Cytoplasmic Fractionation** Treated or non-treated cells were collected and washed twice with ice cold PBS. The cytoplasm was fractionated using a hypotonic buffer solution (20 mM Tris-HCL pH 7.4, 10mM NaCl and 3mM MgCl₂) supplemented with 10% NP40 detergent. A 10 minute centrifugation at 4°C and 3,000 rpm produced the cytoplasmic fraction (supernatant). The pellet (nuclear fraction) was resuspended in a cell extraction buffer (100 mM Tris-HCl pH 7.4, 2 mM Na₃VO₄, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1% SDS, 1 mM NaF, 0.5% deoxycholate and 20 mM Na₅P₃O₁₀); supplemented with 1 mM phenylmethanesulfonylfluoride (PMSF). and centrifuged for 30 minutes at 4°C and 14,000 rpm. The supernatant (nuclear fraction) was isolated and all samples were stored at -80°C prior to SDS-PAGE analysis.

**Immunofluorescence** Cells were fixed to slides with ice cold methanol: acetone (1:1) and blocked for one hour using 5% BSA in PBS. Staining with BCA2 rabbit polyclonal antibody (LifeSpan BioSciences, Inc) was done overnight at 4°C followed by rabbit TRITC-labeled secondary antibody (Sigma Aldrich Croporation) for 1 hour at room temperature. Cells were
washed with PBS in 5 minute intervals, 5 times, followed by nuclei counterstained with 4’6-diamidino-2phenylindole (DAPI) for 5 minutes and then more washes. Photomicrographs were taken at x63 magnification using a Leica DM4000 microscope and 5.0 Openlab Improvision software to document the signals.

**Proteomics: Database Searching** Tandem mass spectra were extracted and charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.0) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1), in the laboratory of Dr. Paul Stemmer, Proteomics Core, Wayne State University-School of Medicine. Mascot was set up to search the SwissProt_2013_04 database (selected for Homosapiens, unknown version, 20253 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search a subset of the SwissProt_2013_04 database also assuming trypsin. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Mascot and X! Tandem as a fixed modification. Dehydrated of the n-terminus, glu->pyro-Glu of the n-terminus, ammonia-loss of the N-terminus, gln->pyro-Glu of the N-terminus, oxidation of methionine, acetyl of the n-terminus and phospho of serine, threonine and tyrosine were specified in X! Tandem as variable modifications. Glu->pyro-Glu of the N-terminus, gln->pyro-Glu of the n-terminus, oxidation of methionine, acetyl of the N-terminus and phospho of serine, threonine and tyrosine were specified in Mascot as variable modifications. (Courtesy of Dr. Paul Stemmer)

**Proteomics: Criteria for Protein Identification** Scaffold (version Scaffold_4.1.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein
Identifications. Identified peptides that were present at greater than 80.0% probability, as per the Peptide Prophet algorithm, were acknowledged (Keller et al., 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 80.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be distinguished based on just MS/MS analysis were grouped according to the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. (Courtesy of Dr. Paul Stemmer)

**Statistical Analysis** Western blot densitometry analysis was performed using ImageJ analysis software (NIH). All phosphorylated proteins were normalized to corresponding total protein expression and BCA2 to β-actin. Fold change was calculated based on the vehicle control/untreated lane and error bars are mean ± SEM for repeated experiments. Statistical analyses of the MTT and colony formation assays were carried out using an unpaired parametrical t test with Welch’s correction to compare the means of six observations for each combination treatment to the single agent condition in both the colony formation and the MTT assays, followed by one-way ANOVA to compare the combination treatments to single agent and then to the control. All statistical tests were two-tailed and $P < 0.05$ was considered to be statistically significant. GraphPad Prism 6 Software was used.
CHAPTER 3

RESULTS

Regulation of Metformin Response by Breast Cancer Associated Gene 2 (BCA2)

Adapted from published material in Neoplasia, 2013

Although metformin is currently being assessed in Phase II and III cancer related clinical trials, the benefit of AMPK activation in vitro and in vivo remains controversial in various cancer types, due to a need for more detailed mechanistic studies (Dowling et al., 2012; Pollak, 2012). The data presented in this dissertation show that BCA2 has a negative regulatory effect on the tumor suppressing, cellular stress sensing kinase, AMPK. On the endogenous level, BCA2 inhibits both basal and inducible levels of AMPKα1 phosphorylation at Thr172 and therefore its activation. This inhibition was dependent on its E3 ligase specific RING domain and was also affected by S132/S133 mutations in its predicted Akt phosphorylation site (Amemiya et al., 2008). In addition, the chemical activation of AMPK by metformin or AICAR also increased BCA2 and pAkt protein levels, suggesting the presence of a possible cell survival feedback mechanism. Furthermore, the tumor growth-inhibitory benefit of metformin was significantly improved once BCA2 was silenced (via siRNA) or destabilized (via PI3/Akt kinase inhibition), compared to treatment with metformin alone, demonstrating that suppression of BCA2 function enhances metformin’s anti-cancer efficacy.
Results

BCA2 is an endogenous inhibitor of AMPKα Thr172 phosphorylation and therefore activation

To study the potential relationship between BCA2 and AMPK involved pathways, the basal expression of each protein in a panel of four breast cancer cell lines was first assessed (Fig. 8). An inverse relationship of BCA2 to pAMPKα1 (Thr172) was observed: those cell lines with higher levels of BCA2, like MDA MB 468 and Hs578t, had lower pAMPK α1 (Thr172) levels when compared to the other two examined, namely MCF7 and MDA MB 231, which contain relatively lower amounts of BCA2, but higher levels of pAMPK α1 (Thr172) (Fig. 8). It has previously been reported that BCA2 contains a predicted AKT phosphorylation site and that AKT-mediated phosphorylation at this site is responsible for stabilization of the BCA2 protein (Amemiya et al., 2008). Levels of pAkt (S473) indeed correlated positively to BCA2 (Fig. 8). These data provided grounds to further investigate the inverse relationship between BCA2 and pAMPK, with the hypothesis that BCA2 may be an endogenous inhibitor of AMPK activation in breast cancer cells.
Figure 8. The assessment of basal protein expression levels in the four indicated human breast cancer cell lines.

Exponentially growing cells were lysed for Western blotting analysis with specific antibodies to BCA2, pAMPK (Thr172), total AMPKα1, pAKT (S473), total AKT and β-actin (loading control).
To provide direct support for this hypothesis, BCA2-specific siRNA was utilized to inhibit the expression of BCA2 in MDA MB 231 cells, followed by measurement of subsequent levels of endogenous pAMPKα1 (Thr172). Suppression of BCA2 occurred in a siRNA concentration-dependent manner (reaching 80% inhibition; Figure 9A); importantly, this was accompanied by an 11-fold increase in phosphorylated AMPKα1. Total AMPKα1 protein levels, however, remained relatively unchanged (Figure 9A). To further confirm that the increase of pAMPK was active, the phosphorylation status of acetyl-CoA carboxylase (ACC), a direct downstream target of AMPK was assessed (Davies et al., 1990). Knock down of BCA2 protein resulted in a concentration-dependent increase (up to 7-fold) in levels of p-ACC (S79), mirroring pAMPK, while levels of total ACC were unchanged compared to the control (Figure 9A). The non-silencing scrambled siRNA negative control had no effect on either BCA2 protein expression or AMPK signaling (Figure 9A, lanes 2 vs. 1). When this experiment was repeated in two other breast cancer cell lines, MCF7 and MDA MB 468 (Figure 9B and C), inhibition (68% and 42%, respectively) of BCA2 expression by its siRNA again resulted in increased levels of pAMPKα1 (2.5- and 1.4-fold, respectively; Figure 9 B-C).
Figure 9. Knockdown of BCA2 by siRNA results in AMPK activation.

A. Immunoblot of MDA MB 231 cells (grown in a 6-well plate 2ml/well) transfected with BCA2 siRNA (x-axis, µg) for 72 hrs (3µg = 0.1µM, 5µg = 0.2µM and 7µg = 0.27µM). Scrambled, nonsilencing siRNA, and untreated cells were used as controls. Cell extracts were used for Western blots with specific antibodies as those in Fig.8 as well as pACC (Ser 79) and total ACC. Densitometry analysis is representative of mean ± SEM for three independent experiments. p-AMPK was normalized to total AMPKα, pACC to total ACC and BCA2 to β-Actin. Fold change was calculated compared to the control. B, C. MCF7 (B) and MDA MB 468 cells (C) were transfected with 5 µg of either BCA2 siRNA or scrambled control for assessment of the effect on pAMPK as done in part A.
More evidence of BCA2 as an endogenous AMPK inhibitor is shown in Figure 10, where the BCA2 gene was subcloned into a pCMV-tag2B vector construct and herein utilized to co-transfect HEK293T cells together with a pCMV6-XL5 AMPK α1-subunit containing construct. HEK293T cells express low basal levels of AMPKα1 and BCA2 (Figure 10, lane 1), making them a good model for co-transfection experiments. Co-transfection of the AMPKα1 subunit and the pCMV empty vector increased cellular levels of pAMPKα1 (Figure 10, lanes 2 vs. 1), while co-transfection of AMPKα1 and BCA2 had little or no increase in the basal level of pAMPKα1 (Figure 10, lanes 3 vs. 2 vs. 1), confirming that BCA2 inhibits basal levels of AMPKα1 activation. To determine whether BCA2 could also inhibit induced AMPKα activation, the co-transfected cells were treated with AMPK activators metformin or AICAR. Metformin is an indirect activator of AMPK, while AICAR is an analog of AMP and can therefore mimic its cellular effect and directly bind its β regulatory subunit, activating AMPK in culture (Corton et al., 1995). A robust induction of pAMPKα1 was observed in cells co-transfected with AMPKα and pCMV empty vector after treatment with metformin or AICAR (Figure 10, lanes 4 and 6 vs. 2). Importantly, this induction was almost completely inhibited by transfected BCA2 (Figure 10, lanes 5 vs. 4, and 7 vs. 6). Again, BCA2 transfection had little inhibitory effect on total AMPKα1 (Figure 10). Therefore, these transfection studies reveal that BCA2 is an inhibitor of both basal and induced AMPK phosphorylation/activation. Also observed in response to AMPK activators, was an increase in BCA2 levels (Figure 10, lanes 4 and 6 vs. 2), suggesting that AMPK signaling may trigger BCA2 up-regulation as a feedback mechanism (see Figure 14-20).
Figure 10. BCA2 inhibits AMPKα1 phosphorylation and activation

Immunoblot of HEK293T cells co-transfected with BCA2 wt and AMPKα1 (2.5 µg each). After a 48 hr transfection, cells were either treated with metformin (20 mM) or AICAR (1 mM) for 6 hr, or left untreated. Cell extracts were used for Western blots with specific antibodies as previously stated. FLAG was used as a measure of transfection efficiency.
Requirement of the BCA2 RING domain and S132/S133 phosphorylation sites for inhibition of AMPK activation

To gain insight into the molecular basis by which BCA2 suppresses AMPK activation, the requirement of its RING domain and S132/S133 phosphorylation sites, residues within its predicted Akt-binding domain, were analyzed. The RING domain of BCA2 is critical for its E3 ligase function and a cysteine mutation to alanine, at positions 228 and 231, renders the protein “ligase dead” and therefore ubiquitination negative (Burger et al., 2005), while AKT-mediated phosphorylation of BCA2 has been reported to increase its stability (Amemiya et al., 2008; Bacopulos et al., 2012). The serine 132 and 133 residues mutated to alanine would therefore increase its ubiquitination/autoubiquitination ability and subsequent degradation by the proteasome. It should be noted that these serines were predicted to be phosphorylated by Akt, but may be susceptible to phosphorylation by other kinases in addition to Akt, which needs to be further characterized.

HEK293T cells were first co-transfected with the AMPKα1 subunit and wild-type (wt) or a mutant (mt) variant of BCA2, followed by examination of the effect on basal p-AMPKα1 levels by Western blotting. Again, an increase in basal pAMPK α1 levels was seen when AMPKα1 was co-transfected with the pCMV empty vector control, which was inhibited in the presence of BCA2 wt (Figure 11A, lanes 1-3).
Figure 11. The requirement of the BCA2 RING domain and AKT phosphorylation sites for inhibition of AMPK (HEK293T cells)

A. Immunoblot of HEK293T cells (grown in 100 mm dishes) co-transfected with AMPKα1 and BCA2 wt, RING mt or S132/S133 mt for the assessment of basal pAMPK levels. B. Immunoblot of HEK293T cells co-transfected with a constant amount of AMPKα1 (1 µg) and increasing amounts of BCA2 wt or mt (1 or 5 µg) treated with metformin (20 mM) for 6 hr.
However, co-transfection of the BCA2 RING mt failed to inhibit an increase in pAMPK α1 (Figure 11A, lanes 4 vs. 3 and 2), indicating that the RING domain, and therefore the E3 ligase function of BCA2, is required for negative regulation of AMPK activation. The co-transfection of BCA2 S132/S133 mt still retained a partial inhibitory effect on pAMPK α1 levels in this experiment, compared to BCA2 wt and RING mt transfected cells (Figure 11A, lane 5 vs. 1-4), suggesting that this mutant still confers enough stability (as shown by the level of FLAG) and therefore has a partial inhibitory activity, perhaps due to its RING domain still being intact. Levels of FLAG and total AMPKα proteins were used as a measure of transfection efficiency and that of β-Actin as the loading control (Figure 11A).

To further assess the requirement of the BCA2 RING domain and predicted AKT phosphorylation site, co-transfected HEK293T cells were treated with metformin (Figure 11B). While transfection of BCA2 wt (1 and 5 µg) inhibited the induction of pAMPK α1 by metformin (Figure 11B, lanes 2 and 5 vs. 1), the BCA2 RING mt failed to do so (Figure 11B, lanes 4 and 7); in fact, a large increase in pAMPK α1 was seen in cells transfected with 5 µg of RING mt BCA2 (lanes 7 vs. 4 and 1), confirming that the RING domain of BCA2 is critical for its ability to inhibit both basal and inducible levels of pAMPK α1, suggesting that the RING mt acts as a dominant negative inhibitor of endogenous BCA2 function. However, the transfection of BCA2 S132/S133 mt also inhibited AMPKα1 phosphorylation in a dose-dependent manner (Figure 11B, lanes 3 and 6 vs. 1) and this inhibition was even greater than that seen by BCA2 wt (Figure 11B lanes 3 vs. 2 and 6 vs. 5) in this experiment. As the serines predicted to be phosphorylated by Akt within this domain of BCA2, the results with this site mt were not entirely expected. While they may in part be explained by the intact RING domain of this construct, as seen under
basal conditions in Figure 11A, the molecular mechanism for such a large inhibitory effect this mutant produces on inducible AMPK remains unclear and will be further investigated in the near future.

Confirming these results in human breast cancer cells was done using MDA MB 231 cells transfected with increasing concentrations (1-5 µg/well) of wt BCA2 to observe the effect on basal pAMPKα1 levels. In Figure 12A, it can be seen that with increasing amounts of transfected BCA2 wt, basal AMPK phosphorylation is prevented, with some slight activation seen in the 1 µg lane (lane 3), but subsequent decrease with 3 and 5 µg of BCA2 wt (Figure 12A, lanes 4 and 5 versus 3). The same experiment was repeated in MDA MB 231 cells transfected with 1 and 3 µg of BCA2 wt, RING mt, S132/S133 mt or pCMV empty vector control, followed by treatment of AICAR and measurement of the effect on induced pAMPK α1 (Figure 12B). The transfection of BCA2 wt had little inhibitory activity on inducible pAMPK levels (probably due to already high basal levels of endogenous BCA2 and AMPKα proteins; Figure 12B, lanes 2 vs. 1), while the transfected S132/S133 mt was again able to partly inhibit the induction of AMPK activation (Figure 12B, lanes 3, 5 vs. 1). Interestingly, also observed was the decrease in endogenous BCA2 protein in the presence of transfected S132/S133 mt compared to the RING mt (lanes 3 and 5 vs. 4 and 6). This may be explained by the still robust ubiquitination ability of this mutant (Amemiya et al., 2008) and therefore targeted degradation of endogenous BCA2. Importantly, the BCA2 RING mt not only fully abolished the BCA2 inhibitory effect, but also enhanced AICAR-mediated AMPK activation in a dose-dependent manner (Figure 12B, lanes 4, 6 vs. 1, 2). Taken together, our data demonstrate that BCA2 E3 ligase activity is essential for its inhibitory function on both basal and inducible AMPK activation.
Figure 12 The requirement of the BCA2 RING domain and AKT phosphorylation sites for inhibition of AMPK (MDA MB 231 cells)

A. Immunoblot of MDA MB 231 cells transfected with 1, 3 and 5µg of BCA2-FLAG wt to observe the effect on pAMPK. B. MDA MB 231 breast cancer cells co-transfected with increasing concentrations of BCA2 wt and mt (1 or 3 µg), to detect changes in inducible (by AICAR) pAMPK levels. The prepared cell extracts in each experiment were used for Western blots with the specific antibodies as indicated.
Requirement of PI3/AKT kinase signaling for BCA2-mediated AMPK inhibition

To determine the role of AKT signaling on BCA2-mediated AMPK suppression, the chemical inhibitor of the PI3/AKT kinase signaling pathway, LY294002, was used. Treatment of MDA MB 231 cells with LY294002 caused a dose-dependent inhibition (> 90%) of pAkt at S473 associated with decrease levels of BCA2 protein (about 60%, Fig. 13A). Importantly, this was also accompanied by a concentration-dependent increase in pAMPK α1 levels (up to 4-fold; Figure 13A, lanes 2-5 vs. 1). Moreover, in a kinetics experiment, 3-6 hr inhibition of pAkt (up to 90%) in MDA MB 231 cells occurred and was associated with BCA2 decrease (>75%), and an up to 2-fold increase in pAMPKα1 levels, which reached its highest point (almost 4.5 fold) at 24 h (Figure 13B, lanes 2-5 vs. 1). However, again there was an increase in BCA2 levels following AMPK activation and return of pAkt at 6-24 h (Figure 13B), consistent with the idea that a potential feedback pathway is present (see Figure 14-20). These data demonstrate that the dose- and time-dependent inhibition of AKT activity leads to a decrease in BCA2 levels and subsequently, significantly increased levels of AMPK activation.
Figure 13. AKT inhibition decreases endogenous BCA2 protein levels, followed by increased AMPK activation.

MDA MB 231 cells were treated with LY294002 in either a dosing study (A, 12 hr time point) or a kinetics experiment (B, 10 μM). The included figures are representative of experiments done in triplicate and the included densitometry is mean ±SEM of phosphorylated proteins (normalized to total) and BCA2 to β-actin, compared to vehicle control (set to 1) for fold change expression. The prepared cell extracts in each experiment were used for Western blots with specific antibodies to pAKT (S473), BCA2, pAMPKa (Thr172), total AMPKa1, and β-actin as a loading control. LY = LY294002
Treatment of breast cancer cells with an AMPK activator increases endogenous BCA2 protein levels

In the previous experiments (Figures 10-13), an increase in endogenous levels of BCA2 in response to the activation of AMPK was noted. To test if AMPK signaling can in turn lead to up-regulation of the BCA2 protein, MDA MB 231 cells were treated with various concentrations of the direct AMPK activator, AICAR, for 2, 4 or 8 h, followed by measurement of the effect on BCA2 by Western blotting (Figure 14). A clear dose- and time-dependent increase in BCA2 protein levels was observed, which corresponded to the induction of AMPK activation, as measured by specific phosphorylation of pAMPK and pACC (Figure 14), demonstrating that BCA2 increase is a consequence of AMPK activation. Since AKT can phosphorylate and stabilize BCA2 (Amemiya et al., 2008; Bacopulos et al., 2012), we determined whether AICAR treatment could also activate AKT. Indeed, levels of pAkt were increased in MDA MB 231 cells in an AICAR concentration- and time-dependent fashion, correlating well with that of the observed BCA2 protein increase (Figure 14).
Figure 14. Activation of AMPK by AICAR increases BCA2 protein levels in MDA MB 231 breast cancer cells.

Immunoblot of MDA MB 231 cells treated with AICAR at the indicated concentrations and time points.
When MDA MB 231 cells were treated with metformin in a kinetics experiment, a time-dependent increase in BCA2 protein levels were again observed, in association with an induction of AMPK activation (Figure 15A). Furthermore, kinetics experiments utilizing MCF7 cells treated with metformin (Figure 15B) or MDA MB 468 cells treated with AICAR (Figure 15C), also produced increased levels of BCA2 and pAkt proteins, associated with AMPK activation. Finally, the use of the chemical AMPK inhibitor, Compound C, not only suppressed activation of AMPKα1, but also inhibited the metformin-induced increase in BCA2 and pAkt in MDA MB 231 cells (Figure 16), suggesting the involvement of AMPK activity in a potential regulatory feedback mechanism with pAkt and BCA2 (see Figure 20).
Figure. 15 Activation of AMPK, by metformin or AICAR, increases BCA2 protein levels in multiple breast cancer cell lines.  
A. Immunoblot of MDA MB 231 cells treated for up to 12 hr with 20 mM metformin.  
B. Immunoblot of MCF7, and  
C. of MDA MB 468 cells treated with 20 mM of metformin for up to 24 hours.
Figure 16. Metformin mediated increase in BCA2 does not occur in the presence of the AMPK inhibitor, Compound C.

MDA MB 231 cells were pre-treated with 20 µM of Compound C for 12 h (lanes 4-5) or untreated (lanes 2-3), followed by treatment with 20 mM metformin for 4 or 8 h. The prepared cell extracts in each experiment (representative of three repeats) were then used for Western blots with specific antibodies as indicated.
To determine whether the metformin-mediated increase in BCA2 occurred on the transcriptional level, BCA2 mRNA levels and promoter activity were assessed in both co-transfected HEK293T and MDA MB 231 cells before and after metformin or AICAR treatment (Figure 17). From these experiments we were able to conclude that AMPK activation increases levels of BCA2 protein only, but not mRNA. We also determined that BCA2 does not bind the α subunit of AMPK directly (Figure 17).
Figure 17. BCA2 mRNA or promoter activity do not increase in response to metformin or AICAR and BCA2 does not inhibit AMPK by binding the α subunit directly

A. B. HEK293T or MDA MB 231 cells were co-transfected with a 1kb region of the BCA2 promoter and Renilla luciferase as a control, followed by treatment with metformin (20 mM, 6 hr) or AICAR (1 mM, 6 hr). Optimal levels for luciferase expression were defined by initially transfecting different ratios of BCA2 to Renilla and 1:100 was used. Data are normalized to Renilla and expressed as Relative Luciferase Units (RLU) of BCA2/control. Means and standard error of 3 experiments done in triplicates are shown, of which no significant change in promoter activity was observed in response to either treatment in either cell line. C. Quantitative PCR indicating no significant fold change in BCA2 using MDA MB 231 breast cancer cells. Data are normalized to β actin internal control. Graph depicts the mean and error bars of 2 experiments done in triplicates. D. HEK293T cells were co-transfected with equal amounts (2.5 µg) of AMPKα and FLAG-tagged BCA2 and immunoprecipitation was performed using 700 µg of protein and FLAG antibody. All AMPKα was found to be in the unbound protein portion.
Inhibition of BCA2 significantly enhances the ability of metformin to inhibit breast cancer cell growth

If BCA2 is an endogenous inhibitor of AMPK phosphorylation/activation, it is logical to assess the growth inhibitory potential of metformin in combination with BCA2 inhibition. To do so, breast cancer MDA MB 231 (Figure 18A), MDA MB 468 (Figure 18B) and Hs578t (Figure 18C) cells were pretreated with BCA2 siRNA or negative control scrambled siRNA for 72 h, followed by treatment with metformin at concentrations ranging from 1-30 mM for an additional 24 h. Cell growth status was then analyzed by MTT assay. Again, BCA2 siRNA, but not control siRNA, inhibited the growth of MDA MB 231 breast cancer cells (59.2 vs. 2.5%; Figure 18A). The combination of metformin and the control scrambled siRNA resulted in metformin dose-dependent growth inhibition, 5.5, 31.0 and 41.2% for 5, 15 and 30 mM respectively (Figure 18A, bars 3-5). Importantly, the addition of metformin to BCA2 siRNA-transfected cells resulted in an even more significant reduction in growth, compared to metformin plus scrambled siRNA, reaching 82.8% (p < 0.001) at 5 mM, 86.0% (p < 0.001) at 15 mM and 92.6% (p < 0.001) at 30 mM (Figure 18A, bars 7-9). MDA MB 468 and Hs578t cells (Figure 18B and C) showed also favorable and statistically significant results when combining BCA2 siRNA with metformin, compared to metformin alone. BCA2 siRNA alone was capable of inhibiting cell growth by 68.4% in MDA MB 468, followed by up to 89.7% inhibition with the addition of metformin (p < 0.001) (Figure 18B). Hs578t, while not as sensitive to such treatment (31.1% inhibition with BCA2 siRNA), also reached up to 79% growth inhibition with the addition of metformin (p < 0.05) (Figure 18C).
Figure 18. Inhibition of BCA2 enhances the anti-proliferative effect of metformin.

A. MTT assay using MDA MB 231 cells transfected with BCA2 siRNA (0.75 µg in a well of a 96-well plate = 0.5µM) for 72 hr followed by metformin treatment at of 5, 15 and 30 mM for 24 hr. Scrambled, non-silencing siRNA (0.75 µg) and untreated MDA MB 231 cells were used as controls. Included figure is representative of three repeats and the data are mean ± SEM of those and based on an average of 6 observations for each condition in each experiment (two-tailed t test with Welch’s correction comparing the means of combination treatment to metformin alone). B, C. MDA MB 468 (B) and Hs578t cells (C) were used for reproducibility of those results shown in part A and are as previously described for the indicated concentrations of metformin.
Similarly, a soft agar, anchorage-independent, colony formation assay was performed to further confirm the efficacy of metformin plus BCA2 inhibition using breast cancer MDA MB 231 cells, which were treated with either metformin alone, the PI3 Kinase/AKT inhibitor LY294002 alone (which leads to BCA2 destabilization), or their combination (Figure 19). The results show that the metformin and LY294002 combination is more effective than either agent alone, reaching 57.9 and 74.3% inhibition vs. 22.5 and 40.2% with 10 and 20 mM metformin alone vs. 14.8% with 10 µM LY294002 alone (bars 5, 6 vs. 2-4, p < 0.001; Figure 19). Although Akt has been implicated in a number of cancer cell survival pathways and is therefore not specific to BCA2, previous published works, together with our positively correlated data herein, indicate it to be important for BCA2 stability. This experiment, furthermore, suggests that the disruption of the BCA2-mediated feedback regulatory loop may be a new avenue for improving metformin efficacy in breast cancer patients with aggressive breast tumors that overexpress BCA2.
Figure 19. AMPK activation and Akt inhibition supresses MDA MB 231 colony formation

Soft agar colony formation assay using MDA MB 231 cells. Metformin (M) and LY294002 (LY) were added at concentrations shown for 6 days. Images (inserted) shown are a representative of 6 independent wells from the same treatment. Bar graph is representative of repeated experiments and the data are mean ± SEM based on an average of 6 wells per condition per experiment (two-tailed t test with Welch’s correction comparing means of the combination treatment to metformin alone) ***p<0.001.
Discussion

In conclusion, BCA2 is an endogenous inhibitor of AMPK activation and the inhibition of BCA2 therefore increases the efficacy of metformin (Figure 20). Investigated was if, and how, BCA2 regulates the tumor suppressing AMPK signaling pathway. Toward this goal, BCA2 was identified as an inhibitor of an upstream event controlling AMPK activation (Figure 20 and see following discussion). Furthermore, the data presented suggest the existence of a potential feedback mechanism, whereby AMPK activation increases BCA2 protein expression (Figure 20). Although the existence of such a phenomenon adds to the controversy surrounding the use of the AMPK activator metformin for the treatment of breast cancer, it provides newfound awareness for the limitations that this type of therapy may have.
At the basal level, AKT works to stabilize BCA2 so that it is free to ubiquitinate protein substrates, including those controlling phosphorylation/ dephosphorylation of AMPKα1 at Thr 172, targeting them for degradation via ubiquitin-proteasome pathway, resulting in inhibition of AMPK signaling in breast cancer cells. The activation of AMPK by metformin or AICAR, however, increases BCA2 protein levels, inciting cancer cell survival involving PI3K/Akt. Therefore, the balance of AMPK and PI3K/AKT/BCA2 might control breast cancer cell sensitivity to metformin therapy and the tumor cell life-death switch.
Immunoprecipitation experiments have revealed that BCA2 does not bind the AMPK α1 subunit directly (Figure 17), leading to the proposition that its negative effect is a result of BCA2 E3 ligase-mediated degradation of a target protein that is located upstream of AMPK and is responsible for regulating the (de)phosphorylation of the α1 subunit (Figure 20). On this account, and in conjunction with Wayne State University’s Proteomics Core, titanium dioxide (TiO2) phosphopeptide enrichment using MDA MB 231 cells treated with BCA2 specific siRNA or control siRNA, followed by mass spectrometry was performed to identify potential leads that would have a place in the regulatory mechanism outlined herein. The preliminary results of this work are discussed in the next chapter. Furthermore, data in Figures 11 and 12 also suggest that the BCA2 target protein is a substrate of its E3 ligase activity since the RING domain is required for its inhibitory effect. Consistent with this argument, if this were a direct effect on the α1 subunit, one would expect the RING mt to have a similar effect to the wt, as this would be the substrate-binding ubiquitination site. Also supportive in this regard is that no change in total AMPKα was observed (Figures 11-12).

Phosphorylation is possibly the most common occurring post-translational protein modification, often resulting in changes in protein conformation and stability, and BCA2 is no different in this regard. The fact that the transfected BCA2 S132/S133 mt also produced an inhibitory effect on basal pAMPKα1 in HEK293T cells (Figure 11A) points to the primary importance of an intact RING domain for its inhibitory activity. Furthermore, the even greater inhibitory effect of this mutant protein seen in the presence of metformin/AICAR (Figure 11B and 12B) also suggests the need for a more detailed characterization of these serine mutations made within the predicted Akt phosphorylation site. It is possible that other kinases may be in
competition for phosphorylation of BCA2 at these sites and delineating the function of each residue under such conditions that induce AMPK activation, as in Figure 11B and 12B, will be determined in future work. Also observed was that the level of endogenous BCA2 in breast cancer cells transfected with S132/S133 mt after AICAR treatment was lower than that with BCA2 wt and RING mt (Figure 12B). This could also be due to the ubiquitination ability of this mutant (Amemiya et al., 2008), which would target the endogenous BCA2 protein for degradation. The detailed mechanisms behind this phenomenon need to be investigated.

Therefore, the BCA2 mutant variant analysis data draws at least one main conclusion: an event upstream of AMPK, regulating its (de)phosphorylation and activation, is likely the substrate of BCA2 E3 ligase activity (Figures 11A and 20). The importance of AKT in suppressing AMPK activation via BCA2 was confirmed in Figure 13 where the use of a PI3/AKT kinase inhibitor produced a correlative effect between pAkt inhibition, BCA2 protein decrease and AMPK activation. These data suggest that BCA2-mediated inhibition of AMPK activation on the basal level is AKT-dependent.

Moreover, these data show that the use of metformin and AICAR not only activates AMPK signaling, but also produces a subsequent increase in BCA2 protein levels (Figures 14-15). This increase in BCA2 was further shown to be specific to AMPK activation, as it does not occur in the presence of the AMPK inhibitor, Compound C (Figure 16). Given what is already known about BCA2 and its dependence on such a classic growth promoting signal as AKT, it is logical to suggest that the observed BCA2 increase in response to AMPK activation by metformin or AICAR is related to a potential cell survival mechanism, a to-be-determined feedback loop ignited by AKT, and may incite cancer cell growth (Figure 20). This argument
was supported by increased levels of pAKT and BCA2 proteins in AICAR- or metformin-treated breast cancer cells (Figures 14-15). Directly opposing these results are those of Zakikhani et al., who reported inhibition of pAkt levels in response to metformin (Zakikhani et al., 2010). However, it is important to note that the difference in experimental conditions between Zakikhani et al.’s work and the current study may affect what results are observed. For instance, Zakikhani et al. performed metformin treatment under serum-starved conditions for 72 hours, whereas the experiments done herein utilize complete media containing 10% serum. In addition, for the purpose of these molecular studies, AMPK activation at shorter time points is sufficient to observe the increase in pAkt and BCA2. Furthermore, literature in support of AMPK activating–pAkt results can be seen in various cancer types (Kuznetsov et al., 2011; Leclerc et al., 2010; Sengupta et al., 2007; Tao et al., 2010). The reason for these discrepancies is still unclear, however, published reports in agreement with both observations, are in existence. To confirm the regulatory role of BCA2 on AMPK activation, three breast cancer cell lines were treated with metformin in the absence or presence of BCA2 inhibition. Shown is that through the inhibition of BCA2, either directly by siRNA, or indirectly by PI3/AKT kinase inhibition, the desirable anti-cancer effect of metformin is significantly enhanced (Figures 18-19).

While some preliminary clinical data are available that test metformin’s anti-tumor effect in breast cancer patients, it is yet to be proven that metformin improves the overall current clinical outcome for breast cancer victims. In the laboratory setting, the mechanisms underlying its reported preclinical anti-cancer activity are still unclear (Campagnoli et al., 2012; Decensi et al., 2010; Evans et al., 2005; Goodwin et al., 2008; Hadad et al., 2011). Most of the best available therapies are beneficial to specific tumor types, for example, anti-estrogens for
hormone responsive breast cancers (1998; Jordan, 2003) or herceptin for Her2 positive cases (Dent et al., 2013). Taking into account the mixed reports in the literature on the effects of AMPK activation in a cancer context, it is important to consider that metformin use may also have beneficial or adverse effects depending on the cancer type and background, including the overexpression of BCA2. Therefore these data convey that the screening of breast tumors, particularly those with a more aggressive phenotype, for BCA2 may not only provide a strong predictor of which patients may respond to metformin-based therapies, but also suggest that the combination of metformin and a BCA2 inhibitor may be a more effective anti-cancer strategy than metformin alone in which the desirable reprogrammed cellular energy homeostatic state, characteristic of AMPK activation, could be maintained.
CHAPTER 4

RESULTS

Preliminary search for candidate BCA2 substrate proteins controlling AMPK activation and BCA2 small molecule inhibitors

The search for BCA2 protein substrates controlling AMPK (de)phosphorylation and activation

As a newly identified negative regulator of AMPK activity in breast cancer cells, solving for the missing factor, on which BCA2 exerts its inhibitory function, is an important aspect in the complete characterization of the governing mechanism outlined within the last chapter. As an E3 ligase, one would expect its inhibitory function to be carried out by way of ubiquitination (and therefore degradation by 26S proteasome) of some upstream event controlling (de)phosphorylation of AMPK. Since BCA2 does not bind to the α subunit directly (Figure 17), identifying this highly sought after piece of information required the assistance of Wayne State University’s proteomics core and a large-scale mass spectrometry (MS) experiment. MDA MB 231 breast cancer cells were treated with either BCA2 specific siRNA or control siRNA in three independent experiments and a small aliquot of each preparation was used in Western blots to verify the BCA2 knock down (Figure 21) and the remaining samples were sent out for titanium dioxide (TiO₂) phosphopeptide enrichment and MS analysis.
Figure 21. Verification of BCA2 knockdown prior to TiO$_2$ enrichment and mass spectrometry

The confirmation of three individual siRNA experiments is shown. Small aliquots of each experiment were validated by western blotting (30µg). Dry pellet samples of each result shown in this figure were sent to the proteomics core for further analysis.
In preparing for this study, the hypothesis was that an AMPK upstream kinase was the target of BCA2 E3 activity and because many phosphorylated proteins are present in low amounts, enrichment before and after the knockdown of BCA2 (and before MS analysis) by TiO₂ beads was required. Protein identification was determined by using the X! Tandem software, which matched identified mass spectra with peptide sequences in the SwissProt_2013_04 database selected for Homo sapiens (see materials and methods section). Identified peptides that were present at greater than 80.0% probability, as per the Peptide Prophet algorithm, were acknowledged (Keller et al., 2002) and protein probabilities assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003) (see Materials and Methods). All results and statistical analysis discussed within are preliminary and were displayed and organized in Scaffold Proteome Software. Of particular interest were those phosphorylated proteins detected to be comparatively increased following the knockdown of BCA2 to control siRNA. At a threshold of 80%, 692 total proteins were identified in this study, which included a mixture of those that increased or decreased following knockdown of BCA2. Of these, 36 were specific to BCA2 siRNA inhibition, 160 to the control and 496 overlapped and remained relatively unchanged (Figure 22).
Figure 22. Venn Diagram Summary of Mass spectrometry results

The total of 692 protein identified can be broken down as depicted here.
Statistically significant changes were found in seven different phospho-proteins, and although these changes refer to a decrease in their level after BCA2 knockdown, their identities are presented in Table 1. Those phospho-proteins found to be increasingly present after treatment with BCA2 siRNA are recorded in Table 2. Furthermore, any other phospho-proteins found to subsequently be changed in phosphorylation status are listed in Table 3 and all happen to be decreased. Many of the proteins turned out to have overlapping roles in cytoskeletal organization, filament crosslinking, mRNA processing, transcription, chromatin remodeling and organization. However, when analyzing the data in terms of which phospho-proteins may best fit the hypothesized regulatory mechanism discussed in Chapter 3, only two may be suitable. Serine/threonine-protein phosphatase 2A regulatory subunit delta isoform (2A5D or PP2A) and Inositol 1,4,5-trisphosphate receptor type 3 (ITPR3) may function in the upstream (in)activation of AMPK and should therefore be considered as potential ubiquitination targets of BCA2.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPD54</td>
<td>- Tumor protein D54</td>
</tr>
<tr>
<td></td>
<td>- cell proliferation</td>
</tr>
<tr>
<td>PPHLN</td>
<td>- Periphilin-1</td>
</tr>
<tr>
<td></td>
<td>- epithelial differentiation</td>
</tr>
<tr>
<td>LARP1</td>
<td>- La-related protein 1</td>
</tr>
<tr>
<td></td>
<td>- Down regulation of Ras-MAPK pathway</td>
</tr>
<tr>
<td></td>
<td>- RNA degradation</td>
</tr>
<tr>
<td>NIBL1</td>
<td>- Niban-like protein 1</td>
</tr>
<tr>
<td></td>
<td>- inhibition of apoptosis</td>
</tr>
<tr>
<td></td>
<td>- melanoma cell invasion</td>
</tr>
<tr>
<td>ICLN</td>
<td>- Methylosome subunit plChn</td>
</tr>
<tr>
<td></td>
<td>- Regulates small nuclear ribonucleoprotein biosynthesis</td>
</tr>
<tr>
<td></td>
<td>- platelet activation</td>
</tr>
<tr>
<td></td>
<td>- cytoskeletal organization</td>
</tr>
<tr>
<td></td>
<td>- chloride current regulator</td>
</tr>
<tr>
<td>LMA1</td>
<td>- LIM domain and actin-binding protein</td>
</tr>
<tr>
<td></td>
<td>- Cytoskeleton associated protein</td>
</tr>
<tr>
<td></td>
<td>- inhibits actin filament depolymerization</td>
</tr>
<tr>
<td></td>
<td>- Cross links filaments</td>
</tr>
<tr>
<td>SPTB2</td>
<td>- Spectrin beta chain, non-erythrocytic 1</td>
</tr>
<tr>
<td></td>
<td>- Calcium dependent movement of cytoskeleton at membrane</td>
</tr>
</tbody>
</table>

Table 1. Statistically significant proteins following BCA2 knockdown (all down-regulated)
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Function</th>
</tr>
</thead>
</table>
| ITPR3 | - Inositol 1,4,5-trisphosphate receptor type 3  
- second messenger for intracellular calcium release |
| SEC62 | - Translocation protein SEC62  
- part of a complex involved in protein translocation into the endoplasmic reticulum (ER)  
- Backward transport of proteins from the ER for UP-S degradation |
| NPM | - Nucleophosmin  
- ribosome biogenesis  
- centrosome duplication (together with BRCA2)  
- protein chaperoning  
- histone assembly  
- cell proliferation  
- regulator of p53/ARF  
- mutations are associated with acute myeloid leukemia (AML) |
| MARCS | - Myristoylated alanine-rich C-kinase substrate  
- substrate for protein kinase c (PKC)  
- actin filament corsslinking protein  
- binds calmodulin  
- cell motility  
- phagocytosis  
- membrane trafficking |
| LARP7 | - La-related protein 7  
- negative transcriptional regulator of polymerase II genes  
- a part of the 7SK small nuclear ribonucleoprotein (snRNP) |

*Table 2. Phospho-proteins upregulated following BCA2 knockdown*

Phospho-protein of interest is marked in red.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Function</th>
</tr>
</thead>
</table>
| MBB1A     | - Myb-binding protein 1A  
- represses transcription                                                      |
| HS90B     | - Heat shock protein HSP 90-beta  
- ATPase chaperone  
- regulation of proteins in cell cycle and signal transduction |
| PTSS2     | - Phosphatidylyserine synthase 2  
- cell membrane phospholipid  
- blood coagulation  
- apoptosis                                                    |
| CBX8      | - Chromobox protein homolog 8  
- part of regulatory complex required to keep certain genes repressed during development  
- chromatin remodeling and histone modification                        |
| FLNC      | - Filamin-C  
- actin crosslinking  
- anchoring of membrane proteins  
- structural function at Z lines in muscle cells                          |
| BANF1     | - Barrier-to-autointegration factor 1  
- nuclear assembly  
- chromatin organization  
- gonad development  
- promotes integration of viral DNA into genome                          |
| NIPA      | - Nuclear-interacting partner of ALK  
- component of SCF-type E3 ligase complex, SCF(NIPA), which ubiquitinates and degrades cyclin B1 (CCNB1)  
- down regulation results in early entry into mitosis                        |
| NADAP     | - Kanadapin  
- primarily nuclear localization, previously reported to have a role in kidney anion exchange but now unclear |

*Table 3. Other proteins found to be down regulated following BCA2 knockdown*
<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
</table>
| **1433G** | - Cluster of 14-3-3 protein gamma  
- adaptor protein  
- binds to a large number of proteins by recognition of a phosphoserine or threonine (ex. RAF1 and PKC)  
- highly expressed in muscle tissue |
| **LAP2B** | - Cluster of Lamina-associated polypeptide 2, isoforms beta/gamma  
- nuclear lamina assembly and structural organization of nuclear envelope |
| **HNRH1** | - Heterogeneous nuclear ribonucleoprotein H  
- pre-mRNA processing and alternative splicing regulation |
| **RBM14** | - RNA-binding protein 14  
- general nuclear co-activator  
- RNA splicing modulator |
| **ZBT7A** | - Zinc finger and BTB domain-containing protein 7A  
- represses T-cell Notch pathway to allow for B cell lineage development  
- represses CDKN2A gene, which can arrest cells in G1 and G2 phase |
| **UBP24** | - Ubiquitin carboxyl-terminal hydrolase 24  
- deubiquitinating enzyme |
| **BCLF1** | - Bcl-2-associated transcription factor 1  
- death promoting transcriptional repressor |
| **CLIC6** | - Chloride intracellular channel protein 6  
- chloride ion transport |
| **ACINU** | - Apoptotic chromatin condensation inducer in the nucleus  
- mRNA splicing  
- regulates cyclin A1 in leukemia cells |
| **LMNA** | - Prelamin-A/C  
- part of nuclear lamina |
| **PVR** | - Poliovirus receptor  
- transmembrane glycoprotein, immunoglobulin superfamily  
- natural killer cell adhesion and cell function |

**Table 3. Continued** Other proteins found to be down regulated following BCA2 knockdown
Table 3. Continued Other proteins found to be down regulated following BCA2 knockdown

Proteins of interest are marked in red.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
</table>
| MAP1B   | - Microtubule-associated protein 1B  
- microtubule assembly  
- nervous system development and function |
| NUCKS   | - Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1  
- phosphorylation substrate of casein kinase II, cyclin dependent kinases and Cdk1 |
| ZN830   | - Zinc finger protein 830  
- unconfirmed function in cell cycle of embryo |
| SGPP1   | - Sphingosine-1-phosphate phosphatase 1  
- regulates intra and extracellular levels of S1P |
| ABCF1   | - ATP-binding cassette sub-family F member 1  
- transport of various molecules across membrane |
| SNUT2   | - U4/U6.U5 tri-snRNP-associated protein 2  
- pre-mRNA splicing  
- cytokinesis and the spindle checkpoint |
| **2A5D**| - Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform  
- one of four major Ser/Thr phosphatases  
- negative control of cell growth and cell division |
| CDC20   | - Cell division cycle protein 20 homolog  
- regulatory protein of the cell cycle  
- required for chromosome separation and progression for APC/C ligase activity into anaphase |
| KTN1    | - Kinetin  
- kinesin receptor involved in kinesin driven vesicle trafficking |
| HTSF1   | - HIV Tat-specific factor 1  
- general transcription factor in elongation  
- cofactor for Tat-enhanced transcription of the HIV-1 virus |
<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
</table>
| DDX21    | - Nucleolar RNA helicase 2  
           - unwind double stranded RNA  
           - cofactor for JUN-activated transcription  
           - rRNA processing |
| AT2B1    | - Plasma membrane calcium-transporting ATPase 1  
           - intracellular calcium homeostasis |
| CHAP1    | - Chromosome alignment-maintaining phosphoprotein 1  
           - proper alignment of chromosomes at metaphase and segregation during mitosis |
| UBP37    | - Ubiquitin carboxyl-terminal hydrolase 37  
           - deubiquitinating enzyme of cyclin A to promote S phase entry  
           - antagonizes APC/C during G1/S |
| UBE2O    | - Ubiquitin-conjugating enzyme E2 O  
           - catalyzes ubiquitin transfer to proteins |
| FA21A    | - WASH complex subunit FAM21A  
           - WASH complex is present at the surface of endosomes and involved in actin polymerization |
| GAPD1    | - GTPase-activating protein and VPS9 domain-containing protein 1  
           - endocytosis  
           - GLUT4 trafficking in response to insulin stimulation  
           - EGFR trafficking and degradation  
           - GEF activity for Rab5 and GAP activity for Ras |

*Table 3. Continued* Other proteins found to be down regulated following BCA2 knockdown
PP2A has been reported to inhibit the activation of AMPK and is therefore overactive in some cancer types (Park et al., 2013; Seshacharyulu et al., 2013). Because the number of phosphopeptides associated with its expression was found to decrease following BCA2 knockdown in our MS screen, if BCA2 plays a role in keeping PP2A active on a basal level in breast cancer cells, it may be indirect; perhaps by directly targeting an inhibitory kinase of PP2A and thereby inhibiting AMPK. Furthermore, while this may be one of the ways in which cancer cells regulate the anti-tumor activity of AMPK, the effect of this particular PP2A regulatory subunit delta isoform on AMPK activity, and especially its relationship to BCA2, remains to be determined.

AMPK activation by CaMKK occurs in an AMP independent manner and is triggered by a rise in Ca$^{2+}$ concentration (Hawley et al., 2005; Woods et al., 2005). One such way in which cellular Ca$^{2+}$ concentration may rise is as a consequence of the binding of inositol triphosphate to ITPR3, which in turn initiates the activation of this receptor’s second messenger Ca$^{2+}$ release function. Therefore, it is tempting to postulate that BCA2 may inhibit AMPK activation in breast cancer cells by ubiquitination and degradation of ITPR3, which would prevent Ca$^{2+}$ mediated activation of AMPK by CaMKK. Yet others have shown Ca$^{2+}$ to mediate the activation of PP2A, and therefore inhibition of AMPK, suggesting a two-fold function of intracellular Ca$^{2+}$ on AMPK activity (Davare et al., 2000; Janssens et al., 2003). As a receptor tyrosine kinase, degradation would occur by way of the lysosome and the endocytic pathway. Indeed, BCA2 has previously been indirectly implicated in the endocytosis of another tyrosine kinase receptor, EGFR (Miyakawa et al., 2009). Whether or not ITPR3 is a substrate of BCA2 activity remains to be determined and the status of PP2A and CaMKK need to be better defined.
in regards to the BCA2 mediated inhibitory effect of AMPK. Importantly though, the preliminary results of this proteomics experiment have certainly paved the way for doing so. Furthermore, it would be interesting to assess the position of CaMKK in the positive feedback mechanism reported in response to metformin treatment of breast cancer cells. Considering the mixed reports on CaMKK facilitated activation of AMPK promoting or inhibiting tumor cell growth, clarifying its role in the current context is an important aspect for the further development of AMPK as a molecular drug target.

**The preliminary search for DSF analogs as potential BCA2 inhibitor**

Previous work has suggested that DSF inhibits BCA2 autoubiquitination activity in an *in vitro* assay using the recombinant protein (Brahemi et al., 2010; Kona et al., 2011). Because a multitude of anti-tumor effects have been described for DSF, it cannot be considered a BCA2 inhibitor solely. On this account, several “zinc-affinic” DSF analogs were synthesized and assessed for their ability to inhibit both breast cancer cell proliferation and BCA2 autoubiquitination *in vitro* (Brahemi et al., 2010). The screening of these compounds led to the conclusion that some analogs were relatively more potent than others, with the DSF analog, D24, and dithio(peroxo)thioate, DPT001, being most potent (IC$_{50}$ in the sub-micromolar range). Conversely, the structurally similar dithiocarbamates and benzisothiazolones (no disulfide bond) were all inactive (Figure 23). These data are presented in Table 4 (Brahemi et al., 2010).
Figure 23. Analogs with BCA2 inhibiting potential by way of zinc-ejection

Series of compounds structurally related to DSF were synthesized so that SAR relationships to BCA2 and cancer cell growth inhibition could be performed.
Table 4. DSF analog and carbamo(dithioperoxo)thioate activity in human breast cancer versus normal cell lines

Novel lead compounds are marked in red.
Subsequent SAR analysis found the N(C=S)S-S motif to be a common denominator for this difference (see Figure 5). Following this discovery, a group of 77 N(C=S)S-S containing compounds (listed in Table 5) were received from the NCI for further testing in breast cancer cells as potential BCA2 inhibitors. The evaluation of all 77 in MCF7 and MDA MB 231 cells has produced 15 leads that are active in the micromolar range in both cell lines (IC50 less than or around 1 µM) and are summarized in Table 6. Whether they are capable of specifically inhibiting BCA2 autoubiquitination, however, remains to be determined. Moreover, as the data in Chapter 3 suggest, the combination of a BCA2 inhibitor plus metformin may be more effective than metformin alone and therefore experiments testing the combination of these drugs with metformin may prove to be a valuable direction for future translational *in vivo* work. Since its reported discovery in 2005, much has been learned about the regulation of BCA2 and its binding partners, but still the need for more information in regards to its downstream substrates and a better understanding of why it is overexpressed in the diseased state exists. Only then can the synthesis and screening of novel compounds be further developed for its specific inhibition. Currently, a first generation of BCA2 inhibitors does exist and while the design of more specific agents is needed, access to the additional NCI compounds outlined in this chapter is certainly a good start.
Table 5. NCI compounds containing the “disulfonyl-methanethione” group

Novel lead compounds are marked in red.
<table>
<thead>
<tr>
<th>NCI Drug Number</th>
<th>MCF7 IC50 ±SD (µM)</th>
<th>MDA MB 231 IC50 ± SD (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1771 – H/7</td>
<td>0.06 ± 0.5</td>
<td>0.06 ± 0.007</td>
</tr>
<tr>
<td>16847 – T/2</td>
<td>0.03 ± 0.002</td>
<td>0.09 ± 0.005</td>
</tr>
<tr>
<td>49512 – Y/2</td>
<td>1 ± 0.03</td>
<td>1 ± 0.05</td>
</tr>
<tr>
<td>59637 – G/2</td>
<td>0.04 ± 0.3</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>66933 – L/3</td>
<td>0.3 ± 0.07</td>
<td>0.3 ± 0.05</td>
</tr>
<tr>
<td>68090 – S/1</td>
<td>0.07 ± 0.2</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>160617 – R/1</td>
<td>0.05 ± 0.1</td>
<td>0.09 ± 0.003</td>
</tr>
<tr>
<td>342008 – H/1</td>
<td>0.05 ± 0.01</td>
<td>0.4 ± 0.02</td>
</tr>
<tr>
<td>402538 – Y/6</td>
<td>0.3 ± 0.01</td>
<td>0.9 ± 0.003</td>
</tr>
<tr>
<td>403854 – G/2</td>
<td>0.05 ± 0.003</td>
<td>0.9 ± 0.05</td>
</tr>
<tr>
<td>62291 – V/2</td>
<td>0.05 ± 0.002</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>645616 – P/1</td>
<td>0.2 ± 0.005</td>
<td>0.6 ± 0.02</td>
</tr>
<tr>
<td>645619 – S/1</td>
<td>0.04 ± 0.05</td>
<td>0.04 ± 0.003</td>
</tr>
<tr>
<td>298135 – S/1</td>
<td>0.2 ± 0.01</td>
<td>0.5 ± 0.006</td>
</tr>
<tr>
<td>290661 – T/1</td>
<td>0.5 ± 0.02</td>
<td>0.6 ± 0.007</td>
</tr>
</tbody>
</table>

**Table 6. Lead NCI compounds and their respective activity in breast cancer cells**

MCF7 and MDA MB 231 cells were tested against each compound. All have IC$_{50}$ concentrations in the micromolar range with a small difference (<10 fold) in potency between the two breast cancer cell lines in select cases.
CHAPTER 5

RESULTS

The need for novel molecular targets in the treatment of cancer goes without saying. BCA2, although first isolated from an invasive breast cancer cell line, is also expressed in prostate cancer cells and perhaps in many other cancer cell types making it a great candidate for therapeutic intervention. In the current Chapter, data presented in Chapter 3 are expanded to include prostate cancer cells, where a similar negative correlation of BCA2 and pAMPK in human prostate cancer cells is described. In addition, using several human breast and prostate cancer cell lines, a decrease in BCA2 protein levels during reduced serum conditions, (indicative of G1 phase) and reappearance once the cells are synchronized with the re-addition of 10% FBS (indicative of progression into S phase), inhibitable by cycloheximide, is described. This phenomenon also occurs at the BCA2 mRNA level. Furthermore, BCA2’s cytoplasmic localization is more prominent in aggressive breast tumor cell lines, as opposed to the less aggressive, estrogen receptor-positive, breast cancer cells. BCA2 nuclear and cytoplasmic expression levels were also found to be altered during the serum reduced conditions, as well as under metformin or AICAR treatment conditions.

Results

BCA2 and pAMPK (Thr172) are inversely expressed in prostate cancer cells and metformin treatment increases the expression of both proteins
To investigate the potential relationship between BCA2 and AMPK activation, first we assessed the basal expression of these proteins in a panel of four prostate cancer cell lines (Figure 24). An inverse relationship between BCA2 and pAMPKα1 (Thr172) was observed: those cell lines with higher levels of BCA2, like LNCaP and DU145, have lower pAMPK α1 (Thr172) levels when compared to the other two examined, namely C4-2B and PC3, which contain relatively lower amounts of BCA2, but higher levels of pAMPK α1 (Thr172) (Figure 24).
Figure 24. Relationship of BCA2 to pAMPK in prostate cancer cells

The assessment of basal BCA2 and pAMPK protein expression levels in the four indicated human prostate cancer cell lines. Exponentially growing cells were lysed for Western blotting analysis with specific antibodies to BCA2, pAMPK (Thr172), total AMPKa1, and β-actin (loading control).
Previous work (Buac et. al., 2013) has revealed an increase in BCA2 levels in breast cancer cells after treatment with metformin. To determine if this also occurs in prostate cancer cells, PC3 cells were treated with 20 mM metformin for up to 12 h (Figure 25A) and the effect on pAMPK and BCA2 protein levels observed. AMPK activation occurred as early as 2 hours in these cells, which remained high for the course of the time course experiment (Figure 25A). BCA2 levels increased slightly at 2 h and further increased at 12 h (Figure 25A). A similar result was seen when LNCaP cells were treated with metformin (data not shown). The use of the chemical AMPK inhibitor, Compound C, in PC3 and LNCaP cells suppressed basal levels of pAMPKα1 and also showed some inhibition of basal level of BCA2 expression (Figure 25 B and C). Therefore, prostate cancer cells exhibit an inverse correlation between BCA2 and pAMPK protein levels and metformin treatment can increase levels of both, but compound C can inhibit BCA2 protein levels in these cells.
Figure 25. Metformin mediated activated of AMPK also increase BCA2 protein levels, inhibitable by compound C

A. Immunoblot of PC3 cells treated with metformin (20 mM) at the indicated time points. B-C. PC3 (B) and LNCap cells (C) treated with the AMPK inhibitor, compound C (20 µM) for up to 20 h.
BCA2 RNA and protein expression changes in response to serum reduction (G₁/S phase-dependent)

To determine whether BCA2 protein expression may be cell cycle-dependent, we reduced the serum concentration of growth media for prostate and breast cancer cells that express high levels of BCA2 from 10% to 1% FBS (G₁ phase) for up to 72 or 96 hours and then observed the effect on BCA2 protein expression by Western blotting (Figure 26). Panels in Fig. 26A and B are of LNCaP and C4-2B prostate cancer cells and show a clear decrease in BCA2 protein expression when these cells are in serum reduced conditions (G₁ phase) (Figure 26A and B, lanes 4 and 5 vs. 1). After 24 hours of 10% FBS release (S phase), BCA2 protein expression returned to a level similar to that of the exponentially growing control (Figure 26A and B, lane 6 vs. 1). Furthermore, MDA MB 231 and MCF7 breast cancer cells produced similar results under these conditions. Serum starvation in MDA MB 231 and MCF7 cells was performed for up to 96 hours (Figure 26C and D), since these cells were not as sensitive to serum withdrawal as the prostate cell lines used. Once again, a decrease was observed in BCA2 protein levels during the expected G₁ phase in MDA MB 231 cells (Figure 26C, lanes 2-4 vs. 1), followed by an increase of the protein once FBS is added once again (S phase) for a 24-48 hour release period (lanes 5 and 6). Figure 26D depicts the same effect in MCF7 cells. Cyclin A was used as an S phase marker and β-actin or PARP was the loading control. Therefore these results suggest that BCA2 protein expression is low under reduced serum conditions and may indicate a correlation of BCA2 expression and progression from the G₁ to S phase of the cell cycle.
Figure 26. BCA2 protein is sensitive to G1/S phase arrest

LnCaP (A) and C4-2B (B) prostate cancer cells were serum starved for up to 72 hours, indicating G1 phase, and reintroduction of 10% serum for up to 24 hrs is denoted as S phase. Breast cancer MDA MB 231 (C) and MCF7 cells (D) were serum-starved for up to 96 hours (G1) and then grown in 10% serum-containing medium for up to 48 hrs (S Phase), showing similar sensitivity to that seen in prostate cancer cells. This was followed by Western blot assay using specific antibodies to BCA2, Cyclin A (as an S phase marker) and β-Actin or PARP (as a loading control).
During the $G_1$ phase of the cell cycle, DNA transcription is minimized and no further production occurs until the cells progress successfully in S phase (Orlando et al., 2008). To assess if BCA2 mRNA levels are also $G_1$/S phase-related, breast cancer MDA MB 231 cells were serum starved, followed by addition of complete medium containing 10% FBS, as in Figure 27C. RNA was then extracted for PCR amplification and the cDNA prepared and analyzed. Figure 27A depicts these results, where BCA2 mRNA levels can be seen to drop off after 72 hours when cells were in low serum-containing medium and to an even lower level after the 96 hour starvation (Figure 27A, lane 2, 3 versus 1), with $\beta$-actin used as control. After serum was added back into these cells for 24 or 48 h, BCA2 mRNA reappeared and reached level similar to that of the exponentially grown cells (Figure 27A, lanes 4 and 5 vs. 1).

To further confirm that the increase in BCA2 protein seen during the re-addition of 10% FBS (S phase) (Figure 26) was due to new protein synthesis, prostate cancer LNCaP (Figure 27B) and breast cancer MDA MB 231 cells (Figure 27C) were again serum starved, followed by release into S phase by re-addition of serum in the growth medium for 24 or 48 h, with or without the protein synthesis inhibitor cycloheximide. Again, BCA2 protein level was increased when cells crossed were released from the low serum conditions and so the $G_1$/S boundary (Figure 27B-C, lanes 2, 3 vs. 1); however, such S phase-associated BCA2 protein increase was completely blocked in the presence of cycloheximide (Figure 27B-C, lanes 4, 5 vs. 2, 3). These results allow us to conclude that the subsequent BCA2 increase following release into S phase by the addition of whole serum may be due to new BCA2 protein synthesis.
Figure 27. BCA2 transcription and protein synthesis at the G<sub>1</sub>/S phase

**A.** mRNA analysis of BCA2 (~198 b.p = base pairs) in MDA MB 231 cells serum starved as in Figure 27. **B-C.** Immunoblot of prostate cancer LnCaP (B) and breast cancer MDA MB 231 cells (C) at G<sub>1</sub>/S with or without cycloheximide. These cells were serum starved for up to 48 hrs and then allowed to proceed into S phase for an additional 48 hours in 10% FBS. Cycloheximide (CHX, 10 µM) was added for 24 or 48 hrs as indicated in the corresponding lanes. β-actin was used as a loading control.
BCA2 localizes to the nucleus and cytoplasm of a breast cancer cell

BCA2 was previously reported to be present in both the nucleus and cytoplasm (Burger et al., 2005). To better characterize its cellular distribution, a panel of breast cancer cell lines was selected and used for fractionation of the nucleus and cytoplasm, followed by assessment of BCA2 localization (Figure 28A and B). Cell lines shown in Figure 28A are those typically classified as triple negative, void of estrogen receptor (ER), progesterone receptor (PR) and Her2, and therefore more aggressive than other breast cancer cells (Frank et al., 2013; Goncalves et al., 2013). BCA2 expression in both the nucleus and cytoplasm was seen, with a higher level in the cytoplasm (Figure 28A, lanes 2, 4, 6 vs. 1, 3, 5) amongst this group of triple negative breast cancer cells. Figure 28B depicts the estrogen receptor-positive cell lines where we see a similar distribution of BCA2 between the nucleus and cytoplasm throughout, although in varying amounts specific to the cell line. For example, DCIS.com cells have more in each compartment than the others (Figure 28B, lanes 3 and 4). Histone 3 (H3) and β-tubulin were used as nuclear and cytoplasmic markers, respectively.

The immunofluorescence data (Figure 28C) also further supports the fractionation results shown in Figure 28A and B. In this experiment we again observe both nuclear and cytoplasmic BCA2 expression in the three cell lines (MDA MB 231, T47D and MCF7) shown (Figure 28C).
Figure 28. BCA2 nuclear and localization studies in breast cancer cells

A-B. Fractionation of nucleus from cytoplasm in a panel of breast cancer cell lines divided as triple negative (A) and estrogen receptor-positive lines (B). β-Tubulin was used as the cytoplasmic control and Histone 3 (H3) as the nuclear control. C. Immunofluorescence data of BCA2 localization in MDA MB 231, MCF7 and T47D breast cancer cells. Cells were counterstained with DAPI for nuclear signal and rabbit-TRITC secondary used for BCA2.
Changes in BCA2 protein localization at the G\textsubscript{1}/S boundary and upon AMPK activation

BCA2 protein levels increase after release of serum starvation conditions and therefore crossing of the G\textsubscript{1}/S border (Figures 26-27). To study where this potentially cell cycle-dependent changes in BCA2 protein reflects changes in distribution between the cytoplasm versus nuclei, breast cancer MDA MB 231 cells were collected under the following conditions: exponentially growing (asynchronized, lanes 1-2; Figure 29); serum starved for 96 h (lanes 3-4); or released into S phase by adding 10\% FBS for 24 or 48 h (lanes 5-8). Cells were then used for fractionation of cytoplasm and nuclei. BCA2 levels were then determined in each fraction by Western blotting. Compared to asynchronized cells, the nuclear but not cytoplasmic BCA2 level in G\textsubscript{1} cells, was greatly decreased (Figure 29, lanes 3, 4 vs. 1, 2). When G\textsubscript{1} and S preparations were compared, nuclear BCA2 increased after 24 h of release from G\textsubscript{1}, but decreased again at 48 h; however the cytoplasmic BCA2 protein level was increased at 24 h and remained high after 48 h of crossing the G\textsubscript{1}/S boundary (Figure 29, lanes 5-8 vs. 3, 4). The levels of pAMPK, but not total AMPK were increased in both cytoplasmic and nuclear fractions after 48 h release from G\textsubscript{1} blockade (Figure 29, lanes 7-8 vs. 3-4). Interestingly, again an inverse relationship between BCA2 and pAMPK in both cytosol and nuclei was seen. For example, in nuclear fractions of G\textsubscript{1}, and 48 h after release preparations, a low level of BCA2 and relative high pAMPK were observed while in the nuclear fraction of the 24 h release sample, high BCA2 and decreased pAMPK were detected. These data suggest that both cytoplasmic and nuclear BCA2 protein respond to cell cycle synchronization and progression.
Figure 29. Changes in BCA2 localization during the cell cycle in MDA MB 231 breast cancer cells.

MDA MB 231 cells were G_{1}/S phase arrested followed by fractionation and analysis by Western blot. β-tubulin was used as the cytoplasmic control and Histone 3 (H3) as the nuclear control.
Recently reported was that AMPK activation (by either the anti-diabetic agent metformin or the direct AMPK activator AICAR) increases BCA2 protein expression levels in human breast cancer cells via a potential AKT-mediated feedback mechanism (Buac et. al., 2013). To study the cellular localization of BCA2 protein induced by AMPK activation, MDA MB 231 cells were treated with metformin for 4 or 8 h and then fractionated. The results (Figure 30A) show that the increase in BCA2 protein by metformin treatment occurs in both the cytoplasm and nuclei, compared to the control, at 4 and 8 hour time points (Figure 30A, lanes 3-6 vs. 1-2). Similarly, when cytoplasmic and nuclear fractions were prepared from MDA MB 231 cells treated with AICAR, an increase in cytoplasmic BCA2 was apparent, while nuclear BCA2 increased only at 8 hr time point (Figure 30B). The induction of pAMPK by metformin or AICAR occurred mainly in the cytoplasm (Figure 30A-B). Our results support the idea that AMPK signal triggers an increase in cytoplasmic and nuclear BCA2 protein that may play a role in the tumor cell survival.
Figure 30. Changes in BCA2 localization as a result of AMPK activation in MDA MB 231 breast cancer cells

MDA MB 231 cells were either treated with Metformin (A) or AICAR (B), followed by fractionation and Western blot. 20 mM metformin and 1 mM of AICAR were used for the indicated time points. β-Tubulin was used as the cytoplasmic control and Histone 3 (H3) as the nuclear control.
Discussion

BCA2 was discovered almost a decade ago and research efforts to date have culminated in numerous published works linking its expression to breast cancer development and progression (Buac et al., 2013; Amemiya et al., 2008; Bacopoulos et al., 2012; Burger et al., 2006; Burger et al., 2005; Burger et al., 2010; Kona et al., 2012). However, others have also reported an interesting protective role for BCA2/Rabring7 in HIV, where it functions to accelerate the degradation process of viral particles tethered to the cell surface, thereby restricting HIV-1 release from infected cells that are tethrin dependent (Miyakawa et al., 2009). Another recent article, by Aguilar-Hernandez et al., describes the evolutionary process and domain structure of Rabring7/BCA2, largely conserved amongst eukaryotes, but shown in this work to expand to angiosperms and even have ubiquitin binding ability (Aguilar-Hernandez et al., 2013). The collection of such works leads to one comprehensive conclusion: continuing to extensively characterize this protein will not only build upon our knowledge of RING-finger ubiquitin E3 ligases in general, but will also promote the synthesis and development of targeted agents for BCA2 inhibition in those tumor types exhibiting its overexpression.

In this chapter, an inverse relationship between BCA2 and pAMPK in prostate cancer cells (Figure 24) and its cell cycle-dependence have been described (Figure 26 A-D). Serum starvation of breast and prostate cancer cells for 72-96 hours prevents cells from entering the S phase of the cell cycle, arresting them in G1, during which time the amount of DNA is retained and no new synthesis occurs (Chen et al., 2012; Elledge, 1996; Orlando et al., 2008). This could be confirmed by the decrease in mRNA BCA2 levels observed in G1 cells (Figure 27A), further suggesting that the BCA2 change may be regulated on the transcriptional level during the G1/S
transition (Figure 26). Furthermore, the protein synthesis inhibitor, cycloheximide, was extremely potent in reducing BCA2 protein levels during S phase, affirming that the later detected protein is as a result of new protein synthesis in S phase (Figure 27 B-C).

BCA2 was previously found to be present in the nucleus and cytoplasm. Figure 28 shows that its cytoplasmic expression pattern, across a panel of breast cancer cell lines ranging in degree of aggressiveness, tends to correlate with those that are triple negative and therefore more aggressive (Figure 28A). Furthermore, in the less aggressive cell lines assessed (Figure 28B), BCA2 is seen more equally disbursed amongst the nucleus and cytoplasm. We previously investigated the transcriptional control of BCA2 by estrogen receptor and found it to be an estrogen responsive gene, which may in part explain its increased nuclear expression seen in these cell lines (Kona et al., 2012). From the fractionation experiments it may be that the cytoplasmic form of BCA2 is the major contributor to its tumor promoting phenotype and the predominant increase in cytoplasmic BCA2 observed during the S phase of the cell cycle (Figure 26) supports this idea. The decrease in nuclear (and some cytoplasmic) BCA2 during G1 is in agreement with the fact that the nucleus is the site of DNA transcription, which is at a minimum during this phase (Figure 29). Because BCA2 was shown to be a negative regulator of AMPK in MDA MB 231 cells (Buac et al., 2013), and since most of the BCA2 present in this cell line is cytoplasmic, it is again logical to conclude that it is this form of BCA2 that likely contributes to the promotion of breast cancer cell growth. Along these lines, the previously reported (and undesirable) increase in BCA2 protein levels in response to AMPK activators, metformin and AICAR, does indeed appear to be more cytoplasm-related phenomenon (Figure 29B and C),
again pointing to the possibility that this form of BCA2 is more active for its tumor promoting activity than the nuclear expressed protein.
**PUBLICATIONS**


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ABSTRACT

THE BCA2 & AMPK PARADIGM: UNRAVELING THE CANCER CONNECTION

by

DANIELA (BUAC) VENTRO

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Advisor: Dr. Q Ping Dou and Dr. Angelika M. Burger (deceased)
Major: Cancer Biology
Degree: Doctor of Philosophy

Adenosine monophosphate-activated kinase (AMPK), a master regulator of cellular energy homeostasis, has emerged as a promising molecular target in the prevention of breast cancer, and phase II and III clinical trials using the FDA-approved, AMPK activating, anti-diabetic drug metformin are promising in this regard, but the question of why metformin is protective for some women but not others still remains. Breast Cancer Associated Gene 2 (BCA2/Rabring7/RNF115), a novel RING-finger ubiquitin E3 ligase, is overexpressed in >50% of breast tumors. Herein, I hypothesized that BCA2 is an endogenous inhibitor of AMPK activation in breast cancer cells and that BCA2 inhibition would therefore increase the efficacy of metformin. My hypothesis is strongly supported by the finding that BCA2 overexpression inhibited both basal and inducible Thr172 phosphorylation/activation of AMPKα1, while BCA2-specific siRNA enhanced pAMPKα1. Furthermore, the AMPK-suppressive function of BCA2 requires its E3 ligase-specific RING domain, suggesting that BCA2 targets a critical protein controlling (de)phosphorylation of pAMPKα1 for degradation. A large scale proteomics analysis has revealed that PP2A and ITPR3 may be promising leads in this regard and should be
further investigated in relation to AMPK activation by specifically CaMKK. Activation of
AMPK by metformin not only triggered growth a inhibitory signal, but also increased BCA2
protein (but not mRNA) levels, which correlated positively with activation of AKT and could be
curbed by an AMPK inhibitor, suggesting a potential feedback mechanism from AMPK to pAkt
to BCA2. Finally, BCA2 siRNA, or inhibition of its upstream stabilizing kinase AKT, increased
the growth-inhibitory effect of metformin in multiple breast cancer cell lines, supporting the
conclusion that BCA2 weakens metformin’s efficacy in breast cancer cells. My data therefore
suggests that metformin in combination with a BCA2 inhibitor may be a more effective breast
cancer treatment strategy than metformin alone. On this account, a first generation of “specific”
BCA2 inhibitors, as well as DSF analogs, is in existence and all compounds have been evaluated
for their cancer cell anti-proliferative effects and should be further tested in combination with
metformin. The studies performed in this dissertation provide new grounds for the development
of BCA2 as a novel anti-cancer drug target, and in addition, provide awareness for the potential
limitations metformin use in the clinic may have.
Daniela spent her childhood in the former Yugoslavia and immigrated with her family to Windsor, Ontario at the end of 1991, where she grew up. Daniela received her Bachelor of Arts degree from Wayne State University with a major in Biology and minor in English in 2007. She commenced her studies in the cancer biology Ph.D program at the Wayne State University School of Medicine in fall, 2009. She began her studies under the mentorship of Dr. Angelika Burger, studying a newly discovered ubiquitin E3 ligase, BCA2, and its role in promoting breast cancer cell growth. After the passing of her original mentor, Daniela joined the laboratory of Dr. Q Ping Dou in April of 2011 where she continued to study BCA2 and its relationship to AMPK in breast cancer, providing the framework to continue with the development of BCA2 as a molecular drug target and potential marker of metformin response in breast cancer.