Reciprocal Signaling Between BCAR3 and Cas Controls Protein Function and Expression

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A Dissertation Presented to the Graduate Faculty of the University of Virginia
In the Candidacy for the Degree of
Doctor of Philosophy

Department of Microbiology, Immunology, and Cancer Biology

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May 2013

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Abstract

Adaptor proteins play a central role in relaying signals downstream of activated cell surface receptors. Adaptors have evolved as catalytically inert molecules that consist only of domains that facilitate protein-protein interactions. These proteins greatly influence signal transduction by serving as molecular scaffolds that physically and functionally bridge other signaling molecules. Through this ability, adaptors regulate many cell processes in a broad range of biological contexts. Their aberrant expression can therefore deregulate critical functions so to promote pathogenic cell behaviors and human diseases such as cancer. The research performed during the completion of this thesis project describes novel aspects of the physical and functional relationship between two adaptor proteins, Breast Cancer Antiestrogen Resistance-3 (BCAR3) and $p130^{Cas}$ (Cas). These two proteins are components of signaling pathways that regulate cell growth and motility, two processes important to both normal and cancer cells. Moreover, in many human cancers an inverse correlation exists between elevated Cas expression and relapse-free and overall survival times. The work presented here shows that BCAR3 expression augments the activity of Cas signaling pathways, and, reciprocally, Cas acts to positively regulate the expression of BCAR3. We also show that BCAR3 is constitutively bound to Cas and that the two proteins display similar degradation kinetics. These findings suggest that the function of these two proteins are intimately linked and that Cas may cyclically perpetuate its own activity through the promotion of BCAR3 expression. BCAR3 cooperates with Cas to activate c-Src (Src), a tyrosine

kinase associated with malignant growth and development. Therefore, the results described in this thesis may provide important insights into the mechanism of how the aberrant expression of non-catalytic proteins could influence malignant progression. Lastly, these data provide rationale and support for future work with the specific goal of elucidating the physiological and pathological significance of the signaling relationship between BCAR3 and Cas. Such work will help to determine whether these proteins are viable candidates for use as biomarkers to better direct therapy and improve disease outcome.

Acknowledgements

I would first like to thank my mentor Dr. Amy Bouton, Amy, for granting me the many years (and resources) that were necessary to complete this thesis project. Without her intellectual guidance and support, her singular patience and optimism concerning my work, and her dogged determination that I succeed, completing my doctorate would have been a much more difficult, if not an impossible, process. My ultimate success here and my future career are indebted to Amy, not only for the training I received but her unique ability to create an atmosphere of professionalism and familiarity got me through difficult periods of dead-end work and physical setbacks like surgeries to my back, foot, and knee. I would also like to thank my thesis committee: Drs. Sarah Parsons, David Brautigan, Margaret Shupnik, and David Kashatus. Their collective insights and suggestions helped drive my project forward and were instrumental to its final completion, as were those given by past members, Drs. Corinne Silva and Isa Hussaini.

Thanks to everyone who worked on of the 7th floor of Jordan Hall during my time here for creating an amazing work environment. To Sandy Weirich, AJ Roberts, and Virginia Coffey, their work and assistance made finishing this thesis project a much easier task. For their friendship, thanks to Melissa Anderson, Evonne Woodson, Anna Maria Copeland, Bill Newcomb, and Tom Ellison.

A million thanks to Keena Thomas, Ashley Wilson, Allison Batties, Ryan Llewellyn, Michael Gutknecht, Michelle Abshire, Brianne Ray, Huy Ta, Kate Owen, Kat Schornberg, and Randy Schrecengost: present and past members of

the Bouton Lab, first-class co-workers, great resources, and wonderful friends. On a daily basis over the past eight years, I relied on this group of people for support, advice, humor, and victuals. I cannot adequately express my gratitude for them.

Thanks to my good buddy Rob Tacke for his (almost) constant companionship during the seven years we were in school together. The time spent with Rob, his wife Trisha, and sister Sarah in Charlottesville will always be valued.

Thanks to my family – my mother, father, brothers Hank and Joel, and sister-in-law Terese – for all their encouragements over the years.

And thanks to my wife, Libby, for more than I could describe here. Her constant support and motivation were invaluable during the completion of this project.

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Abbreviations:

- PTK – non-receptor protein tyrosine kinase
- RTK – receptor tyrosine kinase
- Crk – CT10 regulator of kinase
- SH2 – Src homology-2
- SH3 – Src homology-3
- BCAR3/NSP2 – Breast Cancer Antiestrogen Resistance-3/novel SH2-containing protein 2
- NSP – novel SH2-containing protein
- Cas/BCAR1 – Crk-associated substrate/Breast Cancer Antiestrogen Resistance-1
- GEF – Guanine nucleotide exchange factor
- GF – growth factor
- ECM – extracellular matrix
- CQ – chloroquine
- AHA – azidohomoalaine
- PBS – phosphate buffered saline
- eIF4E – eukaryotic translation initiation factor 4E
- 4EBP – eIF4E binding partner
- EGF – epidermal growth factor
- EGFR – epidermal growth factor receptor
- PI3K – phosphatidylinositol 3-kinase

- PTP – protein tyrosine phosphatase
- STAT - signal transducer and activator of transcription
- HER2 – human epidermal growth factor receptor 2
- TGF-β – transforming growth factor-β
- PDGFR – platelet-derived growth factor receptor
- GAP – GTPase activating protein
- VEGFR – vascular endothelial growth factor receptor
- Csk – C-terminal Src kinase
- SYF – $Src^{-/-}$ $Yes^{-/-}$ $Fyn^{-/-}$
- pTyr/pY – phosphotyrosine
- pSer – phosphoserine
- Pro – proline
- BSA – bovine serum albumin
- Dox – doxycycline
- Tet – tetracycline
- MMTV – mouse mammary tumor virus
- IHC – immunohistochemistry
- UPS – ubiquitin proteasome system
- kDa – kilodalton
- PP2A – protein phosphatase 2A
- Shep1 – SH2 domain-containing Eph receptor-binding protein 1

- NEDD9 – neural precursor cell expressed, developmentally down-regulated 9
- FXR2 – fragile X mental retardation syndrome-related protein 2
- FMRP – fragile X mental retardation protein
- CHX – cycloheximide
- mRNA – messenger RNA
- siRNA – small interfering RNA
- MFI – mean fluorescence intensity
- C.I. III – calpain inhibitor III
- WT – wild-type
- GST – glutathione S-transferase
- TUBEs – tandem ubiquitin binding entities
- SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis
- DMSO - dimethyl sulfoxide
- FN – fibronectin
- PFS – progression-free survival
- MFS – metastasis-free survival
- E2 – estrogen
- 4-OHT – 4-hydroxytamoxifen
- CSS – charcoal stripped serum
- CDS – coding sequence
- miRNA – microRNA

Chapter 1. Introduction*

*This Chapter contains text adapted from:

Cas and NEDD9 Contribute to Tumor Progression through Dynamic Regulation of the Cytoskeleton
Michael S. Guerrero, J. Thomas Parsons, Amy H. Bouton
Genes & Cancer. 2012 May; 3(5-6): 371–381.

Preface: This introductory chapter begins with a brief review of the molecular biology of adaptor protein function in order to place this class of proteins within the proper context of cell physiology. This chapter mainly focuses on the adaptor proteins BCAR3 and Cas, with a specific emphasis on protein domain structure and organization to give the reader a better understanding of how these two proteins act to facilitate signal transduction. As these proteins have been shown to cooperatively activate the protein tyrosine kinase c-Src, a review of the structure and function of this signaling molecule is also provided. In addition, both the established and potential roles that these proteins play in tumorigenic processes are discussed.

1.1 – Adaptor Proteins

The primary function of adaptor proteins is to recruit other signaling proteins, including additional adaptors, to propagate and transduce appropriate signals downstream of activated cell surface receptors and cytoplasmic non-receptor protein tyrosine kinases (PTKs). This activity is mediated by the creation of multi-protein complexes that act as signaling nodes that drive many

fundamental cell processes. The formation of these protein complexes is facilitated by the unique domain composition of adaptor proteins. This class of proteins is defined by the presence of multiple protein-binding domains and an absence of intrinsic enzymatic activity (Flynn 2001). These protein-binding domains are the sites where protein-protein interactions take place, and the specificity of these associations is mediated by the sequence, structure, and post-translational modification (primarily phosphorylation) of such domains.

The first recognized adaptor protein, CT10 regulator of kinase (Crk), was originally identified as the transforming gene product of the avian CT10 retrovirus (Sriram and Birge 2010). The discovery of this unique viral oncogene, v-*Crk*, whose product consists only of non-catalytic Src homology (SH) domains, yet elevates intracellular phosphotyrosine levels (Mayer, Hamaguchi et al. 1988), led to the identification and characterization of several cellular Crk homologues, prototypes of a novel class of proteins – adaptors (Matsuda and Kurata 1996, Hossain, Dubielecka et al. 2012). Crk proteins contain a single Src homology-2 (SH2) domain and one or two Src homology-3 (SH3) domains (Sriram and Birge 2010). SH2 and SH3 domains are conserved protein modules found in many signaling proteins that bind to specific regions of phosphotyrosine residues and proline-rich motifs, respectively (Pawson and Scott 1997). Along with SH2 and SH3 domains, adaptor proteins can contain a wide variety of protein binding modules each recognizing specific, conserved peptide sequences and structures. These modules include, but are not limited to, WW domains, phosphotyrosine binding (PTB) domains, pleckstrin homology (PH) domains (which can serve as

platforms for both phosphoinositide binding as well as protein-protein binding), leucine zippers, and 14-3-3 protein ligands (Mitsuuchi, Johnson et al. 1999, Flynn 2001, Scott and Pawson 2009, Hrdinka, Draber et al. 2011, Scheffzek and Welti 2012). And while adaptor proteins consist simply of linked protein-binding domains, they strongly influence the activity of other signaling proteins, thus affecting many signaling cascades and biological processes. Specifically, adaptor proteins act to spatially and temporally organize activated signaling networks downstream of growth factor (GF) binding to its cognate receptor and integrin engagement to extracellular matrix (ECM). In so doing, they regulate biological processes such as development, immune cell signaling, proliferation, survival, apoptosis, adhesion, and motility (Tikhmyanova, Little et al. 2010, Hrdinka, Draber et al. 2011, Guerrero, Parsons et al. 2012, Tsuda and Tanaka 2012, Wang, Sheng et al. 2012). Not only are these proteins necessary to transduce signals from the cell surface, but their overexpression can also catalyze the formation of active signaling complexes in the absence of an external stimulus. Therefore, it is not surprising that the aberrant expression and/or activity of adaptors has been implicated in playing a pathological role in human diseases such as cancer, diabetes, Parkinson's disease, and Alzheimer's disease (Ravichandran 2001, Giubellino, Gao et al. 2007, Kabbage and Dickman 2008, Sriram and Birge 2010, Hossain, Dubielecka et al. 2012, Wallez, Mace et al. 2012, Wang, Sheng et al. 2012, Ogawa, Yamazaki et al. 2013). Given connections to such a broad spectrum of disease, research focused on the regulation of adaptor protein activity and expression, like the work performed

during the completion of this thesis, may improve our understanding of the initiation and/or progression of certain human diseases. Such work could also assist in the development of improved therapeutic tools.

Since the discovery of the v-*Crk* oncogene 25 years ago, a plethora of additional adaptor proteins have been identified and characterized, and through their study much has been learned about the molecular and cellular biochemistry of both normal and disease processes. Two adaptor proteins, Breast Cancer Antiestrogen Resistance-3 (BCAR3) and $p130^{Cas}$ (also known as BCAR1, hereafter referred to as Cas), are the focus of this thesis. BCAR3 and Cas were identified in a random search for genes involved in antiestrogen resistance, mediated by retroviral insertion mutagenesis (van Agthoven, van Agthoven et al. 1998). The ectopic overexpression of BCAR3, a previously unknown protein, or Cas, a known regulator of cytoskeletal and adhesion dynamics, sustains proliferation in the presence of the antiestrogens tamoxifen and ICI 182,780 in previously sensitive, estrogen receptor (ER)-positive breast cancer cell lines (van Agthoven, van Agthoven et al. 1998, Brinkman, van der Flier et al. 2000). Adjuvant treatment with antiestrogens, which competitively inhibit estrogen-ER binding, is routinely used to treat ER-positive breast cancer with a high degree of success, yet up to one-third of ER-positive breast cancers acquire resistance to these drugs over time (Musgrove and Sutherland 2009). Therefore, the biochemical and cellular functions of proteins like BCAR3 and Cas generate a great deal of interest, as a better understanding of the mechanisms of drug resistance could lead to the development of new biomarkers to better predict

therapeutic response and to the identification of novel therapeutic targets to better treat refractory disease.

Remarkably, despite the inherent randomness of the original genetic screen, BCAR3 and Cas are binding partners that synergistically act to promote the activity of the non-receptor tyrosine kinase c-Src (Src) (Gotoh, Cai et al. 2000, Riggins, Quilliam et al. 2003). The transient overexpression of either BCAR3 or Cas also enhances the migratory and invasive potential of normal and tumor cells (Klemke, Leng et al. 1998, Brabek, Constancio et al. 2004, Schrecengost, Riggins et al. 2007). Given the promotion of cellular processes characteristic of tumor aggressiveness, the specifics of BCAR3 and Cas function are the subject of much investigation. The research presented in the following chapters of this thesis addresses novel aspects of the functional interrelationship that exists between these two proteins. Through this work, we describe a unique, functionally reciprocal relationship between these adaptors that controls both protein function and expression. As such, dysregulation of this relationship has a high potential for influencing human disease, specifically breast cancer. First, this introductory chapter provides the reader with a structural and functional review of BCAR3, Cas, and Src.

1.2 – Breast Cancer Antiestrogen Resistance-3 (BCAR3)

1.2.1 – *Discovery*

Fifteen years ago, four papers independently reported the discovery of a novel SH2 domain-containing protein. The human protein was named BCAR3

(van Agthoven, van Agthoven et al. 1998), NSP2 (novel SH2-containing protein 2)(Lu, Brush et al. 1999), or Shep2 (SH2 domain-containing Eph receptor-binding protein 2) (Dodelet, Pazzagli et al. 1999), and the murine homologue was termed AND-34 (Cai, Clayton et al. 1999).

BCAR3, the focus of the first paper to be published, was identified in a retroviral-mediated mutagenesis screen designed to search for novel genes whose products are involved in the establishment of antiestrogen resistance (van Agthoven, van Agthoven et al. 1998). The paper reported the discovery of a 90kDa protein with an amino-terminal SH2 domain. Indeed, following its isolation and cloning, ectopic expression of BCAR3 was shown to induce resistance to the antiestrogens tamoxifen and ICI 182,780 in estrogen-dependent, ER-positive breast cancer cell lines. Furthermore, the authors demonstrated that BCAR3 mRNA is expressed in a group of breast, ovarian, and endometrial cancer cell lines and, intriguingly, that mRNA levels inversely correlate with ER status. This finding provided evidence that BCAR3 expression (and possibly protein levels) may negatively track with a biomarker that is used during the prognosis and selection of therapy for certain cancers. However, whether BCAR3 protein expression correlates with any prognostic or predicative markers of human breast cancer remains to be determined; this is further addressed in the discussion. BCAR3 mRNA expression was also verified in a number of normal human tissues, including heart, placenta, skeletal muscle, spleen, prostate, testis, ovary, small intestine, colon, and fetal kidney (van Agthoven, van Agthoven et al. 1998).

Shortly after the publication of BCAR3, a second group reported the discovery of a new family of adaptor proteins (Lu, Brush et al. 1999). An expressed sequence tag (EST) database was screened for sequences homologous to SH2 domains of known adaptor proteins in an effort to identify novel proteins involved in growth factor and integrin signaling. An EST, homologous to the SH2 domain of the adaptor protein Shc, was found and cloned, and the full-length cDNA was named *NSP1* (novel SH2-containing protein 1). The EST library was rescreened using the *NSP1* sequence and two related cDNAs were identified, *NSP2* and *NSP3* (Lu, Brush et al. 1999). This publication established a unique family of adaptors, the novel SH2-containing protein (NSP) family. All three proteins contain an amino-terminal SH2 domain, a medial proline/serine-rich region, and a carboxyl-terminal domain with an amino acid motif frequently found in proteins with “guanylate exchange activity” (Lu, Brush et al. 1999). The authors noted that, during the preparation of their manuscript, the sequence of NSP2 had been previously published as BCAR3. Consistent with the work in the first paper, NSP2/BCAR3 mRNA expression is reported in human heart, placenta, lung, skeletal muscle, kidney, and fetal lung and kidney (Lu, Brush et al. 1999). Although this paper only focused on NSP1, it did reveal two key features later shown to be shared across the NSP family: binding to the cytoplasmic domain of stimulated cell-surface receptor tyrosine kinases (RTK), and a physical association to Cas. In response to epidermal growth factor (EGF), NSP1 was shown to associate with the EGF-receptor

(EGFR). A high degree of constitutive NSP1/Cas binding was also demonstrated (Lu, Brush et al. 1999).

Shep2 was discovered in a yeast two-hybrid screen designed to identify proteins involved in Ephrin (Eph) receptor signaling (Dodelet, Pazzagli et al. 1999). The expression and activity of Eph RTKs, and prevalence of their ephrin ligands, are associated with many aspects of human cancer (Pasquale 2010). In this study, the phosphorylated cytoplasmic domain of the Eph receptor EphB2 was the "bait," and the first "prey" isolated was a protein the authors name Shep1 (SH2 domain-containing Eph receptor-binding protein 1). The sequence of Shep1 was used to screen existing EST data sets and a second, closely related protein was identified, Shep2. As in the above paper, Shep2 was found to be the same protein as the recently identified BCAR3. In agreement with the analysis in the NSP paper, Shep1 and Shep2 were shown to contain an amino-terminal SH2 domain, a proline/serine-rich medial region, and a carboxyl-terminus with "weak, but significant homology" to the Cdc25 homology domain of Ras family GEFs (Dodelet, Pazzagli et al. 1999). These authors also showed that 1) the Shep1 SH2 domain associated with phosphotyrosine residues of both EphB2 and EphA4 and 2) that a Shep1 GEF domain GST fusion protein pulled-down R-Ras and Rap1A in the extracts of cells transfected with these Ras family GTPases. However, in *in vitro* exchange assays, this Shep1 GEF domain construct did not promote GDP/GTP exchange (Dodelet, Pazzagli et al. 1999).

The mouse homologue, AND-34, was identified in a study set up to examine the molecular mechanisms that guide negative selection in the thymus

(Cai, Clayton et al. 1999). AND-34 mRNA was found as an up-regulated transcript in thymic stromal cells of AND TCR (T cell receptor) transgenic RAG-$2^{-/-}$ H-2^{d} mice following *in vivo* CD3ε cross-linking. Additional analysis of thymic epithelial cell lines demonstrated that levels of both AND-34 transcript and protein are increased in response to interleukin-1 (IL-1) and tumor necrosis factor (TNF), cytokines also elevated in the CD3ε cross-linking experiment. Further characterization revealed a 93kDa protein that contains an amino-terminal SH2 domain, a proline-rich region, and a carboxyl-terminal domain with sequence homology to the Cdc25 family of Ras GEFs (Cai, Clayton et al. 1999). AND-34 was found to constitutively associate with Cas, and the open reading frame (ORF) of AND-34 was shown to share 86% sequence identity to BCAR3, and 93% homology at the amino acid level. The authors concluded from these data that AND-34 is involved in adhesion and cytokine signaling. NSP2/Shep2/AND-34 will be referred to as BCAR3 throughout the remainder of the thesis.

All together, these reports describe a previously unknown signaling molecule that induces a drug resistant phenotype in breast cancer cell lines, forms a constitutive association with Cas, and is a member of a novel protein family defined by a conserved domain structure: an amino-terminal SH2 domain, a medial proline/serine-rich region, and a carboxyl-terminal domain with sequence homology to the Cdc25 domain of Ras GEFs. Since the discovery of BCAR3 and the first descriptions of its basic domain structure, the majority of published literature has focused on the molecular mechanism of antiestrogen

resistance with an emphasis on the identification of domain-specific binding partners.

1.2.2 – *Basic Structure and Domain Organization of BCAR3 and Interacting Proteins*

Like all adaptors, BCAR3 consists solely of modular protein-binding domains. As discussed above, BCAR3 and the NSP family proteins are distinguished by the presence of an amino-terminal SH2 domain, an adjacent proline/serine-rich region, and, for want of a better term, the carboxyl-terminal Cdc25 GEF-homology domain (Figure 1.1). In this section, each domain/region is described and known binding partners are discussed.

The SH2 domain is located between amino acids 154 and 253 (van Agthoven, van Agthoven et al. 1998) and deletion of this region abolishes the ability of BCAR3 to promote cell growth in the presence of antiestrogens (Felekkis, Narsimhan et al. 2005). This inability to induce the drug-resistant phenotype is evidence of an SH2 domain-binding protein involved in proliferative and survival signaling. An *in vitro* analysis using extracts of cells that had been stimulated with EGF, demonstrated that EGFR associates with full-length BCAR3 and an SH2 domain fragment (Oh, van Agthoven et al. 2008). Furthermore, this same study showed that either BCAR3 knockdown, mediated by small interfering RNA (siRNA), or the transient expression of an SH2 domain fragment blocks EGF-induced DNA replication. While these experiments suggest a key role for BCAR3 in mediating the mitogenic response to EGF, a direct or indirect physical

association between BCAR3 and EGFR has not been shown *in vivo*. To date, this remains the only published report of a BCAR3/RTK interaction and additional studies are needed to verify if such an interaction takes place in the cell. A more recent study demonstrated that in response to adhesion, BCAR3 binds to the receptor-like protein tyrosine phosphatase (PTP) PTPα in an SH2/phosphotyrosine-directed manner (Sun, Cheng et al. 2012). While the interactions of the BCAR3 SH2 domain are not the focus of this thesis, BCAR3/RTK and BCAR3/PTPα interactions remain an area of great interest. Their continued study will improve our understanding of BCAR3 function through the identification of the specific external cues that BCAR3 acts to transduce, and through the determination of the subcellular distribution of BCAR3 in response to these environmental factors.

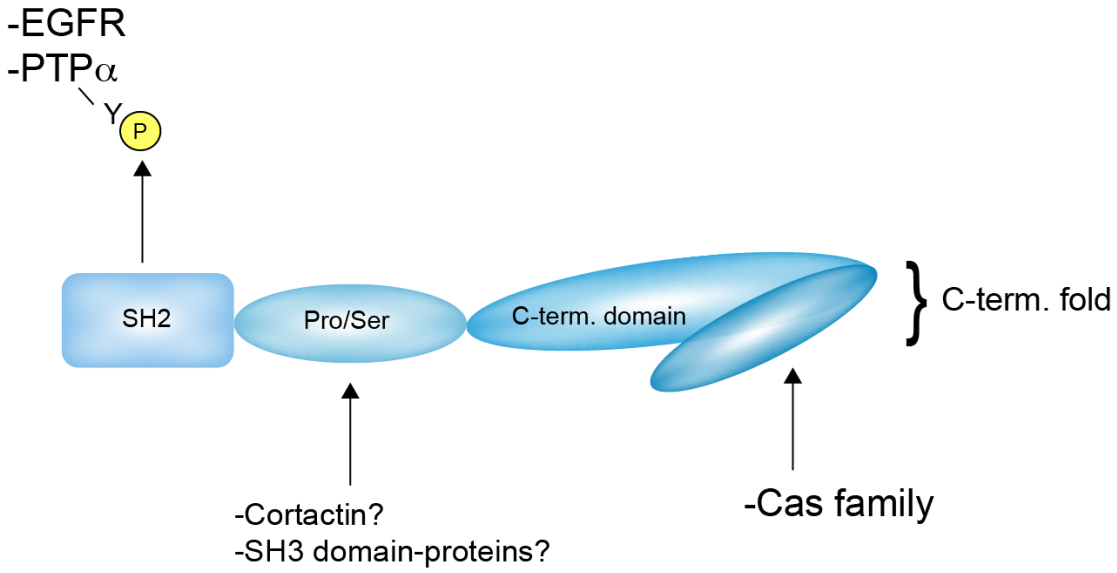

Figure 1.1: BCAR3 domain structure and binding partners.

BCAR3 has three conserved domains that mediate its association with other proteins: an N-terminal SH2 domain, a proline/serine-rich region, and a C-terminal domain that contains a unique structural fold that allows binding sites to Cas family member proteins. Domain-specific binding partners are shown.

Directly adjacent to the SH2 domain is the medial proline/serine-rich region. This motif is a putative ligand for SH3 domain-containing proteins, but so far no interacting partners have been identified (Wallez, Mace et al. 2012). However, Wallez et al. reported a predicted association with the SH3 domain of cortactin, a cytoskeleton modulating protein and Src substrate [(Wallez, Mace et al. 2012) scansite.mit.edu]. The predicted interaction with cortactin is intriguing, as work by our lab has demonstrated that BCAR3 is involved in the reorganization of the actin cytoskeleton in response to both EGF and fibronectin [(Schrecengost, Riggins et al. 2007); Wilson et al., 2013, *manuscript in progress*].

The carboxyl-terminal 281 amino acids of BCAR3 are approximately 30% homologous to the Cdc25 catalytic domain of the Ras subfamily GEF Son of Sevenless (Sos) (Cai, Clayton et al. 1999), and are essential during the conversion to an antiestrogen resistant phenotype (Felekkis, Narsimhan et al. 2005). Several early studies demonstrated that the overexpression of BCAR3 promotes nucleotide exchange in both Ras and Rho family GTPases, including Ral, Rap1, R-Ras, Cdc42, and Rac (Gotoh, Cai et al. 2000, Cai, Felekkis et al. 2003, Cai, Iyer et al. 2003). Furthermore, Rap1 activity has been implicated in playing a key role in BCAR3-mediated cell migration (Riggins, Quilliam et al. 2003). Despite these initial findings, purified BCAR3 or Shep1 displays no nucleotide exchange activity *in vitro* towards Rap1A or Rap2A (Mace, Wallez et al. 2011). Further structural and biochemical analyses showed that this domain is catalytically inert due to the evolution of a unique fold that occludes both the GTPase binding site and the catalytic core. Importantly, it is this native fold that

actually creates the binding site for Cas and Cas family proteins (Mace, Wallez et al. 2011). In this study, neither unbound nor Cas-bound protein demonstrated GDP release *in vitro* (Mace, Wallez et al. 2011). Furthermore, two studies using both *in vitro* pull-down assays and isothermal calorimetric titration did not detect an interaction between purified BCAR3 and Rap1A, or between BCAR3 and RalA or Rap1A when BCAR3 was bound to NEDD9, a Cas family member (Garron, Arsenieva et al. 2009, Mace, Wallez et al. 2011). No other carboxyl-terminal binding partners are known outside of the Cas family proteins, and these studies demonstrate that BCAR3 has evolved a native structural conformation that converted a catalytic subunit into a Cas family protein-binding domain. Currently, the overriding thought is that BCAR3 mediates GTPase activity through the activation of Ras and Rho family specific GEFs, which can occur downstream of Cas signaling.

Early studies first characterized the carboxyl-terminal-mediated interaction between NSP family members and Cas (Gotoh, Cai et al. 2000, Sakakibara and Hattori 2000). Deletion mutants of BCAR3 identified the carboxyl-terminus as the region of Cas interaction (Gotoh, Cai et al. 2000). And the direct interaction between NSP proteins and Cas was first demonstrated through the incubation of purified His-tagged Shep1 with GST-tagged Cas, which had been immobilized on glutathione-Sepharose beads (Sakakibara and Hattori 2000). These two seminal studies are of fundamental importance to the work of this thesis, as the characterization of the direct association suggested a functional association between BCAR3 and Cas as well. The focus of much of the work done during the

development and completion of this project was on how the interaction between BCAR3 and Cas may be involved in their functional regulation.

These structure/function analyses of BCAR3 define a true adaptor protein: a molecule lacking catalytic activity and consisting solely of linked protein-binding domains. Furthermore, the organization of these motifs is in line with a protein that functions to connect membrane-associated receptors to their downstream effectors. The following section describes BCAR3 adaptor function in a cellular and biological context.

1.2.3 – *Biological, Cellular, and Biochemical Functions of BCAR3*

As mentioned above, BCAR3 overexpression promotes drug resistant and migratory phenotypes in cultured cells. Depletion of BCAR3 from highly motile breast cancer cell lines dramatically reduces their migratory and invasive capacities (Schrecengost, Riggins et al. 2007). These findings suggest that BCAR3 functions within a signaling network that regulates cell proliferation, survival, and motility. In line with this idea, BCAR3 plays a role in the mitogenic and haptotactic response to EGF. As discussed above, BCAR3 expression is required for EGF-induced DNA synthesis and is reported to bind to EGFR via its SH2 domain following ligand stimulation in a non-tumorigenic breast epithelial cell line (Oh, van Agthoven et al. 2008). In a highly migratory breast cancer cell line, BCAR3 depletion results in an attenuated cytoskeletal response to EGF, as BCAR3-depleted cells fail to undergo growth factor-mediated cytoskeletal reorganization and exhibit reduced migration towards EGF (Schrecengost,

Riggins et al. 2007). Thus, BCAR3 appears to contribute to two processes central to the development and maintenance of multicellular organisms. However, no developmental defects are observed in BCAR3 knockout mice (Near, Smith et al. 2009), which makes discerning the exact physiological role of BCAR3 difficult.

Because of its ability to establish antiestrogen resistance, a lot of research has examined the effect of BCAR3 on other proteins known to control proliferation and survival. For example, BCAR3 overexpression augments the activity of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, promotes the activation of Rho GTPases like Rac and Cdc42, and induces the expression of cyclin D1 (Cai, Iyer et al. 2003, Felekkis, Narsimhan et al. 2005). The chemical inhibition of either PI3K or Rac reverses BCAR3-induced resistance, and the expression of a dominant negative version of Rac negates the effect on cyclin D1 (Cai, Iyer et al. 2003, Felekkis, Narsimhan et al. 2005). Interestingly, the overexpression of either NSP1 or Shep1 activates PI3K/Akt, Rac, and Cdc42 to a similar degree as BCAR3, but both proteins are ineffectual as mediators of antiestrogen resistance (Near, Zhang et al. 2007). This demonstrates that while PI3K and Rac are necessary components of the mechanism of BCAR3-mediated resistance, their activities are clearly not sufficient to establish the full resistant phenotype.

Shortly before work on this thesis began, our lab published a paper showing that BCAR3 enhances Cas-mediated Src kinase activation and cell migration (Riggins, DeBerry et al. 2003). Others demonstrated that Cas

overexpression induces cell migration, antiestrogen resistance, and augments the capacity of Src to promote cell invasion through matrigel (Klemke, Leng et al. 1998, Brinkman, van der Flier et al. 2000, Brabek, Constancio et al. 2004). Moreover, Cas binds to Src, also a known regulator of cell proliferation, and serves as both a substrate and a potent activator of the kinase (Nakamoto, Sakai et al. 1996, Burnham, Bruce-Staskal et al. 2000, Nasertorabi, Tars et al. 2006). Based on these observations, and in addition to the direct physical association between BCAR3 and Cas, we became interested in further analyzing the physical and functional relationship between BCAR3, Cas, and Src. Specifically, how does BCAR3 enhance Src kinase activity through Cas? And what are the functional effects of BCAR3-mediated Src activation? Given this focus on Cas and Src, the remaining sections of this introductory chapter provide the reader with an overview of the structure and function of these two proteins.

1.3 – p130^{Cas}

1.3.1 – *Discovery*

Five years prior to the isolation and characterization of the human *BCAR* genes, Cas (Crk-associated substrate; p130) was discovered as a highly tyrosine phosphorylated 130kDa protein in v-*Crk* and v-*Src* transformed rat 3Y1 fibroblasts (Sakai, Iwamatsu et al. 1994). Moreover, this study showed that Cas forms complexes with both Crk and Src, and demonstrated that phosphorylated Cas localizes to cellular membranes, indicative of a molecule involved in the transduction of signals downstream of membrane-associated proteins. Indeed,

early research showed Cas to be an adaptor protein that forms complexes with a large number of signaling proteins upon integrin engagement to the ECM, including the PTKs Src and focal adhesion kinase (FAK), the adaptor molecules Crk and Nck, and the GEFs Sos and C3G (Schlaepfer and Hunter 1996, Vuori, Hirai et al. 1996). Following adhesion, Cas is phosphorylated on numerous tyrosine residues, primarily by Src but also, in part, by FAK (Petch, Bockholt et al. 1995, Vuori and Ruoslahti 1995, Schlaepfer, Broome et al. 1997). Cas bridges the function of these signaling proteins through its unique modular domain structure. One biological result of such integration is the induction of cell migration and invasion (Klemke, Leng et al. 1998). As a direct, constitutive association between BCAR3 and Cas had been established early, the literature describing Cas signaling pathways helped to functionally place BCAR3 activity within previously established protein networks. Cas is the founding member of the Cas protein family that also includes NEDD9/Hef1, EFS, and CASS4; their conserved domain structure defines this family.

1.3.2 – *Basic Structure and Domain Organization of Cas and Interacting Proteins*

Cas contains four domains: an amino-terminal SH3 domain; a large substrate domain that contains 15 YxxP repeats that, when phosphorylated, provide docking sites for SH2 domain-containing proteins; a serine-rich four-helix bundle; and a highly conserved carboxyl-terminal domain that contains binding sites for the Src family of protein tyrosine kinases and a second four-helix bundle that promotes dimerization with BCAR3 and NSP family members (Fig. 1.2).

The amino-terminal SH3 domain is essential in regulating many Cas-dependent processes, as this site mediates many heterologous protein interactions. Proteins that directly modulate Cas tyrosine phosphorylation, and thus Cas-mediated signaling, dock at the SH3 domain. The carboxyl-terminal poly-proline regions of FAK and the FAK family member, proline-rich tyrosine kinase 2 (Pyk2) bind to the SH3 domain of Cas (Polte and Hanks 1995, Lakkakorpi, Nakamura et al. 1999). The protein tyrosine phosphatases, PTP1B and PTP-PEST also bind here and dephosphorylate Cas to negatively affect downstream signaling (Wisniewska, Bossenmaier et al. 2005). There is also evidence that Cas directly interacts with the Rap1 and R-Ras GEF C3G (Kirsch, Georgescu et al. 1998). These SH3 domain interactions serve to positively and negatively regulate Cas-mediated signaling.

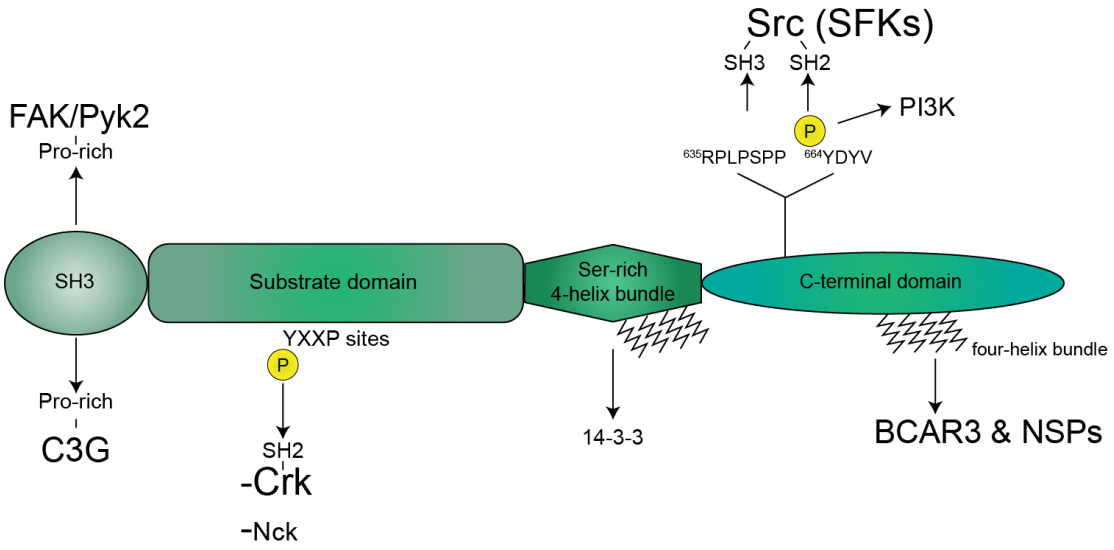

Figure 1.2: Cas domain structure and binding partners.

Cas has four conserved domains that mediate its associations with other proteins: an N-terminal SH3 domain, a substrate domain containing multiple YxxP motifs that are phosphorylated by SFKs, a serine-rich four-helix bundle, and a C-terminal domain that contains binding sites for SFKs and members of the novel SH2-containing protein (NSP) family. Domain-specific binding partners are shown.

Phosphorylation of tyrosine residues within YxxP sites in the substrate domain create binding sites for SH2 domain-containing proteins, particularly Crk. Binding to Crk serves to functionally connect Cas to active Rho and Ras family GTPases through the recruitment of family-specific GEFs (Vuori, Hirai et al. 1996, Schlaepfer, Broome et al. 1997, Kiyokawa, Hashimoto et al. 1998, Klemke, Leng et al. 1998, Burnham, Bruce-Staskal et al. 2000, Tamada, Sheetz et al. 2004). In this manner, the substrate domain facilitates the downstream signaling of Cas following focal adhesion association. An interesting feature of the substrate domain is its dynamic structure. The adjoining amino- and carboxyl-terminal regions serve to localize Cas to focal adhesions, effectively anchoring Cas to the cytoskeleton. Studies have demonstrated that the physical stretching of matrix induces a similar lengthening of the substrate domain and leads to a dramatic increase in the Src-dependent phosphorylation of YxxP motifs (Tamada, Sheetz et al. 2004, Sawada, Tamada et al. 2006). The plastic nature of this domain appears to allow for the concealment or the presentation of these Src substrates. This is thought to be one mechanism of how Cas signaling is regulated following changes in extracellular matrix composition (Sawada, Tamada et al. 2006, Tikhmyanova, Little et al. 2010).

The sequence and unique structure of the serine-rich four-helix bundle mediates the binding of 14-3-3 proteins (Briknarova, Nasertorabi et al. 2005). The association between Cas and 14-3-3 proteins is dependent on adhesion and the phosphorylation of these serine residues (Garcia-Guzman, Dolfi et al. 1999).

Although the activities of 14-3-3 proteins have pleiotropic effects on cell behavior, no biological consequence of their association to Cas has been identified.

The carboxyl-terminal domain is of particular importance to the work described in this thesis, as it contains the sites of both Src and BCAR3 interaction. The SH3 and SH2 domains of Src bind to a carboxyl-terminal proline-rich region and to the phosphotyrosine (pY) residues pY664 and pY666, respectively (Nakamoto, Sakai et al. 1996)(Fig. 1.4B). The SH3 domain interaction is established first. This brings the kinase in close proximity to the adjacent tyrosine residues, which are then phosphorylated by Src to create the SH2 domain-docking site (Nakamoto, Sakai et al. 1996, Burnham, Bruce-Staskal et al. 2000, Pellicena and Miller 2001). The occupation of these two Src domains maintains the kinase in a catalytically active conformation, specifically through the displacement of auto-inhibitory interactions mediated by the SH3 and SH2 domains (see Fig. 1.4B). This leads to increased substrate phosphorylation, including the processive tyrosine phosphorylation of the Cas on YxxP motifs (Burnham, Bruce-Staskal et al. 2000, Pellicena and Miller 2001).

Following the Src-binding motifs is a second four-helix bundle structurally similar to focal adhesion targeting (FAT) domains present in FAK and other proteins (Mace, Wallez et al. 2011). The FAT region of Cas binds to the Cdc25 GEF-homology domain of BCAR3 (Mace, Wallez et al. 2011). The entire NSP family and three of the four Cas family proteins have conserved these regions (Mace, Wallez et al. 2011), which suggests the evolution of family-specific

signaling duplex that may have functionally divergent activities depending on the composite proteins and the given cell context. Indeed, BCAR3 and Shep1 associate with NEDD9 in B cells, and the genetic deletion of Shep1 impairs NEDD9 phosphorylation and B cell development and migration towards cytokines (Cai, Felekkis et al. 2003, Al-Shami, Wilkins et al. 2010, Browne, Hoefer et al. 2010).

1.3.3 – *Biological, Cellular, and Biochemical Functions of Cas*

Unlike BCAR3, the biological and physiological activities of Cas are much more established. At the cellular level, Cas regulates cytoskeletal remodeling and the formation of membrane protrusions, functions that are essential to a wide variety of cellular and biological processes, including cell movement through tissue, cell division, morphogenesis, homeostasis, phagocytosis, and sealing zone formation in osteoclasts (Honda, Oda et al. 1998, Klemke, Leng et al. 1998, Bouton, Riggins et al. 2001, Lakkakorpi, Bett et al. 2003, Brabek, Constancio et al. 2004, Hudson, Bliska et al. 2005). The finding that global deletion of Cas is embryonic lethal highlights the importance of Cas in many developmental processes, as examination of Cas-deficient embryos shows widespread cardiovascular abnormalities and severe growth retardation (Honda, Oda et al. 1998).

To mediate such biological processes, Cas participates in the transduction of biochemical and mechanical signals initiated by ligand binding to growth factor, cytokine, and integrin receptors. One of the best-understood examples of

Cas signaling is the response to integrin engagement. Upon adhesion to the cell matrix, Cas forms a complex with Src and FAK (Mitra and Schlaepfer 2006). As stated above, the interaction between Cas and Src stabilizes the kinase in an active conformation and results in the phosphorylation of Cas within its substrate domain (Burnham, Bruce-Staskal et al. 2000, Nasertorabi, Tars et al. 2006). This creates binding sites for Crk and other SH2 domain-containing adaptors such as Nck (Vuori, Hirai et al. 1996, Schlaepfer, Broome et al. 1997, Burnham, Bruce-Staskal et al. 2000). Cas-Crk complexes activate Rho and Ras family GTPases such as Rac1 and Rap1 via the recruitment of two GEFs, DOCK180 and C3G (Vuori, Hirai et al. 1996, Kiyokawa, Hashimoto et al. 1998, Klemke, Leng et al. 1998, Tamada, Sheetz et al. 2004). These GTPases regulate cytoskeletal dynamics and normal cell processes such as motility, proliferation, and cell division, but can also contribute to tumorigenesis (Hattori and Minato 2003, Vega and Ridley 2008). Interestingly, the ectopic overexpression of Cas drives more Cas into complex with Src, leading to an increase in the overall levels of Src activity in the cell. This leads to enhanced tyrosine phosphorylation of multiple Src substrates, including Cas (Burnham, Bruce-Staskal et al. 2000, Brabek, Constancio et al. 2004). In this way, Cas overexpression is sufficient to induce, prolong, and amplify Src activity to elicit Src- and Cas-mediated signaling in the absence of any exogenous stimuli. The promotion of Cas/Src complex formation represents one possible mechanism by which BCAR3 potentiates Src signaling through Cas.

Like BCAR3, the overexpression of Cas promotes antiestrogen resistance and cell motility (Klemke, Leng et al. 1998, Brinkman, van der Flier et al. 2000, Riggins, Quilliam et al. 2003, Brabek, Constancio et al. 2004). Studies have shown that Cas induces antiestrogen resistance through a mechanism that requires Cas-Src association and Src PTK activity. The establishment of this resistance mechanism also involves the activity of two Src substrates, EGFR and signal transducer and activator of transcription (STAT) 5b (Riggins, Thomas et al. 2006). Cas overexpression also protects MCF7 breast cancer cells from the cytotoxic effects of Adriamycin, and this protective effect is abolished upon inhibition of Src activity (Ta, Thomas et al. 2008).

The ability of Cas to promote enhanced motility is also Src-dependent, as the co-expression of a kinase-dead version of Src blocks the Cas-dependent increase in cell migration (Riggins, Quilliam et al. 2003). The pro-migratory effects of Cas have been demonstrated in *in vivo* models of tumor cell dissemination. For example, lung metastases are significantly reduced following tail vein injection of Cas-depleted, HER2-overexpressing breast cancer cells compared to control counterparts (Cabodi, Tinnirello et al. 2006). The pulmonary dissemination of Cas-deficient cells is also significantly impaired in a transgenic model of TGF-β-driven breast cancer (Wendt, Smith et al. 2009). Lastly, Cas phosphorylation has been shown to be an essential event during Rap1-dependent, EGFR-induced pancreatic cancer cell metastasis (Huang, Anand et al. 2012).

An association between Cas and cancer has existed since its discovery as a mediator of cell transformation by the v-Src and v-Crk oncoproteins (Sakai, Iwamatsu et al. 1994); subsequent work has shown that Cas expression contributes to tumorigenesis in mice (Cabodi, Tinnirello et al. 2006) and humans (Cabodi, del Pilar Camacho-Leal et al. 2010, Tikhmyanova, Little et al. 2010). Research links alterations in Cas function and expression to many cancers, including those of the breast, prostate, colon, lung, brain, and several hematopoietic malignancies (Tikhmyanova, Little et al. 2010). Moreover, high Cas expression is associated with high tumor grade, advanced-stage disease, decreased relapse-free survival, and/or increased mortality in patients with breast, ovarian, prostate, and non-small cell lung cancer (Dorssers, Grebenchtchikov et al. 2004, Nick, Stone et al. 2011, Fromont, Rozet et al. 2012, Huang, Deng et al. 2012).

Collectively, these data describe an adaptor protein that is involved in the regulation and promotion of a wide variety of normal and tumorigenic processes. This work also establishes that the kinase activity of Src is both an essential regulator and effector of Cas signaling networks.

1.4 – c-Src

1.4.1 – *Discovery*

The proto-oncogene c-*Src* is the cellular counterpart of viral v-*Src*, the first identified oncogene and the causative genetic agent of Rous sarcoma virus (RSV)-mediated tumor formation. Cellular *Src*, c-*Src*, was discovered by probing

normal avian DNA with a collection of DNA sequences generated from v-*Src* (Stehelin, Varmus et al. 1976, Martin 2001). The c-Src protein (Src), the first recognized tyrosine kinase, regulates many fundamental cell processes, including differentiation, adhesion, migration, proliferation, and survival (Martin 2001, Parsons and Parsons 2004). Src is the archetypal member of the Src family of protein tyrosine kinases (SFKs). Several of these kinases, including Src, Yes, and Fyn are ubiquitously expressed but many others are expressed only in a certain cell types and tissues (Engen, Wales et al. 2008). Although non-transformative, the overexpression of Src promotes aggressive behaviors in tumor cells and its heightened expression and/or activity is implicated in the development and malignant progression of many human cancers (Irby and Yeatman 2000, Kim, Song et al. 2009). The catalytic activity of Src is regulated by transitory intra- and intermolecular interactions; these interactions occur between specific regions and protein-binding domains (SH3 and SH2) within Src, and between Src and other heterologous proteins.

1.4.2 – *Basic Structure and Domain Organization of Src and Interacting Proteins*

Src, and all SFKs, contains five key features that are important to kinase function, the myristoylated amino-terminus (also known as the SH4 domain); the amino-terminal SH3 domain; the centrally located SH2 domain; the catalytic SH1 domain; and a regulatory carboxyl-terminal tyrosine residue (Fig. 1.3).

The amino-terminus of Src is myristoylated and this modification serves to stabilize Src at the membrane through mediating an interaction with the lipid

bilayer (Buss and Sefton 1985). This post-translational modification localizes Src to regions proximal to transmembrane receptors. This positioning is essential to Src function as Src acts to transduce signals from activated cell surface receptors to an array of cytoplasmic signaling proteins (Kypta, Goldberg et al. 1990, Tice, Biscardi et al. 1999).

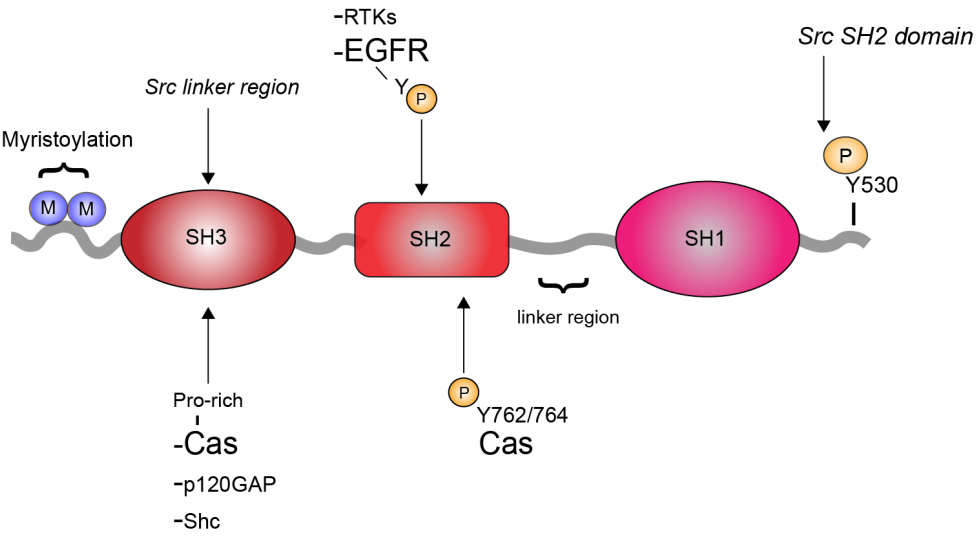

Figure 1.3: Src domain structure and binding partners.

Src has five conserved regulatory regions and domain structures that mediate its localization, activity, and association with other proteins: an N-terminal myristoylated tail, an SH3 domain, an SH2 domain, the catalytic SH1 domain, and a C-terminal regulatory tyrosine. Domain-specific binding partners are shown and intramolecular interactions are in italics.

The SH3 domain dually acts to bind substrate and to regulate kinase activity. The SH3 domain binds to a number of signaling proteins, including the adaptor protein and proto-oncogene Shc, the Ras GTPase-activating protein p120GAP, and Cas (Weng, Thomas et al. 1994, Briggs, Bryant et al. 1995, Nakamoto, Sakai et al. 1996, Ravichandran 2001). As discussed above, the interaction between the SH3 domain of Src and the carboxyl-terminal poly-proline region of Cas is the first step in a process that “opens” Src in an active conformation. In its “closed” or inactive conformation, the SH3 of Src domain is bound to a 14-nucleotide linker region between its SH2 and SH1 domains, which assists in maintaining the kinase in its inactive state (Xu, Harrison et al. 1997). The interaction with Cas initiates Src activation through the displacement of this interaction.

Like the SH3 domain, the SH2 domain of Src is involved in both mediating protein-protein interactions to promote signal transduction and in regulating kinase activity. During growth factor signaling, the SH2 domain binds to phosphotyrosine residues on a number of RTKs, such as EGFR (*in vitro*), platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR) (Kypta, Goldberg et al. 1990, Tice, Biscardi et al. 1999, Kim, Song et al. 2009). The intramolecular association between the SH2 domain and a carboxyl-terminal tyrosine (Y530 in human Src; Y527 in avian Src), in cooperation with the SH3/linker interaction, keeps the kinase in its “closed” state (Xu, Harrison et al. 1997) (Fig. 1.4A). Both the phosphorylated cytoplasmic tails of RTKs and the carboxyl-terminal phosphotyrosine of Cas are competitive

ligands for Src-SH2 binding, and effectively relieve this inhibitory interaction to configure Src into its active state.

Once in the “open” conformation (Fig. 1.4B), the active conformation is further stabilized through the SH1 domain-dependent phosphorylation of tyrosine 419 (human; 416 in avian Src) within the activation loop (Xu, Harrison et al. 1997, Engen, Wales et al. 2008). This fully active conformation allows for binding of both substrate and ATP within the SH1 domain; the addition of a phosphate group to substrate peptides is catalyzed here.

The final feature of Src kinase is the carboxyl-terminal regulatory tyrosine residue (Y530) that when phosphorylated promotes Src autoinhibition. This tyrosine is phosphorylated by the kinase Csk (C-terminal Src kinase), a master regulator of Src kinase activity (Xu, Harrison et al. 1997, Engen, Wales et al. 2008). Conversely, this tyrosine can be dephosphorylated by the phosphatases PTPα and PTP1B, which are known to functionally interact with BCAR3 and Cas, respectively (Liu, Hill et al. 1996, Yeatman 2004, Sun, Cheng et al. 2012). The activity of Src is tightly regulated through its intramolecular interactions, but the availability of exogenous ligands like those on Cas (Fig. 1.4B) relieve these negative constraints and promote kinase activity. In this way, aberrant changes in the cellular concentration or the mislocalization of such ligands can dramatically alter Src signaling.

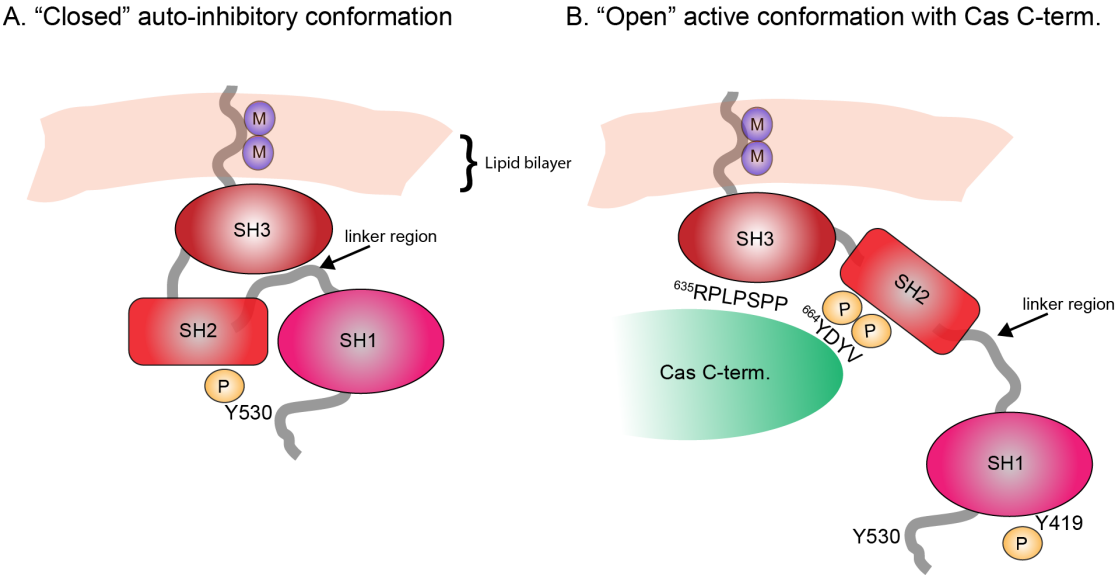

Figure 1.4: Regulatory Src conformations.

A. “Closed” conformation. Src is maintained in an inactive conformation through two intramolecular interactions: the SH3 domain binds to the linker region between the SH2 and SH1 domains, and the SH2 domain binds to phosphorylated tyrosine 530 (Y530). **B.** “Open” conformation. Binding to heterologous proteins, such as Cas, relieves the inhibitory interactions within Src. The C-terminus of Cas contains a poly-proline and an SH2 domain-binding motif (^{664}YDYV). Upon the SH3/poly-proline interaction, Src phosphorylates the YDYV region of Cas and creates a binding site for its SH2 domain. These interactions open up the catalytic domain, allowing Src to autophosphorylate tyrosine 419 (pY419).

1.4.3 – *Biological, Cellular, and Biochemical Functions of Src*

The phenotypes of single Src and SFK knockout mice reveal redundant functions across this family of kinases. Src knockout mice develop osteoporosis due to defects in osteoclast function, and the deletion of other family members results in impaired hippocampal development and mild deleterious effects on immune cell function (Lowell and Soriano 1996). A number of SFK double knockout mice die at birth and together with the single knockout phenotypes, this speaks to functional redundancy during development. Furthermore, the embryos of SYF ($Src^{-/-}$ $Yes^{-/-}$ $Fyn^{-/-}$) mice exhibit severe phenotypic abnormalities and die mid-gestation (Klinghoffer, Sachsenmaier et al. 1999). In culture, embryonic SYF cells are defective in their response to integrin/ECM engagement and display a great reduction in haptotaxis and wound healing, indicating that SFK kinase activity is essential for cell migration during embryogenesis.

The importance of Src in the development of cancer is highlighted by the prevalence of overexpressed or hyperactive Src in many malignancies, including those of the breast, colon, pancreas, lung, and brain (Irby and Yeatman 2000). In culture, drugs that inhibit the activity of Src have been demonstrated to reverse many resistant phenotypes, including antiestrogen resistance (Kim, Song et al. 2009). The pleiotropic effects of Src on cell behavior and fate are due to the role Src plays during the transduction of signals initiated by a wide variety of cell surface receptors to an even greater number of cytoplasmic signaling proteins. The expression and activity of many these receptors are also elevated in human cancers; single agent Src therapies have proven ineffectual in clinical trials, but

combinatorial regimens targeting Src and RTKs have proven more efficacious (Zhang and Yu 2012). Therefore, the identification of new biomarkers that would enable the pre-selection of patients whose tumors would respond well to Src inhibition are needed to identify patient populations that would benefit from such treatment (Zhang and Yu 2012).

1.5 – The BCAR3, Cas, and Src Signaling Axis

Ultimately, the development of refractory and metastatic disease is the major cause of cancer mortality. Perturbations in the underlying cellular processes of proliferation, survival, and motility are the root of these advanced disease states, yet their controlled activity in response to environmental stimuli is essential to biological processes necessary for multicellular life, such as cell morphogenesis and differentiation, and the maintenance of tissue homeostasis. In this introductory chapter, the roles of three proteins (BCAR3, Cas, and Src) in the regulation of these fundamental cellular processes are discussed. BCAR3, Cas, and Src appear to be part of a cooperative signaling axis that facilitates signal transduction downstream of a number of physical and chemical extracellular cues. Prior to the start of this thesis, work from our lab demonstrated that the overexpression of BCAR3 augments the ability of Cas to promote Src kinase activity and Src-dependent migration of fibroblasts towards both fibronectin and serum (Riggins, Quilliam et al. 2003). The constitutive association between BCAR3 and Cas had been demonstrated (Cai, Clayton et al. 1999), and the ability of Cas to bind to and activate Src was well established (Burnham,

Bruce-Staskal et al. 2000, Pellicena and Miller 2001). A large part of this introduction is dedicated to describing the essential roles of both Cas and Src in normal and tumorigenic processes. Therefore, a protein like BCAR3 that has been shown to activate Cas/Src signaling became one of particular interest to our lab.

In untransformed cells, BCAR3 has been shown to be an essential component of cell machinery that mediates the proliferative response to EGF (Oh, van Agthoven et al. 2008) and in the response to cell adhesion to ECM (Sun, Cheng et al. 2012). As described in the above sections, Cas and Src are similarly involved in the transduction of such chemical and physical stimuli. In breast cancer cells, the ectopic overexpression of either BCAR3, Cas, or a constitutively active version of Src induces antiestrogen resistance (van Agthoven, van Agthoven et al. 1998, Brinkman, van der Flier et al. 2000, Morgan, Gee et al. 2009). Furthermore, BCAR3 and Cas are implicated in the generation of migratory and invasive phenotypes in normal and tumor cells (Klemke, Leng et al. 1998, Brabek, Constancio et al. 2004, Schrecengost, Riggins et al. 2007).

While much of the research on the relationship between BCAR3 and Cas, including the work described in this thesis, has been performed in the context of breast cancer cell signaling and behavior, this signaling duplex has been linked to the function of other cells as well. For example, BCAR3 binds to both Cas and its family member NEDD9 in B-lymphocytes and induces Cdc42 activation (Cai, Felekkis et al. 2003). Although not directly tested, the authors speculate that BCAR3/Cas-family interactions are important in the regulation of lymphoid cell

adhesion and motility. Furthermore, the BCAR3 family member Shep1 also binds to NEDD9 in B-cells and promotes its tyrosine phosphorylation (Browne, Hoefer et al. 2010). In Shep1-deficient B-cells, the level of tyrosine phosphorylated NEDD9 is greatly reduced, concomitant with decreased marginal zone B-cell maturation and migration towards chemoattractants (Browne, Hoefer et al. 2010). These studies suggest that BCAR3 and Shep1 are involved in B-cell development and function through the regulation of Cas and NEDD9 tyrosine phosphorylation (possibly through the negative influence on some phosphatase, but more likely through the promotion of SFK activity). The deletion of Shep1 in mice leads to a high incidence of perinatal lethality, and a dramatic reduction in both Cas tyrosine phosphorylation and Src kinase activity is observed in the brain cells of these mice (Roselli, Wallez et al. 2010). Collectively, these studies provide evidence that NSP and Cas family members cooperate with Src (or other SFKs) to regulate the development and function of many tissue-specific cells. Thus, studying the mechanisms of this conserved NSP/Cas family/SFK signaling axis may assist in developing a better understanding of many normal and pathogenic processes.

1.6 – Research Objectives and Significance

When work on this thesis began, the mechanism of BCAR3-mediated antiestrogen resistance remained incompletely understood. The original goal of this thesis project was to further elucidate this resistance mechanism. We hypothesized that BCAR3 induces resistance through the positive manipulation

of proliferative and survival signaling downstream of Cas and Src. However, despite the ability of other labs to reproduce this effect (Felekkis, Quilliam et al. 2005, Near, Zhang et al. 2007), we were unsuccessful in recapitulating the BCAR3-mediated antiestrogen resistant phenotype (for details see the Appendix). Therefore, this hypothesis was unable to be tested.

A secondary objective of this research was to further characterize the relationship between BCAR3 and Cas, with the goal of understanding how these proteins cooperate to influence Src activity. We sought to address several key questions regarding the contribution of BCAR3 to the regulation of the BCAR3/Cas/Src signaling axis. Does the elevated expression of BCAR3 in breast cancer cells influence the activation of endogenous Cas and Src? Does BCAR3 promote Cas tyrosine phosphorylation? Is the manipulation of BCAR3 protein levels sufficient to alter Src kinase activity? And if so, what are the functional consequences? In Chapter 3 we show that BCAR3 acts as a positive regulator of Cas and Src signaling, as Cas/Src complexes, Src activity, Cas tyrosine phosphorylation, and Cas/Crk complexes all change as a function of BCAR3 expression. Furthermore, we demonstrate that BCAR3, through Src, regulates the dynamics of cell adhesion and spreading following integrin/ECM engagement, two processes essential to motility.

As our understanding of the regulation of the BCAR3/Cas/Src signaling axis became more complete, the identification of BCAR3 functions independent of this pathway became an area of increasing interest. Specifically, what are the Cas-dependent and Cas-independent functions of BCAR3? The data presented

in Chapter 4 reveal that the complete pool of BCAR3 protein present in two invasive breast cancer cell lines is found in association with Cas. Surprisingly, we also discovered that Cas is required for BCAR3 protein synthesis, indicating that BCAR3 function is dependent on Cas. This finding is particularly intriguing, as it suggests that Cas controls the expression of its modifier to potentiate its own activity.

The aberrant expression and activity of Cas and Src have been linked to tumorigenesis and poor disease outcome. Therefore, based on the work presented in this thesis and together with additional work by our lab and others, the adaptor proteins BCAR3 and Cas could prove to be useful biomarkers to better determine prognosis and direct therapy. The following chapters and the appendix describe this work in full, and further discuss the implications of the BCAR3/Cas/Src signaling axis in normal and pathological cell processes. Finally, the BCAR3/Cas/Src signaling axis described here could be a representative model for an NSP/Cas family/SFK signaling axis that has broader implications in human development and disease.

Chapter 2. Materials and Methods

Cell culture and generation of MCF7 clones expressing tetracycline-inducible BCAR3 **–** COS-1 green monkey kidney cells and BT549 breast cancer cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and passaged in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin. Tetracycline-regulated MCF7 clones stably expressing Myc-BCAR3-pTre2-Puro were generated by transfecting plasmid DNA into "Tet-off" MCF7 cells (BD Biosciences Clontech, Palo Alto, CA) and selecting with 0.75 µg/mL puromycin. Individual clones were isolated, and regulated protein expression was verified by immunoblot and immunofluorescence in the presence or absence of 1-2 µg/mL doxycycline (Dox). Stable clones were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 4 mM L-glutamine, 100 µg/mL G418 and 0.75 µg/mL puromycin. Cells were grown in the presence or absence of 1-2 µg/mL of Dox for experimental analysis. Src inhibitor SU6656 was purchased from Sigma (St. Louis, MO) and used at 10 µM where indicated. For experiments described in Chapter 4, BT549 and MDA-MB-231 breast cancer cell lines, obtained from the ATTC, were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS),

Antibodies **–** The 4G10 phosphotyrosine (pTyr) monoclonal antibody was purchased from Millipore (Billerica, MA). Myc monoclonal antibody 9E10 is from the University of Virginia Lymphocyte Culture Center (Charlottesville, VA). The CasB3b antibody has been described previously (Harte, Hildebrand et al. 1996, Bouton and Burnham 1997). The BCAR3 antibody was purchased from Bethyl Laboratories (Montgomery, TX). The Cas monoclonal antibody 6G11 has been described previously (Bouton and Burnham 1997). The Crk antibody was purchased from BD Biosciences. Src monoclonal antibody 2–17 was a gift of S.J. Parsons (University of Virginia, Charlottesville, VA). Src pY416 polyclonal antibody was purchased from Invitrogen (Carlsbad, CA). FLAG M2 affinity resin, FLAG M5 monoclonal antibody, horseradish peroxidase (HRP)- conjugated monoclonal β-actin antibody, and β-tubulin monoclonal antibody were purchased from Sigma. Protein G-PLUS agarose was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Bax antibody was purchased from Chemicon International (Temecula, CA). Protein A-Sepharose was purchased from Amersham Biosciences (Piscataway, NJ) and HA.11 from Covance (Princeton, NJ). Glutathione Sepharose 4B was purchased from GE Healthcare.

Plasmids **–** The pRK5 full-length Cas and the Src-binding mutant, Cas (P642A,Y668/670F), pFLAG3-BCAR3, pCDNA3-FLAG2AB-paxillin, and pFLAG-cortactin have previously been described (Burnham, Harte et al. 1999, Burnham, Bruce-Staskal et al. 2000, Riggins, Thomas et al. 2006). pCDNA-Src was a generous gift of S. J. Parsons (University of Virginia)(Tice, Biscardi et al. 1999).

Wild-type BCAR3 was sub-cloned into pEGFP-C1 vector (Clontech) and the R748A mutant was made using the QuickChange Site-Directed Mutagenesis Kit (Stratagene).

Transfection, immunoprecipitation, and protein expression – Transient transfection of COS-1 cells was performed using Superfect (Qiagen, Valencia, CA) according to manufacturer's specifications. Transient transfection of MDA-MB-231 cells was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to manufacturer's instructions. Cells were lysed 24 hours post-transfection in modified radioimmune precipitation assay buffer (RIPA; 150 mM/L NaCl, 50 mM Tris [pH 7.5], 1% Ipegal CA-630, 0.5% deoxycholate) supplemented with protease and phosphatase inhibitors (100 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.15 units/mL aprotinin, and 1 mM sodium orthovanadate). Protein concentrations were determined using the bicinchoninic acid assay kit (Pierce, Rockford, IL). A BCAR3 specific small interfering RNA (siRNA) (5'AAAUCAACCGGACAGUUCU3') was synthesized to target human BCAR3 (Dharmacon, Lafayette, CO). ON-TARGETplus SMARTpool siRNAs targeted to human BCAR3 were also purchased from Dharmacon. A set of 4 Cas-specific Stealth siRNAs (siCas1 [HSS114272], siCas2 [HSS114271], siCas3 [HSS114273], and siCas4 [HSS190423]) was purchased from Invitrogen. BT549 and MDA-MB-231 cells were treated with 20 μM control siRNAs, BCAR3-specific or Cas-specific siRNAs using Oligofectamine (Invitrogen) transfection reagent as previously described (Schrecengost, Riggins et al. 2007) or the Lipofectamine

RNAiMax (Invitrogen) reverse-transfection method per manufacturer's specifications. Cells were grown for 48-72 hours before lysis in RIPA buffer. Immunoprecipitations were performed as described previously (Burnham, Bruce-Staskal et al. 2000, Riggins, Quilliam et al. 2003). Proteins were resolved by sodium dodecyl sulfate- polyacrylamide gel electrophoresis, transferred to nitrocellulose, immunoblotted with the indicated antibodies, and detected by HRP-conjugated anti-mouse or anti-rabbit immunoglobulin (Amersham) followed by enhanced chemiluminescence (PerkinElmer, Waltham, MA) or HRP Substrate Luminol Reagent (Millipore).

Tandem Ubiquitin Binding Entity (TUBE) pull-downs – TUBEs were purchased from LifeSensors. Control and Cas-depleted MDA-231 and BT549 cells that had been cultured in the presence of absence of MG-132 (10μM) were lysed in RIPA buffer containing 0.2mg/ml GST-tagged TUBEs. Cell extracts were collected into 1.5mL centrifuge tubes and were incubated on ice for 15 minutes. Following this incubation, the samples were added to 50μL of Glutathione Sepharose 4B (GE Healthcare) and were rotated for 2 hours at 4°C. The TUBE/Sepharose complexes were washed 3 times in 500μL of PBS, and precipitated proteins were eluted in Laemmli buffer and separated by SDS-PAGE electrophoresis, transferred to nitrocellulose and blotted with antibodies specific to BCAR3.

Inhibition of protein degradation pathways – To determine if BCAR3 is degraded by the proteasome in the absence of Cas MDA-MB-231 and BT549 cells were

transfected using Lipofectamine RNAiMax (Invitrogen) with control or siCas oligos and were cultured in DMSO or MG-132 (10μM) for the final 24 hours of a 72-hour assay. Cells were lysed and protein expression was detected by Western blot. Similarly, to determine if lysosomal degradation was responsible for Cas-dependent BCAR3 loss, control and Cas-depleted cells were cultured in vehicle (water), Chloroquine (Sigma), or E-64 (Sigma) alone or in combination for the final 24 hours of a 72-hour knockdown. To show that Cas was cleaved by caspases following MG-132 treatment, BT549 cells were pre-treated with vehicle (DMSO) or the caspase inhibitor z-VAD-fmk (Calbiochem) for 24 hours followed by 24 hours of culture in the presence of MG-132 (10μM). Full-length and the 31kDa carboxyl-terminal fragment were detected by Western blot using the carboxyl-terminal recognizing Cas monoclonal mouse antibody 6G11. Finally, to determine whether BCAR3 is proteolytically degraded following Cas knockdown, BT549 cells that had been transfected with control and Cas-specific oligos were treated with z-VAD-fmk, or the calpain inhibitors ALLM (Calbiochem) and Calpain Inhibitor III (Sigma) for the final 24 hours of a 72-hour knockdown time course; protein expression was assayed by Western blot.

Flow cytometry – To analyze GFP-expressing cells, MDA-MB-231 cells that had been transfected with pEGFP-WT BCAR3 or pEGFP-R748A BCAR3 were washed with PBS, trypsinized, and collected in 5 mL round bottom test tubes (BD). Samples were then stained with the Live/Dead Fixable Violet Dead Cell Stain kit (Invitrogen) according to the manufacturer's instruction. Stained cells

were assayed on a FACSCaliber flow cytometer, and GFP-expressing and Live/Dead-stained cells were detected in the FL1-H and FL5-H channels, respectively. Live/Dead stain was used to determine live cell populations, and the percentage of cells expressing GFP and the mean fluorescence intensity (MFI) of GFP-expressing populations was analyzed on the viable population using FlowJo. Samples were compensated using a non-transfected, unstained sample; a GFP-WT BCAR3 transfected, unstained sample; and a non-transfected, Live/Dead-stained control.

Analysis of the rate of protein degradation – BT549 cells were either transfected with control (0 hours) or siCas oligos for 24, 48, 72, and 96 hours. Following each time point, cells were lysed and the expression of BCAR3 and Cas were assayed by Western blot. Similarly, BT549 cells were treated with Cycloheximide (Sigma) for the same time points and Western blots were performed to detect BCAR3 and Cas protein. For both experiments BCAR3 and Cas protein expression was quantified relative to actin by densitometry. The graphs displaying the rate of degradation were made in Microsoft Excel.

Qualitative reverse transcriptase-PCR (RT-PCR) – RNA was purified from cells 72 hours following reverse-transfection with control or Cas-specific siRNAs using Qiagen RNAprotect Reagent and RNeasy mini kit (Valencia, CA, USA). Preparation of cDNA and the RT-PCR reaction was performed as described previously (Ray, Thomas et al. 2012). TaqMan gene expression assay primers

for PCR were purchased from Applied Biosystems (Carlsbad, CA, USA): BCAR3 (HS00182488_m1) and 18S rRNA (Mm03928990_g1). The ΔΔCT ($2^{-\Delta\Delta CT}$) method was used to quantify relative mRNA levels for BCAR3 as described in User Bulletin #2 (Applied Biosystems) using18S rRNA as the reference and internal standard.

Detection of L-azidohomoalaine (AHA)-labeled proteins – Click-iT AHA and the Click-iT biotin protein reaction buffer kit were purchased from Invitrogen. 72 hours after reverse-transfection with control or siCas siRNAs cells were treated with 25μM AHA for indicated time points. Cells were lysed in RIPA plus protease inhibitors and BCAR3 immunoprecipitations were performed as described above. Biotin labeling was performed on Protein A-Sepharose immune complexes as described in the manufacturer's instructions. Following biotin labeling, immune complexes were washed 3 times in cold RIPA lysis buffer and once in cold Tris-Saline. Biotin-labeled proteins were resolved by SDS-PAGE electrophoresis, transferred to nitrocellulose, and blotted with HRP-conjugated streptavidin (Cell Signaling Technologies) and detected with HRP Substrate Luminol Reagent (Millipore).

Cell adhesion to fibronectin and microscopy **–** 10 cm tissue culture dishes and glass coverslips were coated with 20μg/mL fibronectin (Sigma) for one hour or overnight at 4˚C and then washed with phosphate buffered saline (PBS). BT549 cells were transfected with control or BCAR3-specific siRNAs as described

above. Cells were grown for 72 hours and then trypsinized, counted, and left in suspension for 90 minutes in complete growth media containing serum. Suspension cells were then either lysed or plated onto tissue culture plates and fibronectin-coated coverslips in complete growth media for the indicated lengths of time. Cells plated onto coverslips were washed twice in PBS, fixed with 3% paraformaldehyde for 20 minutes, washed, and mounted onto slides. In parallel, cells were plated immediately following transfection and analyzed 72 hours later as cycling adherent cells. For the Src inhibitor studies, dimethyl sulfoxide (DMSO) or 10 μM SU6656 was added to cells during the 90-minute suspension and subsequent plating steps. ImageJ software (National Institutes of Health) was used to calculate cell area using the freehand tracing tool. 100+ cells were counted per time point for at least three independent experiments.

Densitometry and statistical analysis – Densitometry was performed using the Bio-Rad GS-800 densitometer (Bio-Rad, Hercules, CA) and quantified using ImageQuant TL 2005 (Amersham Biosciences). A two-tailed Students t-test was used for comparisons between the various sample sets. Statistical significance was defined at ≥95% confidence interval or P-value ≤0.05. Bar graphs represent the mean ± standard deviation.

Chapter 3. Breast Cancer Antiestrogen Resistance-3 Regulates Src/p130^{Cas} Association, Src Kinase Activity, And Breast Cancer Adhesion Signaling*

*Adapted from BCAR3 Regulates Src/p130Cas Association, Src Kinase Activity, and Breast Cancer Adhesion Signaling
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J Biol Chem. 2010 January 22; 285(4): 2309–2317.

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3.1 – Abstract

The non-receptor protein tyrosine kinase (PTK) Src is frequently overexpressed and/or activated in a variety of cancers, including those of the breast. Several heterologous binding partners of Src have been shown to regulate its catalytic activity by relieving intramolecular auto-inhibitory interactions. Cas is one such protein and is expressed at high levels in both breast cancer cell lines and breast tumors, providing a potential mechanism for Src activation in breast cancers. The Cas-binding protein BCAR3 is expressed at high levels in invasive breast cancer cell lines, and this molecule has previously been shown to coordinate with Cas to increase Src activity in COS-1 cells. In this report, we show for the first time using gain- and loss-of-function approaches that BCAR3 regulates Src activity in the endogenous setting of breast cancer cells. We further show that BCAR3 regulates the interaction between Cas and Src, both qualitatively as well as quantitatively. Finally, we present evidence that the coordinated activity of these proteins contributes to breast cancer cell adhesion

signaling and spreading. Based on these data, we propose that the BCAR3/Cas/Src signaling axis is a prominent regulator of Src activity, which in turn controls cell behaviors that lead to aggressive and invasive breast tumor phenotypes.

3.2 – Introduction

In normal tissues, the PTK Src exists as a tightly regulated molecule that is responsible for many cellular processes, including proliferation, adhesion, migration, and invasion (Finn 2008). This regulation is often lost in solid tumors, including those of the breast, resulting in the transduction of signals that promote tumor progression (Summy and Gallick 2003). Interactions between Src and other proteins play an important role in the regulation of Src activity. In this chapter, we investigate how altered expression of the adaptor protein BCAR3 coordinates with the Src-binding protein and fellow adaptor Cas to regulate Src activity and Src mediated biological processes.

BCAR3 is a member of the novel SH2-containing protein (NSP) family (Gotoh, Cai et al. 2000) and was first identified in a screen for genes whose overexpression conferred resistance to antiestrogens (van Agthoven, van Agthoven et al. 1998). BCAR3 contains an SH2 domain and a guanine nucleotide exchange factor (GEF)-like domain with homology to the CDC25 family of GEFs. Studies directed toward understanding BCAR3-mediated antiestrogen resistance have implicated the activities of Rac, phosphoinositide kinase-3, and cyclin D1 in this process (Felekkis, Narsimhan et al. 2005, Near,

Zhang et al. 2007). Additionally, BCAR3 appears to promote an epithelial-mesenchymal transition in breast cancer cells (Near, Zhang et al. 2007, Makkinje, Near et al. 2009). Our lab has recently reported that loss of BCAR3 from BT549 breast cancer cells disrupts EGF-induced migration and invasion, coincident with a decrease in EGF-induced tyrosine phosphorylation of its binding partner, the adaptor molecule Cas (Schrecengost, Riggins et al. 2007).

Cas, also known BCAR1, was initially identified as a highly tyrosine phosphorylated protein in v-*Src* and v-*Crk* transformed cells (Sakai, Iwamatsu et al. 1994). The carboxyl terminus of Cas contains not only the binding site for BCAR3 (Gotoh, Cai et al. 2000), but also a bipartite binding site for the SH2 and SH3 domains of Src (Nakamoto, Sakai et al. 1996). It is through this latter interaction that Cas is able to relieve the auto-inhibitory conformation of Src and stimulate its catalytic activity (Burnham, Bruce-Staskal et al. 2000, Pellicena and Miller 2001). Co-overexpression of Cas and Src drives Cas into complex with Src, leading to increased kinase activity (Burnham, Bruce-Staskal et al. 2000, Riggins, Quilliam et al. 2003). This in turn leads to phosphorylation of a variety of substrates, including Cas (Burnham, Bruce-Staskal et al. 2000). Tyrosine phosphorylation of Cas creates binding sites for downstream signaling proteins that serve to activate pathways important for cell proliferation, survival, and migration (Defilippi, Di Stefano et al. 2006).

A number of breast cancer cell lines overexpress Cas and Src, with more invasive and aggressive cell lines also overexpressing BCAR3 [(Riggins, Thomas et al. 2006, Near, Zhang et al. 2007, Schrecengost, Riggins et al. 2007) and

unpublished data]. This provides a unique cellular platform in which to study the integrated functions of these three molecules. In this chapter, BCAR3 is shown to regulate Src activity and adhesion-dependent Cas phosphorylation in breast cancer cells. Further, we demonstrate that the coordinated activity of these proteins contributes to breast cancer cell adhesion signaling and spreading. These data, together with previous work from our group and others, support the idea that BCAR3 functions as an oncogenic cofactor that utilizes Cas and Src to promote more aggressive and invasive breast tumor phenotypes.

3.3 – Results

3.3.1 – *Cas/Src Interactions Are Required for BCAR3-dependent Enhancement of Cas-mediated Src Kinase Activity.*

To assess the molecular requirements for Src activation by Cas and BCAR3, COS-1 cells were transfected with plasmids encoding Src, Myc-tagged Cas, and/or Flag-tagged BCAR3. In each case, plasmids encoding Flag-tagged cortactin were also transfected to provide an exogenous substrate for Src activity in the transfected population. Twenty-four hours post-transfection, cells were lysed and the phosphorylation of cortactin was examined using phosphotyrosine (pTyr)-specific antibodies. Cells overexpressing Src and Cas exhibited increased activation of Src, as measured by the increased phosphorylation of cortactin, compared to cells expressing only vector (Fig. 3.1A, top panel, lanes 1-2). In contrast, no increase in cortactin phosphorylation was observed when BCAR3 and Src were expressed (lane 3). However, when BCAR3 was co-expressed with

Cas and Src, phosphorylation of cortactin was even greater than in the presence of Cas alone (lane 4). These data confirm our previous finding that BCAR3 expression enhances Cas-dependent Src activity (Riggins, Quilliam et al. 2003). Similar results were obtained using paxillin as an exogenous substrate (Fig. 3.1B, top panel, lanes 2 and 5), demonstrating that the enhancement in Src substrate phosphorylation observed in the presence of BCAR3 and Cas was not restricted to cortactin.

To determine whether the augmentation of Cas-dependent Src activity by both Cas and BCAR3 required interactions between Cas and Src, a construct of Cas containing three amino acid substitutions (P642A, Y668/670F) in the Src binding site (Nakamoto, Sakai et al. 1996) was utilized in experiments similar to those described above. This Cas mutant, termed Cas-TM for “triple mutant,” is significantly impaired in binding to Src (Riggins, Thomas et al. 2006). As expected, phosphorylation of the Src substrate paxillin was observed under conditions of Cas overexpression, and this was increased significantly when both BCAR3 and Cas were co-overexpressed (Fig. 3.1B, top panel, lanes 2 and 5). In contrast, expression of CasTM either alone or in combination with BCAR3 failed to promote paxillin phosphorylation (lanes 3 and 6). These data indicate that the interaction between Cas and Src is required for both Cas- and BCAR3/Cas-dependent activation of Src.

Figure 3.1

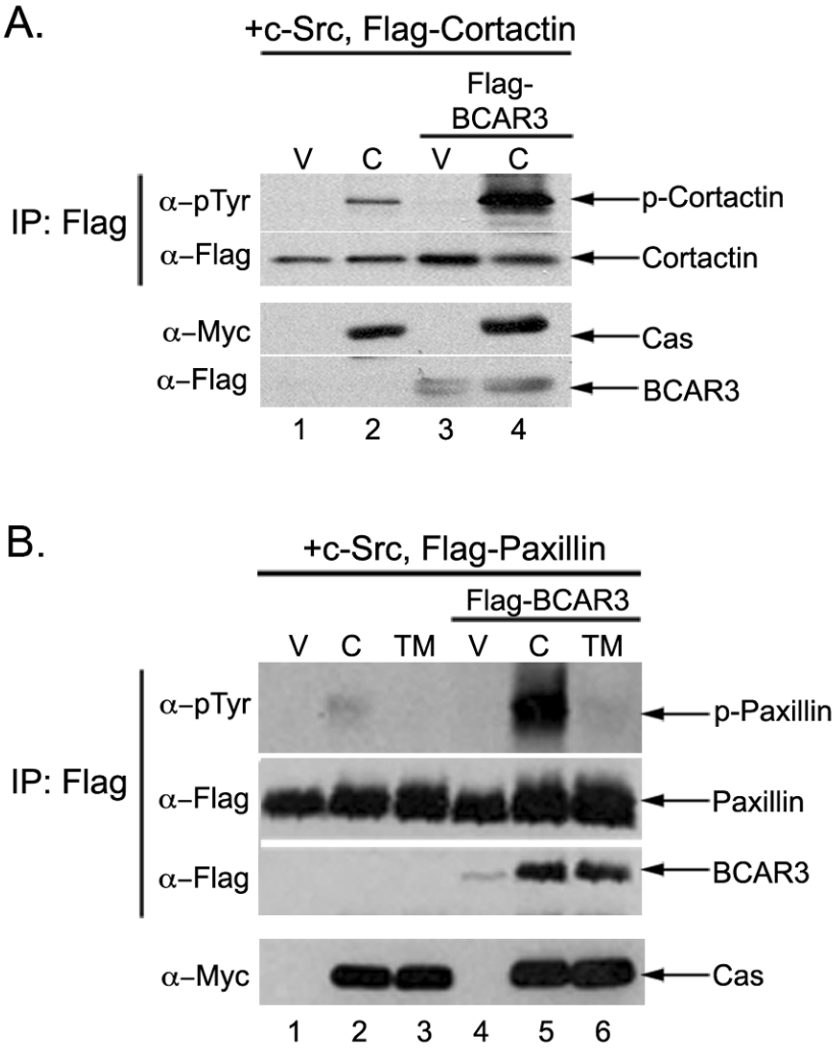

A.
+c-Src, Flag-Cortactin
Flag-
BCAR3
V
C
V
C
IP: Flag
α–pTyr
α–Flag
p-Cortactin
Cortactin
α–Myc
α–Flag
Cas
BCAR3
1
2
3
4
B.
+c-Src, Flag-Paxillin
Flag-BCAR3
V
C
TM
V
C
TM
IP: Flag
α–pTyr
α–Flag
α–Flag
α–Myc
p-Paxillin
Paxillin
BCAR3
Cas
1
2
3
4
5
6

Figure 3.1: BCAR3 augments Cas-mediated enhancement of Src activity. **A.** Co-overexpression of Cas and BCAR3 enhances Cas-dependent Src activity. COS-1 green monkey kidney cells were transfected with plasmids encoding WT Src, Flag-Cortactin, pRK5 vector (V), Myc-Cas (C), and/or Flag-BCAR3. Flag immune complexes were generated from cell lysate 24 hours post-transfection and immunoblotted with pTyr and Flag-specific antibodies (top two panels). Whole cell lysates were immunoblotted with Myc and Flag antibodies (bottom two panels). **B.** BCAR3 requires Cas/Src binding to augment Src activity. COS-1 green monkey kidney cells were transfected with plasmids encoding wild-type Src, Flag-Paxillin, pRK5 vector (V), Myc-Cas (C), Myc-CasTM (TM), and/or Flag-BCAR3. Cells were lysed 24 hours post-transfection and proteins analyzed as described above. Data shown are representative of 2-4 independent experiments. Experiments performed by Natasha Schuh.

3.3.2 – *Loss of Endogenous BCAR3 Expression in Breast Cancer Cells Attenuates Src Activity, Cas Tyrosine Phosphorylation, and the Association between Cas and Src.*

While we have shown above that Cas and BCAR3 regulate Src activity in ectopic expression systems, these regulatory pathways have not yet been investigated in the context of endogenously expressed proteins. Many breast tumors and cancer cell lines that exhibit aggressive and invasive phenotypes express high levels of Cas, BCAR3, and Src, and these proteins can be found in complex with one another [(Schlaepfer, Broome et al. 1997, Cary, Han et al. 1998, Riggins, Thomas et al. 2006, Schrecengost, Riggins et al. 2007) and see Fig. 3.2 for endogenous Cas/Src and Cas/BCAR3 complexes in BT549 cells]. In contrast, cells representative of earlier stage breast cancers generally express BCAR3 at very low or undetectable levels, despite the fact that expression of Cas and Src is often high (Near, Zhang et al. 2007, Schrecengost, Riggins et al. 2007). This dichotomy provides an excellent system with which to examine the effect of BCAR3 expression on Cas/Src interactions and Src activity under physiological conditions.

Figure 3.2

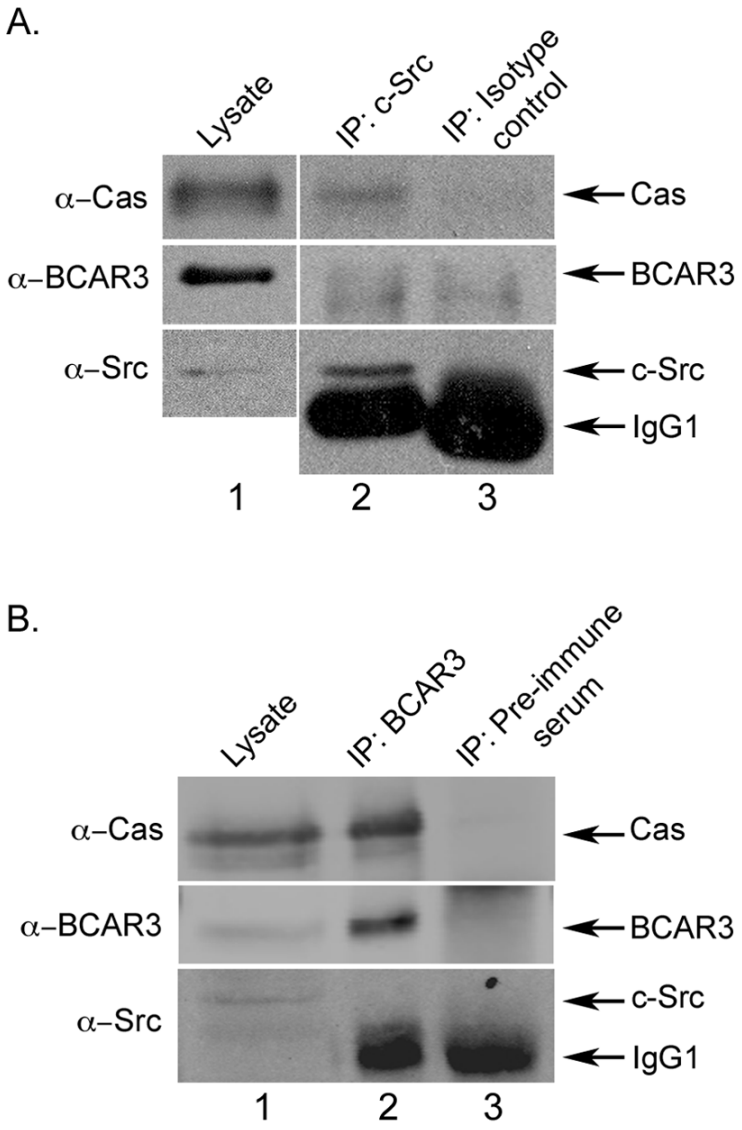

A.
Lysate
IP: c-Src
IP: Isotype control
α–Cas
α–BCAR3
α–Src
Cas
BCAR3
c-Src
IgG1
1
2
3
B.
Lysate
IP: BCAR3
IP: Pre-immune serum
α–Cas
α–BCAR3
α–Src
Cas
BCAR3
c-Src
IgG1
1
2
3

Figure 3.2: Two distinct pools of Cas exist in BT549 cells.

A. Cas and Src are present in a molecular complex. 400 μg of BT549 cell lysate were incubated with Src monoclonal antibody 2-17 (lane 2) or isotype-matched monoclonal antibody HA.11 as a control (lane 3). Immune complexes were collected and immunoblotted with the indicated antibodies. For comparison, 20 μg of cell lysate were immunoblotted (lane 1). The vertical separation indicates different exposure times between lysate and IP lanes. **B.** Cas and BCAR3 are present in a molecular complex. BCAR3 or control (pre-immune serum) immune complexes were generated as described above from 350 μg of BT549 cell lysate, collected on Protein A agarose, and immunoblotted with the indicated antibodies (lanes 2-3). For comparison, 30 μg of cell lysate were immunoblotted (lane 1). Experiments performed by Michael Guerrero.

To determine if BCAR3 is important for the regulation of Src activity in cells expressing high endogenous levels of this protein, BCAR3 expression was reduced by RNA interference in BT549 cells. Following transfection with BCAR3-targeted small interfering RNA (siRNA) oligonucleotides, BCAR3 expression was consistently reduced by 82-99%, as measured by densitometry (Fig. 3.3A, top panel). Depletion of BCAR3 correlated with a concomitant reduction in Src autophosphorylation at tyrosine 419 (Y419 is the autophosphorylation site on human Src, equivalent to Y416 in chicken), a marker for Src activity (Fig. 3.3A, 2nd panel and associated graph).

As Cas is a well-established Src substrate, we next investigated whether the decrease in Src activity exhibited by cells depleted for BCAR3 was accompanied by a loss in tyrosine phosphorylation of Cas. Cas was immunoprecipitated from lysates derived from cells treated with control or BCAR3-targeted siRNAs and the resultant complexes were immunoblotted with a pTyr antibody. Cas tyrosine phosphorylation was reduced by an average of 70% under conditions of BCAR3 knockdown (Fig. 3.3B). Similar results were obtained when BCAR3 was depleted using a second pool of four independent BCAR3-specific siRNAs, confirming that the decrease in Cas phosphorylation was due to depletion of BCAR3 (Fig. 3.4A–C). These results extend our previous study, which showed a reduction in EGF-dependent Cas tyrosine phosphorylation under conditions of BCAR3 depletion (Schrecengost, Riggins et al. 2007).

Cas is an effective activator of Src catalytic activity through its ability to bind to Src and relieve its auto-inhibitory conformation (Burnham, Bruce-Staskal

et al. 2000). We therefore hypothesized that the decreases in Src activity and Cas tyrosine phosphorylation observed under BCAR3-depleted conditions might be the result of a reduction in Cas/Src association. To determine if this was the case, Src was immunoprecipitated from lysates generated from BT549 cells treated with control or BCAR3-targeted siRNAs and the resultant complexes were immunoblotted for Cas. As expected, Cas was seen to associate with Src in cells treated with non-targeting siRNA oligonucleotides (Fig. 3.3C and 3.5A, lane 1). In cells depleted of BCAR3, Cas/Src association was reduced by approximately 60% (Fig. 3.3C, lane 2, Fig. 3.5A, lane 4). This result supports our hypothesis that the observed loss in Src activity is a consequence of decreased Cas association.

Figure 3.3

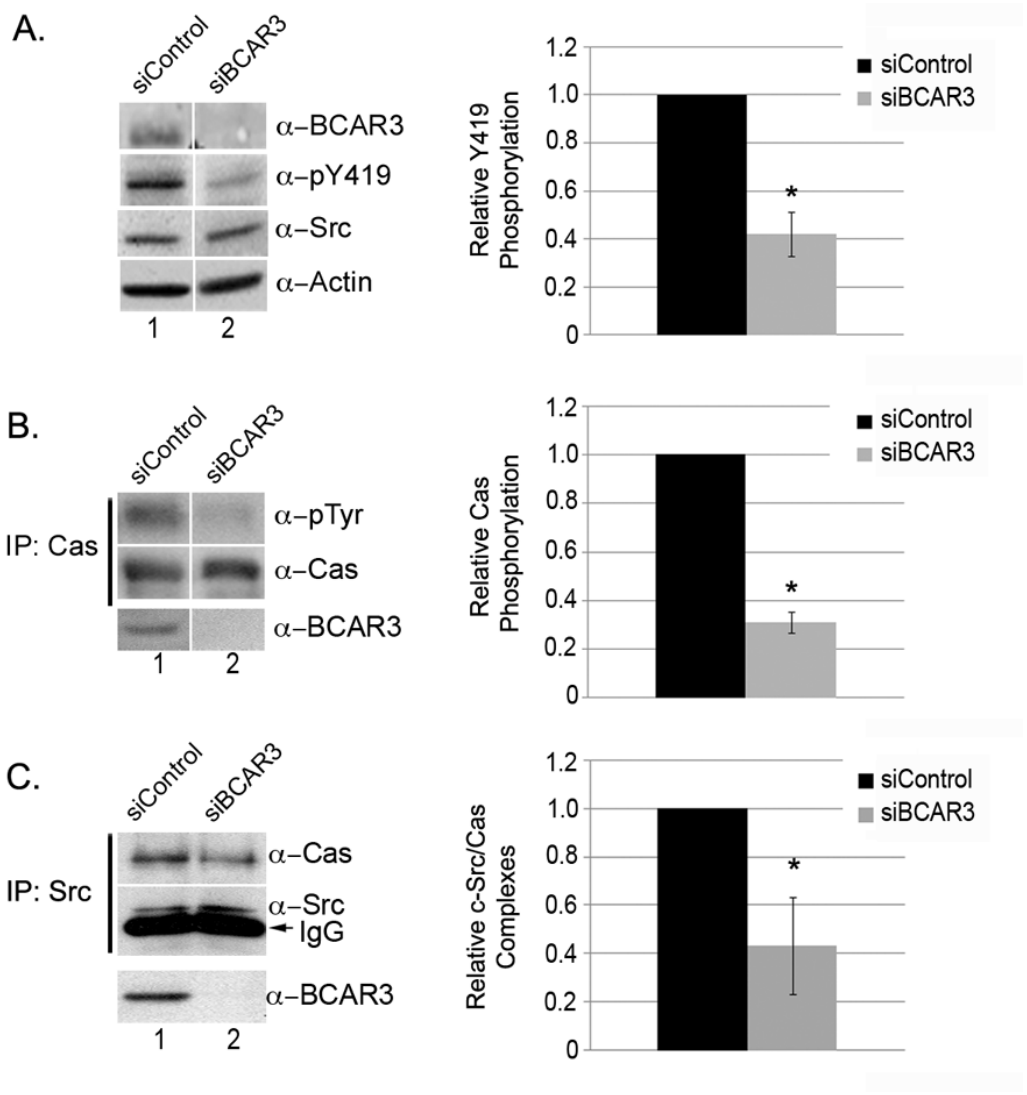

A.
siControl
siBCAR3
α–BCAR3
α–pY419
α–Src
α–Actin
1
2
Relative Y419 Phosphorylation
1.2
1.0
0.8
0.6
0.4
0.2
0
siControl
siBCAR3
*
B.
siControl
siBCAR3
IP: Cas
α–pTyr
α–Cas
α–BCAR3
1
2
Relative Cas Phosphorylation
1.2
1.0
0.8
0.6
0.4
0.2
0
siControl
siBCAR3
*
C.
siControl
siBCAR3
IP: Src
α–Cas
α–Src
IgG
α–BCAR3
1
2
Relative c-Src/Cas Complexes
1.2
1.0
0.8
0.6
0.4
0.2
0
siControl
siBCAR3
*

Figure 3.3: Depletion of BCAR3 in BT549 breast cancer cells disrupts Src activation, Cas phosphorylation, and Cas/Src interactions.

A. BCAR3-depleted BT549 cells have decreased Src activity. BT549 breast cancer cells were transfected with non-targeting or BCAR3-specific siRNA duplexes and grown for 48 hours. Cell lysate was immunoblotted with the indicated antibodies. The mean relative pY419 phosphorylation was determined from four independent experiments. **B.** BT549 cells depleted for BCAR3 have decreased Cas phosphorylation. Cas immune complexes were generated from 72-hour siRNA-treated BT549 cells and immunoblotted with antibodies specific to pTyr and Cas (top two panels). Cell lysate was immunoblotted for BCAR3 to verify loss of BCAR3 expression (bottom panel). The mean relative Cas phosphorylation was determined from four independent experiments. **C.** BT549 cells depleted for BCAR3 have decreased Cas/Src interactions. Src immune complexes were generated from 72-hour siRNA-treated BT549 cells and immunoblotted with Cas and Src antibodies (top panel). Whole cell lysate was also immunoblotted with BCAR3 antibodies (bottom panel). The mean Cas levels in Cas/Src immune complexes was determined from five independent experiments. Two-tailed Student's t-tests were conducted for comparison between siControl and siBCAR3 samples for panels A, B, and C. Bars indicate standard deviation, * indicates significant difference from the mean at ≥95% confidence interval. Within all panels, exposure time is the same; the white vertical lines denote non-contiguous sample lanes. Experiments performed by Natasha Schuh.

Figure 3.4

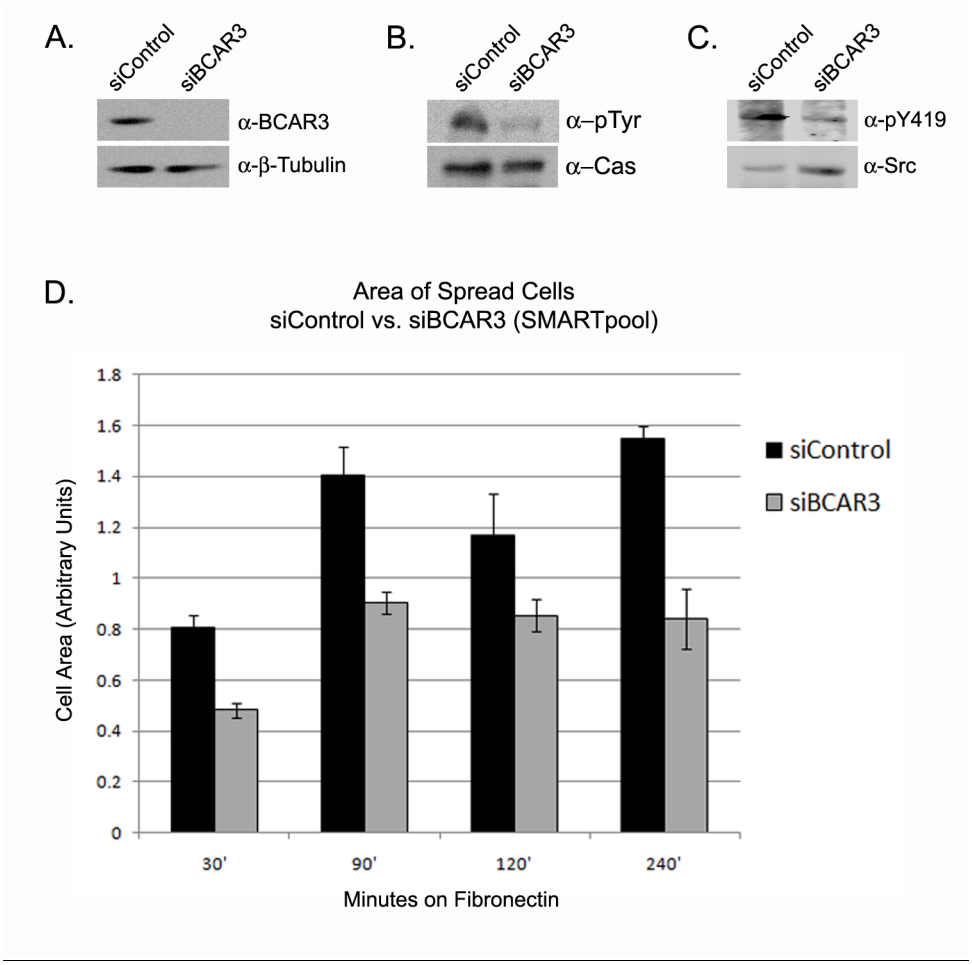

A.
siControl
siBCAR3
α-BCAR3
α-β-Tubulin
B.
siControl
siBCAR3
α–pTyr
α–Cas
C.
siControl
siBCAR3
α-pY419
α-Src
D.
Area of Spread Cells
siControl vs. siBCAR3 (SMARTpool)
Cell Area (Arbitrary Units)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
30'
90'
120'
240'
Minutes on Fibronectin
siControl
siBCAR3

Figure 3.4: Depletion of BCAR3 by SMARTpool siRNA duplexes disrupts Src activation, Cas phosphorylation, and cell spreading. BT549 cells were transfected with non-targeting or BCAR3-specific Dharmacon SMARTpool siRNA duplexes and grown for 72 hours. **A.** Whole cell lysate was immunoblotted with BCAR3 and β-tubulin antibodies. **B.** Cas immune complexes were generated from 72-hour siRNA-treated BT549 cells and immunoblotted with antibodies specific to pTyr or Cas. **C.** Src immune complexes were generated as above and immunoblotted with antibodies specific for pY419 or total Src. **D.** Spreading assays were performed as described in Fig. 3.8. Data shown are the average from 2 independent experiments. Experiments performed by Natasha Schuh.

Figure 3.5

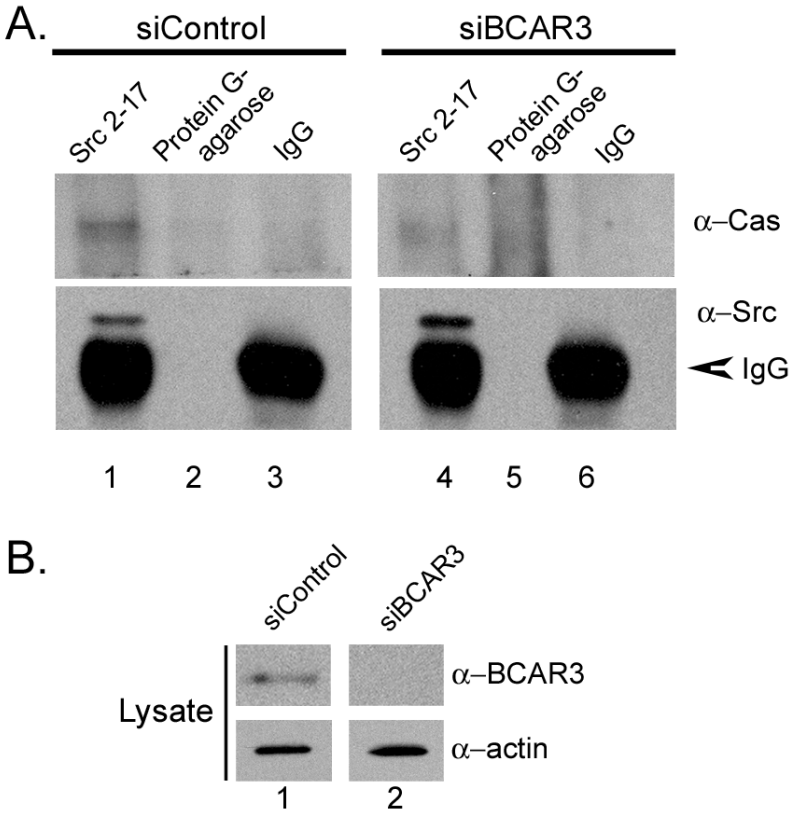

A.
siControl
siBCAR3
Src 2-17
Protein G-agarose
IgG
Src 2-17
Protein G-agarose
IgG
α–Cas
α–Src
IgG
1
2
3
4
5
6
B.
siControl
siBCAR3
Lysate
α–BCAR3
α–actin
1
2

Figure 3.5: Cas interactions with Src are decreased under conditions of BCAR3 depletion.

A. Appears as supplemental figure in original paper; includes bead only and isotype-matched control samples (n = 1). BT549 cells were transfected with control or BCAR3-targeted siRNAs and lysed 72 hrs post-transfection. 250 μg lysate was incubated for 2 hrs on ice with 5μg of the Src specific antibody 2-17 (lanes 1 and 4) or with 5 μg of an isotype-matched IgG (HA.11; Covance MMS-101R) (lanes 3 and 6). Immune complexes were captured by incubation with 25 μl Protein G agarose slurry for 1 hr at 4°C. 250 μg lysate was similarly incubated with 25 μl Protein G agarose (lanes 2 and 4). Proteins were then subjected to PAGE and immunoblotted for Cas (top panels) and Src (bottom panels). **B.** 10 μg of the cell lysates used in panel A were immunoblotted with BCAR3 (top panels) and actin (bottom panels) antibodies to demonstrate BCAR3 depletion. Within each panel, exposure time is the same; the white vertical line denotes non-contiguous sample lanes. Experiment performed by Michael Guerrero.

3.3.3 – *Ectopic Expression of BCAR3 in Breast Cancer Cells Induces Src Activity and Cas Tyrosine Phosphorylation*.

MCF7 cells express low levels of BCAR3 and serve as a model for early-stage breast cancer (Schrecengost, Riggins et al. 2007, Makkinje, Near et al. 2009). To examine the effect of BCAR3 overexpression in this background, MCF7 cell lines were generated that express BCAR3 under a tetracycline-regulated promoter (Tet-off). BCAR3 expression can be turned off in these cells by the addition of 2μg/mL doxycycline (Dox) (Fig. 3.6A, top panel). To determine whether BCAR3 expression alters Src activity in this cell system, Src was immunoprecipitated from lysates and immunoblotted for Y419 phosphorylation. Phosphorylation of Y419 was elevated almost two-fold under conditions of BCAR3 overexpression (Fig. 3.6B), demonstrating that BCAR3 is capable of inducing Src activity when overexpressed in breast cancer cells. To determine whether this increase in Src activity correlated with an increase in BCAR3-dependent Cas phosphorylation, Cas was immunoprecipitated from lysates isolated from cells grown in the presence (endogenous levels of BCAR3) or absence (overexpressed BCAR3) of Dox and the resultant complexes were immunoblotted with an antibody recognizing pTyr. Indeed, Cas tyrosine phosphorylation was increased by three-fold under conditions of BCAR3 overexpression (Fig. 3.6C, compare lanes 1 and 3). Since BCAR3 expression in MCF7 cells has been reported to induce phosphorylation of Cas on serine residues (Makkinje, Near et al. 2009), we confirmed the specificity of the pTyr antibody for phosphotyrosine by pre-incubating the antibody with O-phospho-L-

tyrosine- or O-phospho-L-serine-conjugated bovine serum albumin (BSA) prior to using it for immunoblotting. The Cas pTyr signal was lost under conditions of pre-incubation with the pTyr, but not the phosphoserine (pSer), inhibitor (Fig. 3.7). Finally, to test whether the increase in Cas phosphorylation was directly due to Src activity, the cells were cultured for 24 hours in the presence or absence of 10 μM of the Src family kinase inhibitor, SU6656 (Blake, Broome et al. 2000). SU6656 treatment of BCAR3-overexpressing cells reduced overall Cas phosphorylation and abrogated the BCAR3-dependent increase in Cas phosphorylation (Fig. 3.6C, lanes 2 and 4). These data confirm that the increase in Cas phosphorylation in cells overexpressing BCAR3 is due to increased Src activity.

Because enhancement of Src activity by BCAR3 in COS-1 cells required an interaction between Cas and Src, and given the decrease in Cas/Src association exhibited by BT549 cells depleted of BCAR3 (Fig. 3.3C), we next investigated whether BCAR3 regulates Cas/Src interactions in MCF7 cells. Interestingly, the absolute amount of Cas present in Src immune complexes was not dramatically altered under conditions of BCAR3 overexpression (Fig. 3.6D). However, in contrast to control conditions where multiple species of Cas were seen to associate with Src, virtually all Cas in complex with Src in BCAR3-overexpressing MCF7 cells exhibited a reduced electrophoretic mobility (lane 2). This slower migrating form of Cas has been previously reported to represent a highly phosphorylated species of Cas (Nojima, Morino et al. 1995). Together with the loss-of-function studies in BT549 cells described above, these data support a

role for BCAR3 in regulating Src activity and Cas tyrosine phosphorylation in breast cancer cells via its ability to influence Cas/Src interactions.

Figure 3.6

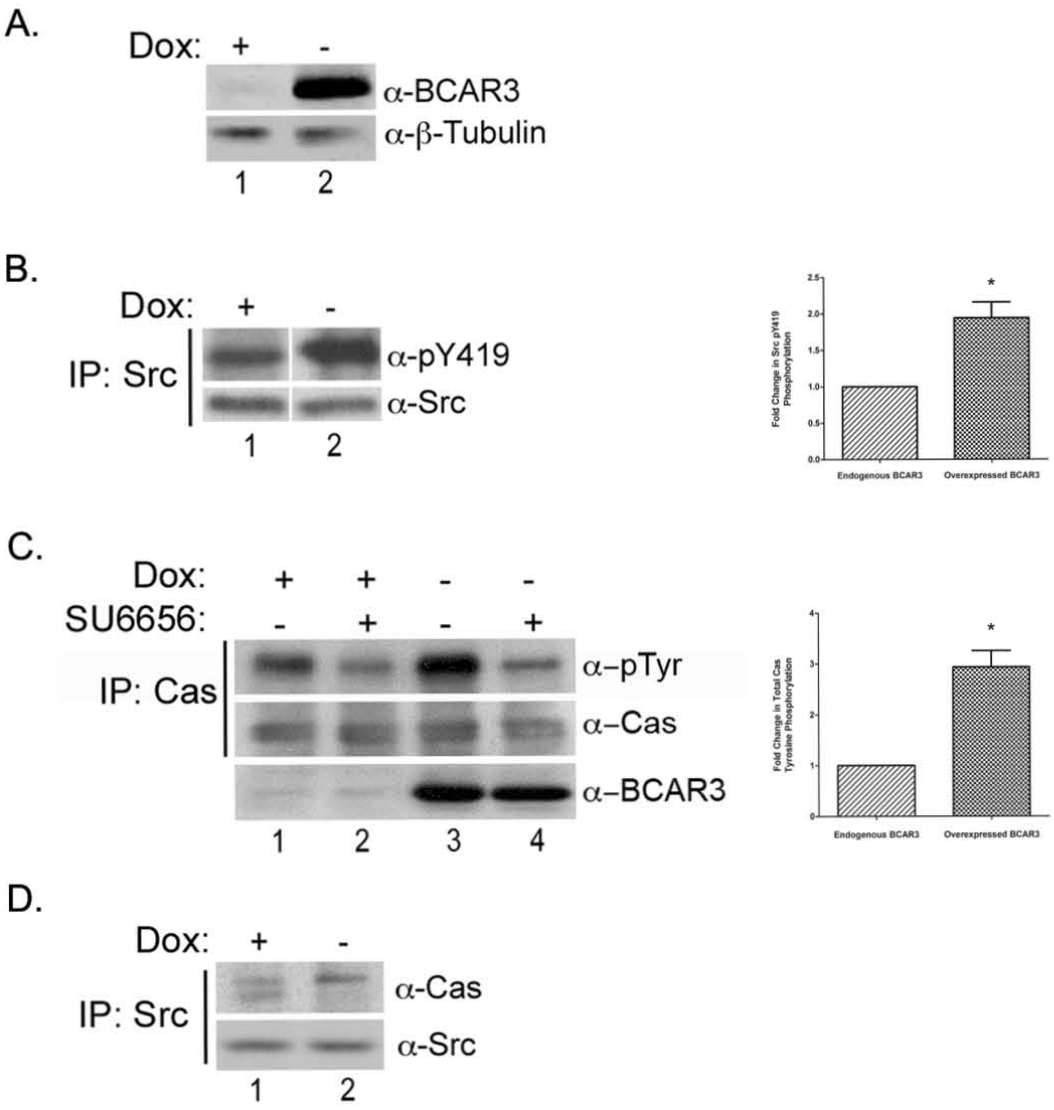

A.
Dox: + -
α-BCAR3
α-β-Tubulin
1 2
B.
Dox: + -
IP: Src
α-pY419
α-Src
1 2
Fold Change in Src pY419 Phosphorylation
2.5
2.0
1.5
1.0
0.5
0.0
*
Endogenous BCAR3
Overexpressed BCAR3
C.
Dox: + + - -
SU6656: - + - +
IP: Cas
α–pTyr
α–Cas
α–BCAR3
1 2 3 4
Fold Change in Total Cas Tyrosine Phosphorylation
4
3
2
1
0
*
Endogenous BCAR3
Overexpressed BCAR3
D.
Dox: + -
IP: Src
α-Cas
α-Src
1 2

Figure 3.6: BCAR3 overexpression in MCF7 cells increases Src activity and Cas phosphorylation.

A. Dox-regulated expression of BCAR3 in Tet-off MCF7 cells. MCF7 cells overexpressing BCAR3 under control of a tet-responsive promoter were grown in the presence or absence of 2μg/ml Dox for 72 hours. Cell lysate was immunoblotted with antibodies specific to BCAR3 and β-tubulin. **B.** BCAR3 overexpressing cells exhibit increased Src activation. Src was immunoprecipitated from MCF7 cells grown in the presence or absence of 2μg/ml Dox for 72 hours and probed with antibodies specific for pY419 and total Src. Graph; pY419 was increased by an average of 1.9-fold ± 0.43 ($p=0.02$, $n=3$). White vertical line denotes non-contiguous sample lanes; the exposure times for the + and - Dox samples in each immunoblot are the same. **C.** BCAR3-dependent Cas phosphorylation is mediated by Src family kinases. MCF7 cells grown in the presence or absence of Dox for 48 hours were treated with vehicle (DMSO) or SU6656 (10 μM) an additional 24 hours. Cas was immunoprecipitated from lysate and blotted with antibodies specific to pTyr and Cas (top two panels). Cell lysate was also probed using BCAR3 antibodies (bottom panel). Graph; Cas phosphorylation increased by an average of 2.9-fold ± 0.64 ($p=0.009$) in the presence of overexpressed BCAR3. Data shown are representative of four independent experiments. **D.** BCAR3 expression regulates Cas/Src interactions. Src immune complexes were generated from MCF7 cells grown in the presence or absence of Dox for 72 hours and immunoblotted with Cas and Src specific antibodies. Experiments performed by Michael Guerrero.

Figure 3.7

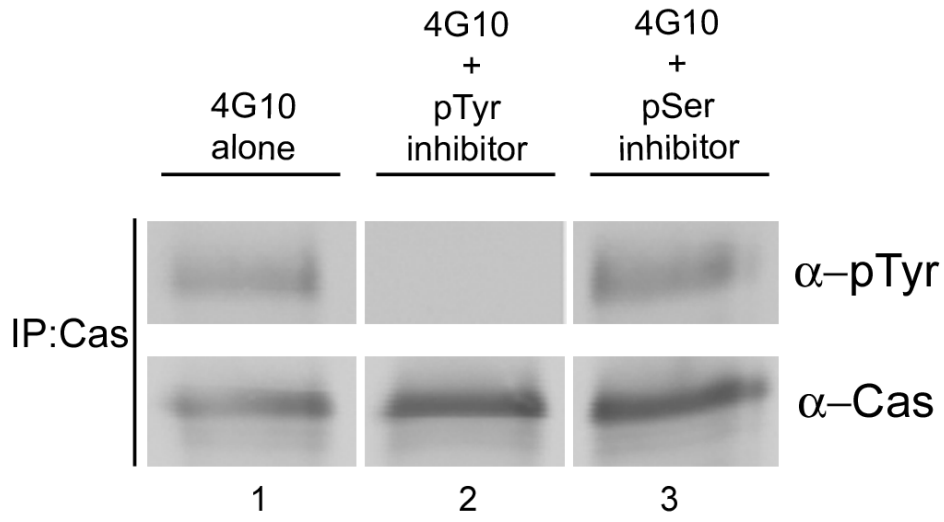


Figure 3.7: **Anti-phosphotyrosine clone 4G10 specifically recognizes phosphorylated tyrosine residues on Cas.**

Cas was immunoprecipitated from MCF7 lysates. Resultant complexes were immunoblotted for total Cas (bottom panels) or tyrosine phosphorylated Cas (top panels) as described for Fig. 3D. To verify the specificity of pY4G10, the antibody was diluted 1:500 (v/v) in 5% BSA/PBS and incubated either alone (lane 1) or in the presence of 5μg of a phosphoserine-conjugated (lane 2) or phosphotyrosine-conjugated (lane 3) BSA (Sigma-Aldrich Corp., P3717 and P3967, respectively) for 1 hr at 37^{o}C. Antibody solutions were then further diluted 1:10 (v/v) in 5% BSA/PBS to achieve a final pY4G10 antibody dilution of 1:5000. Nitrocellulose membranes containing Cas immune complexes were incubated for 1 hr. at room temperature with these antibody solutions, followed by 1 hr. with HRP-conjugated rabbit-anti-mouse antibodies. Experiment performed by Michael Guerrero.

3.3.4 – *BCAR3 Regulates Cell Spreading on Fibronectin and Adhesion-mediated Cas Tyrosine Phosphorylation.*

Since BCAR3 was found to regulate Src activity and Cas tyrosine phosphorylation in cycling, continuously adherent cells, we next investigated whether it played a similar role in response to adhesion and spreading on extracellular matrix, a biological process that is controlled by Cas and Src signaling (Cary, Klinghoffer et al. 2002). BT549 cells were transiently transfected with non-targeting or BCAR3-specific siRNAs and cultured for 72 hours. Cells were trypsinized, left in suspension for 90 minutes, and then plated on fibronectin-coated tissue culture dishes or coverslips for 30-240 minutes. The area of cells on the fibronectin-coated coverslips was determined for each time point. At 30 minutes, control cells expressing BCAR3 had begun to spread and this continued through 240 minutes (Fig. 3.8A, top panel, and B, black bars). BCAR3-depleted cells exhibited delayed adherence and spreading at all time points compared to control cells (Fig. 3.8A, bottom panel and B, grey bars). This defect in spreading was also seen using alternate BCAR3 siRNAs, confirming that the defect in cell spreading was induced by the loss of BCAR3 expression in these cells (Fig. 3.4D).

Because Cas becomes tyrosine phosphorylated in response to cell adhesion (Nojima, Morino et al. 1995, Hamasaki, Mimura et al. 1996), we next investigated whether changes in adhesion-dependent Cas phosphorylation coincided with the delay in spreading in BCAR3-depleted cells. Cas was immunoprecipitated from lysates generated under the treatment conditions

described above and immunoblotted with a pTyr antibody to measure Cas phosphorylation. Tyrosine phosphorylation of Cas in control cells was seen to increase over time in an adhesion-dependent manner, peaking at 120 minutes (Fig. 3.8C, top panel, lanes 1-5). A similar, albeit significantly reduced, adhesion-dependent increase in Cas phosphorylation was seen in cells depleted for BCAR3 (lanes 7-11). These differences in phosphorylation were also observed in continuously adherent cells grown on fibronectin (lanes 6 and 12), confirming our earlier observations in cells plated on plastic (Fig. 3.3B). These data show that BCAR3 expression is required for efficient adhesion-mediated Cas phosphorylation as well as cell spreading in BT549 cells.

Figure 3.8

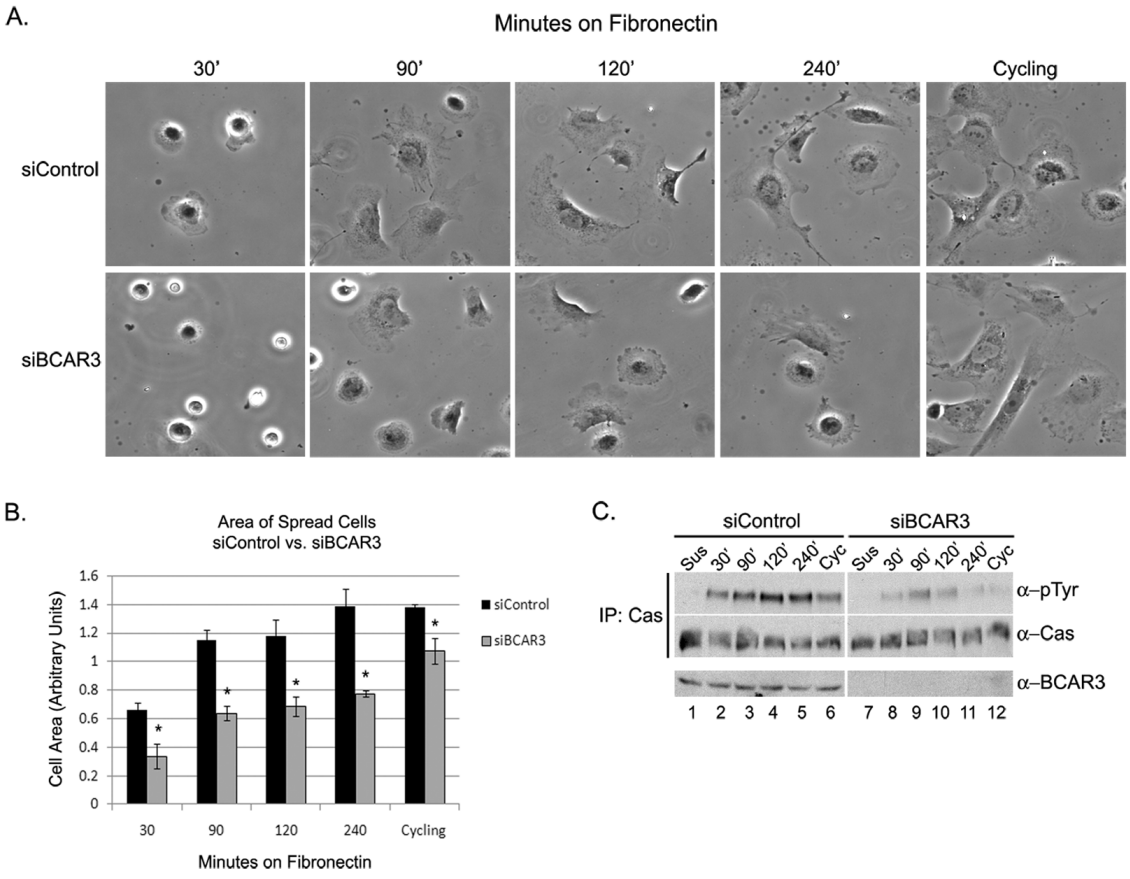

A.
Minutes on Fibronectin
30'
90'
120'
240'
Cycling
siControl
siBCAR3
B.
Area of Spread Cells
siControl vs. siBCAR3
Cell Area (Arbitrary Units)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
30
90
120
240
Cycling
Minutes on Fibronectin
siControl
siBCAR3
C.
siControl
siBCAR3
Sus
30'
90'
120'
240'
Cyc
IP: Cas
α–pTyr
α–Cas
α–BCAR3
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.8: Loss of BCAR3 expression disrupts cell spreading and adhesion-mediated Cas phosphorylation.

A and **B.** BT549 cells depleted for BCAR3 exhibit decreased cell spreading on fibronectin. BT549 cells were transfected with non-targeting or BCAR3-specific siRNA duplexes and grown for 72 hours. Cells were left in suspension for 90 minutes and then plated on fibronectin-coated coverslips in complete growth media with 10% FBS for the indicated lengths of time in normal growth media. A coverslip from cycling, adherent cells was also examined. Mean cell area from three independent experiments was determined by using ImageJ software. Bars indicate standard deviation, * indicates significant difference from the mean at ≥95% confidence interval. **C.** BT549 cells depleted for BCAR3 exhibit decreased adhesion-mediated Cas phosphorylation. BT549 cells were transfected and treated as described above. Cas immune complexes were generated from lysate and immunoblotted with pTyr and Cas antibodies (top two panels). Whole cell lysate was also immunoblotted with antibodies specific to BCAR3 (bottom panel). Data shown are representative of four independent experiments. Within all panels, exposure time is the same; the white vertical line denotes non-contiguous sample lanes. Experiments performed by Natasha Schuh.

3.3.5 – *BCAR3 Regulates Cell Spreading through Src-dependent and -independent Pathways.*

As BCAR3 was shown to be involved in Src signaling (Fig. 3.6B and C), we next investigated whether loss of Src activity could be responsible for the defect in spreading exhibited by cells depleted of BCAR3. BT549 cells were transiently transfected with non-targeted or BCAR3-specific siRNAs and cultured for 72 hours. Cells were then trypsinized, left in suspension in the presence or absence of 10 μM SU6656 for 90 minutes, and then plated on fibronectin-coated coverslips in the continued presence or absence of SU6656 for 30 to 240 minutes. Consistent with our earlier observations, knockdown of BCAR3 resulted in a significant impairment in spreading compared to control cells at all time points (Fig. 3.9A, first and second rows; Fig. 3.9B, black and grey bars). If BCAR3 regulates cell spreading through its ability to enhance Src activity, we reasoned that 1) inhibition of Src in control cells should phenocopy BCAR3 knockdown and 2) the combined knockdown of BCAR3 with Src inhibition should have no greater effect than either treatment alone. Cells treated with SU6656 did in fact show a delay in spreading, albeit to a lesser degree than BCAR3-depleted cells (Fig. 3.9A, third rows; Fig. 3.9B, black striped bars). However, combined BCAR3 knockdown and SU6656 treatment resulted in a delay in spreading that was significantly greater than what was observed for either condition alone (Fig. 3.9B, grey striped bars). With the caveat that Src inhibition and BCAR3 knockdown may not have been complete, these results suggest that Src activity may be only partially required for BCAR3-dependent cell spreading, and vice

versa. Moreover, the fact that combined Src inhibition and BCAR3 depletion did not completely abrogate cell spreading suggests the involvement of additional pathways(s) that may be independent of both of these molecules.

Figure 3.9

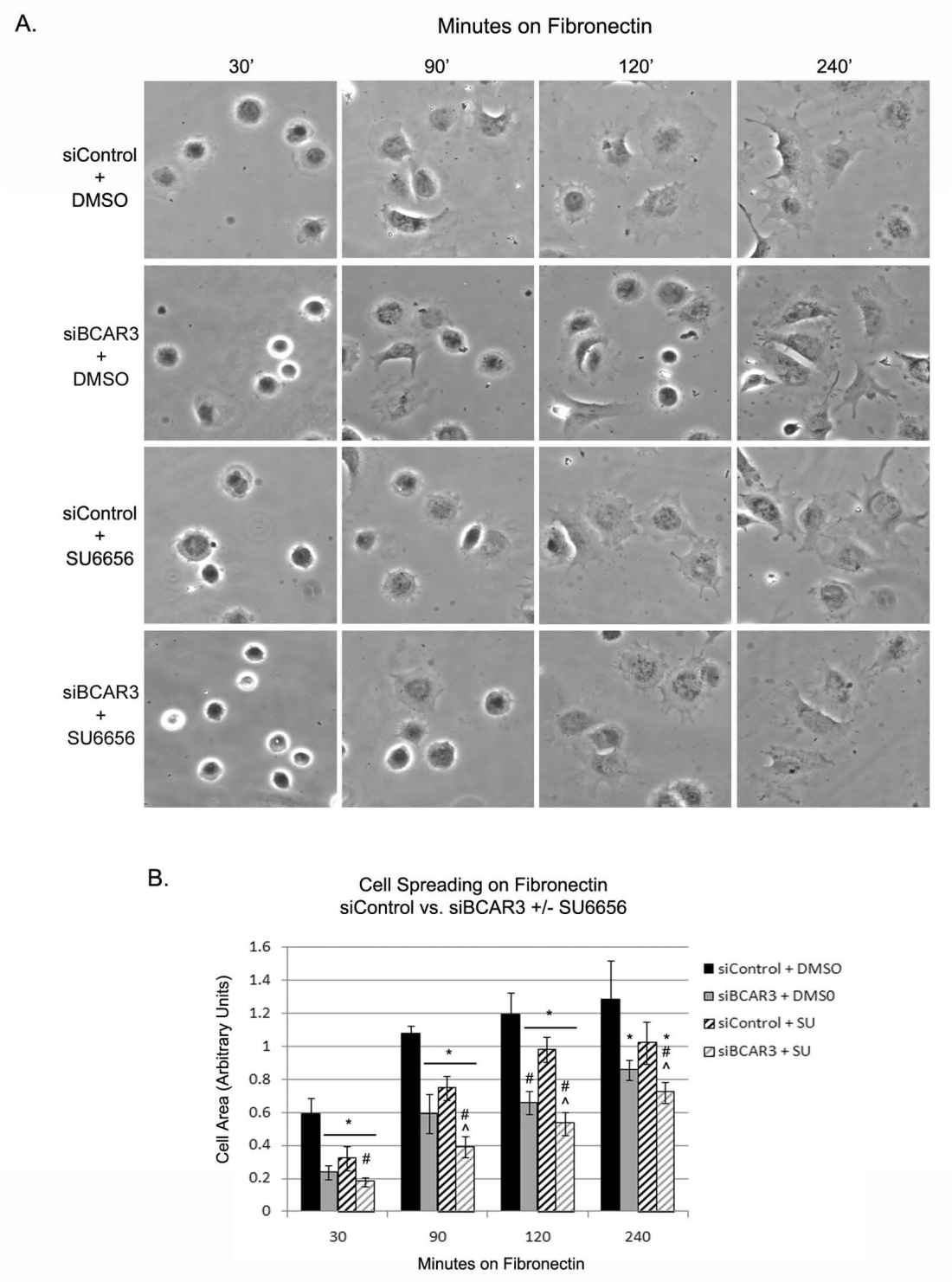

A.
Minutes on Fibronectin
30'
90'
120'
240'
siControl + DMSO
siBCAR3 + DMSO
siControl + SU6656
siBCAR3 + SU6656
B.
Cell Spreading on Fibronectin
siControl vs. siBCAR3 +/- SU6656
Cell Area (Arbitrary Units)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
30
90
120
240
Minutes on Fibronectin
siControl + DMSO
siBCAR3 + DMS0
siControl + SU
siBCAR3 + SU

Figure 3.9: BCAR3 regulates both Src dependent and independent signaling pathways in response to adhesion and cell spreading.

BT549 cells were transfected with non-targeting or BCAR3-specific siRNA duplexes and grown for 72 hours. **A.** Cells were left in suspension for 90 minutes in the presence of DMSO or 10 μM SU6656, and then plated onto fibronectin-coated coverslips in complete growth media with 10% FBS for the indicated lengths of time in normal growth media with or without SU6656. **B.** Mean cell area was calculated from four independent experiments. Two-tailed Student's t-tests were conducted for comparison between the various sample sets. Bars indicate standard deviation. *, #, ^ indicate significant difference from the mean versus control DMSO, control SU6656, and siBCAR3 DMSO, respectively, at ≥95% confidence interval. Experiments performed by Natasha Schuh.

3.4 – Discussion

In this study, we present evidence for the regulation and function of an important signaling network in breast cancer cells involving three proteins – Cas, Src, and BCAR3. Previously, we showed that the co-overexpression of BCAR3, Cas, and Src induces Src activity in untransformed monkey kidney cells (COS-1) (Riggins, Quilliam et al. 2003). The work of this Chapter expands upon this finding by demonstrating that, in BT549 and MCF7 breast cancer cell lines, BCAR3 can regulate Cas/Src interactions, Src activity, Src-dependent Cas tyrosine phosphorylation, Cas/Crk coupling (Fig 3.11 and (Schrecengost, Riggins et al. 2007)), and cell spreading. Collectively, these studies establish BCAR3 as a key regulator of signaling events involving Cas and Src in the setting of breast cancer cells, and for the first time provide insight into why breast cancer cells with elevated BCAR3 expression may exhibit more aggressive behaviors.

3.4.1 – *Molecular Dynamics of the BCAR3/Cas/Src Signaling Axis*

The ability of BCAR3 and Cas to regulate Src activity provides a mechanism through which Src signaling can be both temporally and spatially controlled within the cell. We postulate that BCAR3, which helps to localize Cas to membrane-proximal sites (Riggins, Quilliam et al. 2003, Schrecengost, Riggins et al. 2007, Makkinje, Near et al. 2009), may enhance Cas-dependent Src activity by bringing Cas into proximity of membrane-bound Src (Fig. 3.10). Subsequent association of Cas with Src would then relieve the auto-inhibitory conformation of the kinase, leading to increased catalytic activity in these regions of the cell.

Support for this model comes from the loss-of-function and gain-of-function studies presented above, which show that BCAR3 expression directly impacts Cas/Src interactions and Cas tyrosine phosphorylation. Moreover, we show that this is largely dependent on Src activity since treatment with the Src inhibitor SU6656 blocked the BCAR3-dependent increase in Cas phosphorylation (Fig. 3.6C). Interestingly, it does not appear that BCAR3, Cas, and Src need to be present in a single molecular complex in order to regulate Src activity and functions since BCAR3 was not detected in Cas/Src complexes (Fig. 3.2A) and Src was similarly not present in BCAR3/Cas complexes (Fig. 3.2B). These data indicate that there are two distinct pools of Cas, one that is associated with BCAR3 and one that is associated with Src. Based on these findings we propose that BCAR3 brings Cas into close proximity with Src, where it subsequently promotes association with, and activation of, this kinase. It is this dynamic relationship between pools of Cas that initiates the BCAR3/Cas/Src signaling axis.

Figure 3.10

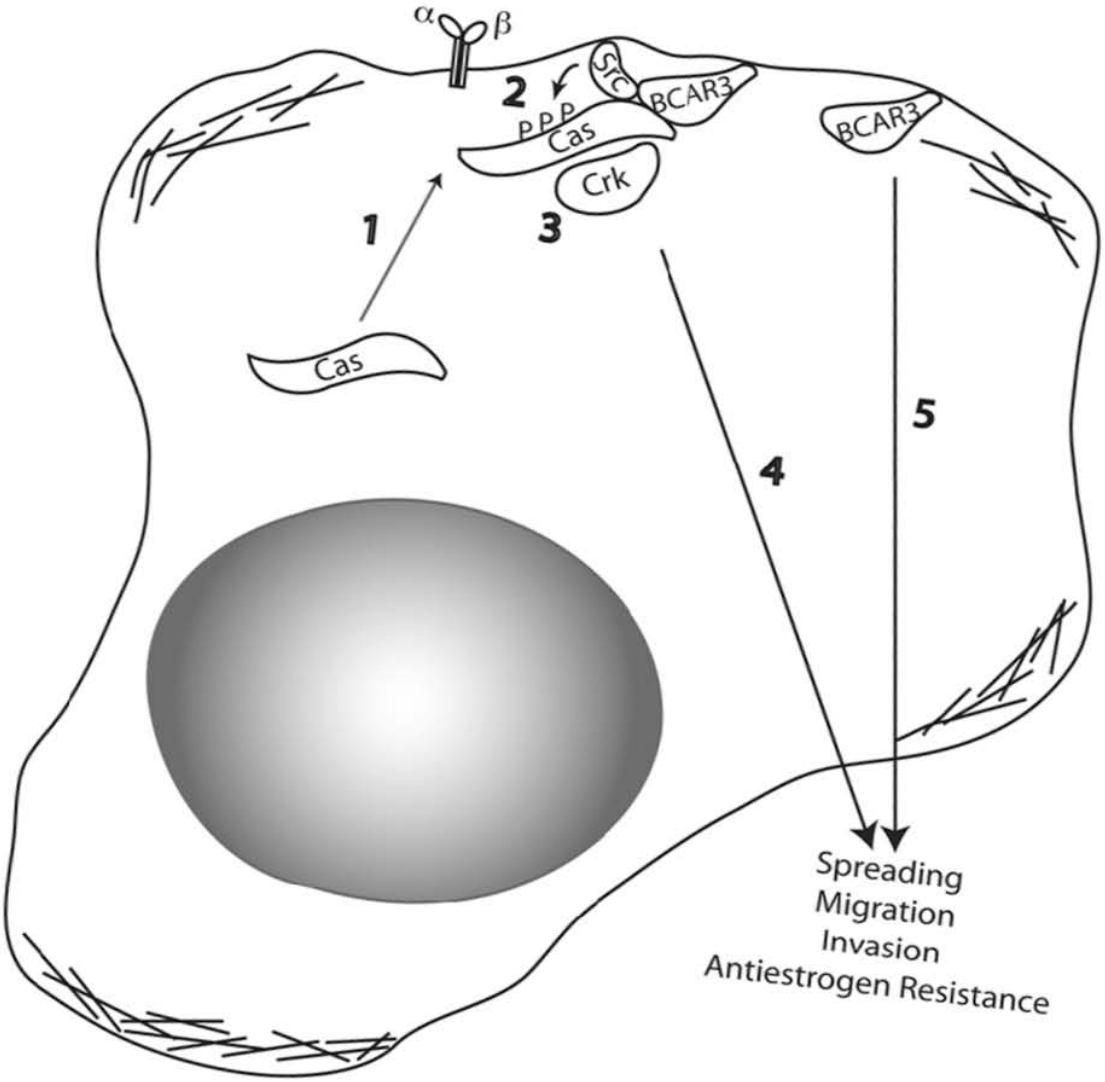

α
β
2
P P P
Src
BCAR3
Cas
Crk
3
1
Cas
BCAR3
4
5
Spreading
Migration
Invasion
Antiestrogen Resistance

Figure 3.10: Proposed mechanism of BCAR3-mediated enhancement of Src activation, cell spreading, and adhesion.

1) Cytosolic-localized Cas is recruited to membrane-proximal regions by BCAR3 through an indirect or direct interaction with BCAR3. Once at the membrane (2), Cas interacts with and activates Src. Src activation results in the phosphorylation of Cas, which leads to the recruitment of substrate-domain binding partners such as Crk (3). Cas/Crk interactions activate downstream signaling pathways important for cell spreading, migration, and invasion (4). BCAR3-dependent (5) and BCAR3-independent (6) Src signaling can activate other signaling pathways important for cell proliferation and survival in the presence of antiestrogens. BCAR3 itself also activates an unidentified Src independent pathway important for cell spreading (7). Model by Natasha Schuh.

While we propose that the recruitment of Cas by BCAR3 to membrane-proximal sites is an important factor in the activation of Src by BCAR3, it may not be the sole determinant. Exactly how BCAR3 is localized and anchored to the membrane is still an incompletely resolved question. More recent work has shown that BCAR3 binds to the transmembrane protein PTPα (Sun, Cheng et al. 2012); this is addressed further in the Discussion (Chapter 5). Indeed, we have previously shown in rat embryo fibroblasts that a carboxyl-terminal deletion mutant of BCAR3 could induce Cas accumulation at lamellipodia without promoting Cas-dependent Src activity (Riggins, Quilliam et al. 2003). The carboxyl terminus of BCAR3 has two known activities that may contribute to its ability to regulate Src activity. First, it contains the sequences necessary for Cas binding. However, while BCAR3/Cas complexes have been detected in a number of breast cancer cell lines (Near, Zhang et al. 2007, Schrecengost, Riggins et al. 2007, Makkinje, Near et al. 2009), we showed previously that BCAR3 was still able to promote activation of Src through a mutant of Cas that could not bind to BCAR3 (Riggins, Quilliam et al. 2003). Second, the carboxyl terminus of BCAR3 contains sequences shown to be important for activating a number of small GTPases, including Rap1 (Gotoh, Cai et al. 2000). Interestingly, constitutively active Rap1 can substitute for BCAR3 in the activation of Src in the COS-1 system (Riggins, Quilliam et al. 2003).

The regulation of Src activity by Cas and BCAR3 appears to be under tight control. Thus, overexpression of BCAR3 to very high levels in MCF7 cells (~35-fold above the level of BCAR3 endogenously expressed in BT549 cells; data not

shown) resulted in only a modest 2-fold increase in Src autophosphorylation (Fig. 3.6B). We propose that the pool of Cas capable of activating Src under these conditions may be a limiting factor, and any BCAR3 expressed above a certain threshold would therefore have little added effect; this model is supported by the finding that the total level of Cas associated with Src was not significantly changed in MCF7 cells overexpressing BCAR3 (Fig. 3.6D). However, there was a marked qualitative difference in Cas found in this complex, indicative of a heavily phosphorylated Cas species (Sakai, Iwamatsu et al. 1994, Nojima, Morino et al. 1995). It is not clear whether this difference is a cause or an effect of the increased Src activity present under these conditions. Regardless, we predict that this modified complex has an increased potential for transducing signals necessary for adhesion signaling, spreading, and migration.

As discussed above, the Cas that is found in association with Src in BCAR3-expressing MCF7 cells is exclusively the slower migrating species, indicative of hyperphosphorylation. Makkinje *et al.* have recently shown that Cas becomes phosphorylated on serine residues in the presence of BCAR3 (Makkinje, Near et al. 2009). While these authors failed to detect tyrosine phosphorylation on Cas by mass spectroscopy in the presence of overexpressed BCAR3, our studies clearly show that tyrosine phosphorylation of Cas is elevated as a function of BCAR3 expression in these cells (Fig. 3.3B and 3.6C). We have not evaluated whether serine phosphorylation of Cas is also elevated under these conditions in our system. However, the increased tyrosine phosphorylation on Cas that was observed as a function of BCAR3 expression in MCF7 cells

coincided with an increase in Cas/Crk complexes (Fig. 3.11, see *Update* below), which become established through interactions between the SH2 domain of Crk and phosphorylated tyrosine residues in the substrate binding domain of Cas (Feller 2001, Chodniewicz and Klemke 2004). Cas tyrosine phosphorylation has also been reported to be elevated when the BCAR3 family member Shep1 is constitutively targeted to the membrane, a process that required Src activity (Dail, Kalo et al. 2004).

3.4.2 – *Biological Activities of the BCAR3/Cas/Src Signaling Axis*

The data presented in this report establish that BCAR3, Cas, and Src function together in a coordinated network when all three proteins are endogenously expressed in breast cancer cells. Each of these proteins has been implicated in a wide variety of cellular processes in breast cancer cells, including cell proliferation, survival, adhesion signaling, and migration (Ishizawar and Parsons 2004, Defilippi, Di Stefano et al. 2006, Schrecengost, Riggins et al. 2007). In this report, we have focused on cell spreading as a component of adhesion signaling and migration dynamics, processes that, when deregulated, can contribute to aggressive and invasive tumor phenotypes. We show for the first time that BCAR3 regulates the kinetics of cell spreading on fibronectin. Coincident with slower spreading in BCAR3-depleted cells, Cas tyrosine phosphorylation, but not overall Cas expression, was reduced. These data suggest that the regulation of Cas phosphorylation by BCAR3 may contribute to cell spreading dynamics in cells expressing this molecule. Cas-null mouse

embryo fibroblasts show a similar spreading defect (Honda, Oda et al. 1998), and expression of a construct of Cas that lacks the heavily phosphorylated substrate-binding domain fails to rescue the spreading and migratory defects (Huang, Hamasaki et al. 2002). As mentioned above, tyrosine phosphorylation of Cas within the substrate binding domain provides binding sites for the small adapter molecule Crk; this interaction in turn regulates cell adhesion, spreading, and migration through the activation of the small GTPases Rap1 and Rac1 (Feller 2001, Chodniewicz and Klemke 2004). Interestingly, forced expression of BCAR3 in MCF7 cells mediates an increase in the formation of Cas/Crk complexes (Fig. 3.11, see *Update* below), while knock down of BCAR3 in BT549 cells results in a reduction in Cas/Crk complexes (Schrecengost, Riggins et al. 2007). Cas/Crk coupling is thus likely to be an important downstream consequence of BCAR3-dependent Src activity and Cas phosphorylation, which in turn contributes to cell spreading, migration, and invasion (Fig. 3.10).

Figure 3.11

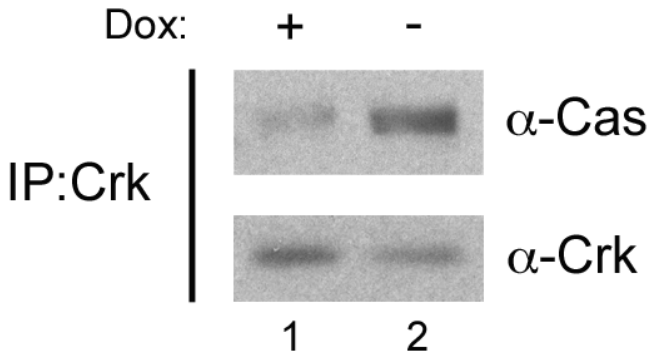


Figure 3.11: BCAR3 overexpression in MCF7 cells induces greater Cas/Crk complexes.

MCF7 Tet-off cells were cultured in the presence of absence of 2μg/ml Dox for 48 hours. Crk was immunoprecipitated from 200μg of total protein, and analyzed via Western blot. Total Crk and Crk-bound Cas were detected by probing immune complexes with antibodies recognizing Crk and Cas, respectively. Experiment performed by Michael Guerrero.

We hypothesize that the regulation of Src activity by BCAR3 contributes in part to the defect in spreading observed when BCAR3 is depleted from breast cancer cells. As was the case for BCAR3 depletion, inhibition of Src activity by SU6656 was shown to delay spreading (Fig. 3.9). However, modulation of Src activity may not be the only mechanism through which BCAR3 functions to regulate cell spreading since combined Src inhibition and BCAR3 depletion resulted in a significantly greater, albeit modest, delay in cell spreading relative to Src inhibition alone. Similarly, it appears from our data that Src may function to control cell spreading through a mechanism that is in part independent of BCAR3. Since combined depletion/inhibition of these molecules failed to completely block cell spreading, pathways independent of both BCAR3 and Src are likely to play some role in controlling this cellular process.

While this study has focused on the role of BCAR3 in Cas and Src signaling during cell spreading, it is important to note that breast cancer cells may exploit this signaling axis for other biological activities. For example, both Cas and BCAR3 induce antiestrogen resistance in estrogen receptor-positive breast cancer cells (Brinkman, van der Flier et al. 2000, Felekkis, Narsimhan et al. 2005, Riggins, Thomas et al. 2006), and Cas and Src play a role in resistance to several chemotherapeutics (Ta, Thomas et al. 2008, Kim, Song et al. 2009). Thus, the expression of BCAR3 and Cas, and the subsequent activation of Src-dependent signaling pathways, may be one mechanism that drives the oncogenic potential, therapeutic response, and metastatic spread of breast tumor cells. Further investigation into the consequences of BCAR3 and Cas expression

and their effect(s) on Src activity may aid in the development of novel therapeutic strategies for patients whose breast tumors overexpress these proteins.

3.4.3 – *Update (March, 2013)*

This chapter was published in the Journal of Biological Chemistry in January of 2010 and the manuscript has been altered and updated for this thesis. At the time that the manuscript was completed, we found that BCAR3 overexpression in the MCF7 Tet-off cells leads to increased Cas/Crk coupling. These data (originally noted as *data not shown*) are included here as Figure 3.11, and will be published in the paper referred to within this thesis as "Wilson et al., 2013, *manuscript in progress*."

Following the acceptance and publication of this paper, we found that the addition of doxycycline to the MCF7 Tet-off cells causes a dramatic reduction in cell proliferation (see Appendix). To verify that the results presented in Figures 3.6 and 3.11 were not due to doxycycline, MCF7 Tet-off cells expressing empty vector were treated with 2μg/mL doxycycline and Cas tyrosine phosphorylation was measured, as described for Figure 3.6C. No doxycycline-induced effect on Cas phosphorylation was observed (Fig. 3.12). This control confirms that the findings in Figure 3.6 and 3.11 are due to the overexpression of BCAR3 and not to any side effect of the drug.

Figure 3.12

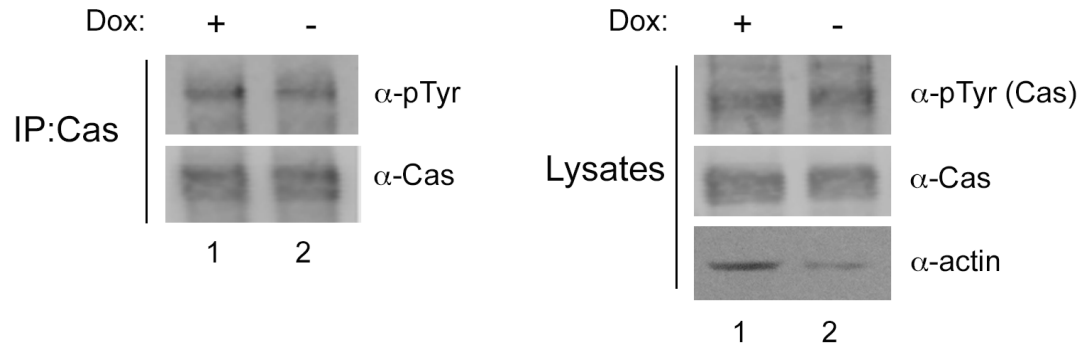


Figure 3.12: Doxycycline (Dox) does not alter the level of Cas tyrosine phosphorylation (pTyr).

MCF7 Tet-off cells stably transduced with empty Myc-pTre2-puro vector were grown in the presence or absence of 2μg/ml Dox for 48 hours. To determine the level of tyrosine phosphorylated Cas, Cas was immunoprecipitated from 500μg of total protein as described in Figure 3.6. Immune complexes and lysate samples were probed with antibodies that recognize total Cas (B3b) and phosphorylated tyrosine residues (4G10). Blots were imaged using the Odyssey Infrared Imaging System (LI-COR). Experiment performed by Michael Guerrero.

Chapter 4. BCAR3 Protein Synthesis Is Regulated by Its Binding Partner $p130^{Cas}$ in the Breast Cancer Cell Lines, MDA-MB-231 and BT549

4.1 – Abstract

The adaptor protein Cas is critically involved in the regulation of a wide variety of cellular processes, most notably in the molecular dynamics of cell motility. BCAR3, a more recently identified adaptor, is also part of the cellular machinery that controls cell adhesion, spreading, and migration. These two proteins are found in complex with one another, and a well-defined role for BCAR3 in the regulation of Cas signaling has been established. In the present study, we initially sought to identify possible Cas-independent functions of BCAR3 and instead found that, by controlling BCAR3 expression, Cas is an indispensable component of BCAR3 signaling. We demonstrate here, using two different cell lines and utilizing siRNA-mediated knockdown of Cas, that BCAR3 protein is lost as a function of Cas knockdown. Furthermore, we show that this loss is not due to the proteolytic degradation of pre-existing protein or to a decrease in steady-state levels of mRNA, but to a reduction in BCAR3 protein synthesis. These data identify Cas as a critical regulator of BCAR3 expression, and serve to expand our knowledge and understanding of the functional interplay between these two proteins.

4.2 – Introduction

The adaptor proteins BCAR3 and Cas integrate the action of multiple signaling molecules to facilitate the transduction of external cues to promote specific cellular responses. BCAR3 and Cas bind directly to each other, and like all adaptors they contain multiple protein-binding domains that allow for the association with a number of heterologous proteins (Vuori, Hirai et al. 1996, Kiyokawa, Hashimoto et al. 1998, Cho and Klemke 2002, Schrecengost, Riggins et al. 2007, Oh, van Agthoven et al. 2008, Mace, Wallez et al. 2011, Sun, Cheng et al. 2012). BCAR3 and Cas have been shown to play a role in migratory, proliferative, and survival signaling downstream of a number of chemical and physical stimuli, such as growth factor/cognate receptor binding or integrin/ECM engagement [(Ojaniemi, Langdon et al. 1998, Cho and Klemke 2000, Sawada, Tamada et al. 2006, Schrecengost, Riggins et al. 2007, Oh, van Agthoven et al. 2008) Wilson et al., 2013, *manuscript in progress*]. Overexpression of either protein has been shown to induce resistance to certain anti-cancer drugs (van Agthoven, van Agthoven et al. 1998, Brinkman, van der Flier et al. 2000, Ta, Thomas et al. 2008), and both proteins have been implicated in the promotion of metastatic behaviors (Schrecengost, Riggins et al. 2007, Wendt, Smith et al. 2009, Tornillo, Bisaro et al. 2011). Moreover, high Cas expression is associated with advanced stage disease and is predicative of poor outcomes in many human cancers (Guerrero, Parsons et al. 2012). Thus, given this link to malignant progression, much research has focused on the coordinate function and regulation of these two binding partners.

Recent evidence, including the work shown in Chapter 3, suggests that a major role of BCAR3 is to modulate Cas function and expression. BCAR3 augments Cas/Src complex formation, Src kinase activity, Src-dependent Cas tyrosine phosphorylation, and Cas/Crk coupling (see Chapter 3; (Schuh, Guerrero et al. 2010) Wilson et al., 2013, *manuscript in progress*). Shep1 similarly affects this Cas signaling pathway in mouse brain tissue (Roselli, Wallez et al. 2010). Increases in BCAR3 and Shep1 expression have also been reported to increase Cas protein levels (Near, Zhang et al. 2007, Roselli, Wallez et al. 2010). Collectively, these studies provide demonstrative evidence that BCAR3 positively regulates the activity of Cas.

In this study, we initially set out to explore potential Cas-independent functions of BCAR3. In the course of performing this work, we found that depletion of Cas expression by siRNA-mediated knockdown leads to a commensurate loss in BCAR3 protein expression. This Cas-dependent control of BCAR3 expression was evident in two different invasive breast cancer cell lines, MDA-MB-231 and BT549. In both cell lines, we show that BCAR3 is found exclusively in complex with Cas. Further analysis into Cas-dependent BCAR3 expression revealed Cas is required for the efficient synthesis of BCAR3 in these cells. These findings not only demonstrate that endogenous BCAR3 function is indeed dependent on Cas, but they introduce the concept that Cas may have the ability to regulate the expression of its own modulator.

4.3 – Results

4.3.1 – *The Expression of BCAR3 Protein Is Dependent on the Expression of Cas.*

To begin to address potential Cas-independent functions of BCAR3, Cas protein levels were depleted by transfection with 4 independent Cas-specific siRNAs in two breast cancer cell lines that express relatively high endogenous levels of both proteins, MDA-MB-231 and BT549 (Near, Zhang et al. 2007, Schrecengost, Riggins et al. 2007). Cells were lysed 72 hours post-transfection and Western blots were performed to confirm Cas knockdown. As expected, Cas expression was dramatically lower in the siCas transfectants compared to their control counterparts (Fig. 4.1A & B; top panels). Surprisingly, upon probing these membranes with BCAR3 antibody, a corresponding and equally dramatic loss of BCAR3 expression was observed in the cells that received Cas-targeted siRNA (middle panels). This appeared to be specific for BCAR3, as FAK and actin levels were unaffected by Cas knockdown (Fig. 4.1C). Interestingly, Src levels appeared to be increased in Cas-depleted cells (fourth panel); a similar result was seen using two other Cas-specific oligos (data not shown). As Cas is a positive regulator of Src activity, this increase in total Src protein following Cas knockdown may be due to the increased stability of inactive Src (Hakak and Martin 1999). The siRNA denoted as “siCas2” is the primary siRNA oligonucleotide used throughout the remainder of this study, and this naming system applies when additional oligos are used in other experiments.

Although unexpected, the loss of BCAR3 expression following Cas knockdown clearly demonstrates that BCAR3 function is dependent on Cas. We then became interested in the mechanism by which Cas governs the expression of BCAR3 and in the dynamics of BCAR3/Cas interactions.

Figure 4.1

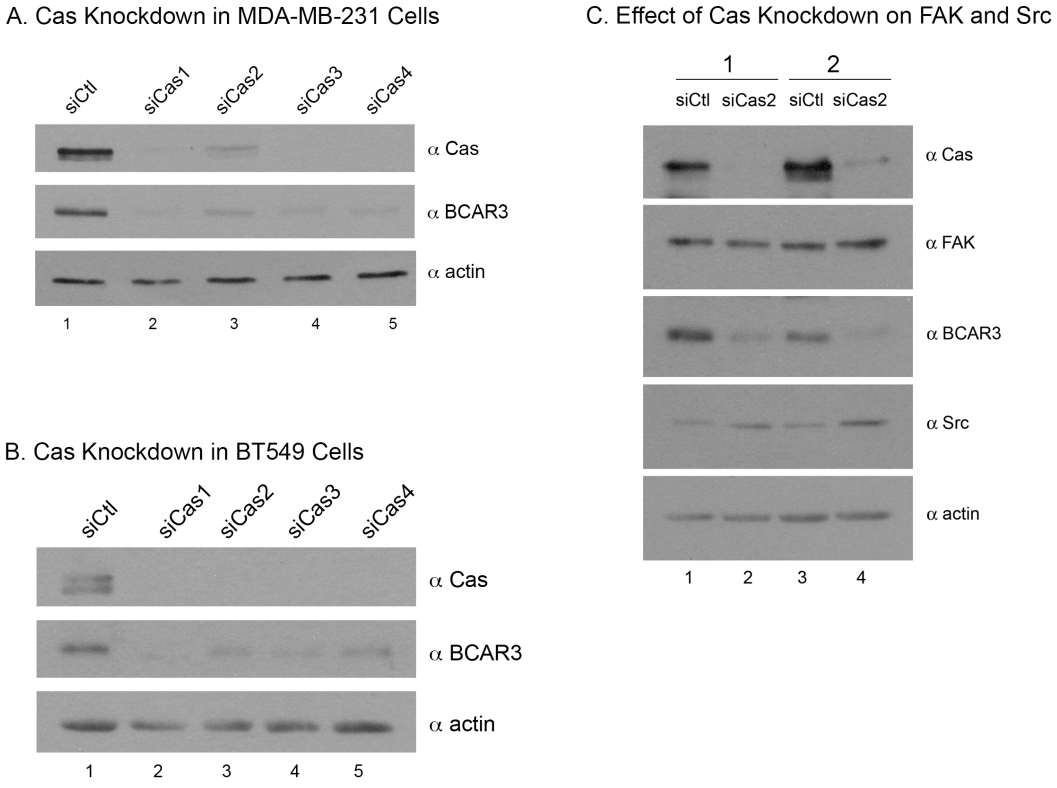


Figure 4.1: Knockdown Cas results in a concomitant loss of BCAR3.

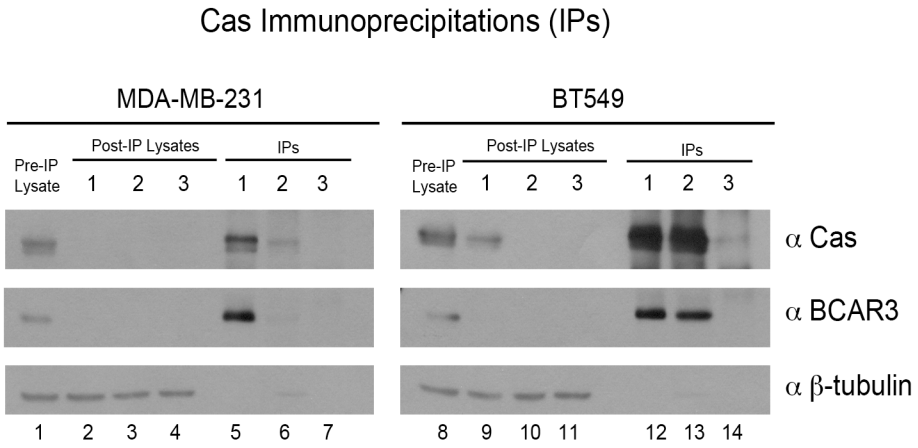
A. and **B.** MDA-MB-231 (A.) and BT549 (B.) cells were transfected with 4 independent Cas siRNA oligos. 72 hours post-transfection, cells were lysed in RIPA and protein extracts were analyzed by Western blot using BCAR3 (middle panel) and Cas (top) specific antibodies. Actin is used as a loading control (bottom). **C.** The effect of Cas depletion on FAK and Src protein levels. Cas was knocked down in BT549 cells as described above, and FAK and Src protein levels were analyzed by Western blot using FAK and Src specific antibodies.

4.3.2 – *The Entire Cellular Pool of BCAR3 Exists in Complex with Cas*

Previous studies describe a direct, physical interaction between BCAR3 and Cas (Cai, Clayton et al. 1999, Gotoh, Cai et al. 2000, Garron, Arsenieva et al. 2009, Mace, Wallez et al. 2011). Given the finding that BCAR3 expression is Cas-dependent (Fig. 4.1), we sought to further characterize this association by determining the respective fraction of each protein found in complex with the other. Lysates of MDA-MB-231 and BT549 cells were cleared of either BCAR3 or Cas by serial immunoprecipitation, and after each pull-down BCAR3- or Cas-depleted lysates were collected and assayed by Western blot to detect populations of unbound protein. Following clearance of Cas, no BCAR3 was detected in the remaining lysate fractions of either MDA-MB-231 (Fig. 4.2A; lane 4) or BT549 cells (lane 11). In contrast, after the immune-depletion of BCAR3, a substantial fraction of Cas remained (Figure 4.2B; top panels, lanes 2 through 4, and 9 through 11). Together, these data indicate that while the complete cellular pool of BCAR3 appears to be associated with Cas, a substantial fraction of Cas remains free of BCAR3.

Figure 4.2

A.


Cas Immunoprecipitations (IPs)
MDA-MB-231
BT549
Post-IP Lysates
IPs
Pre-IP Lysate
1 2 3 1 2 3
1 2 3 4 5 6 7
8 9 10 11 12 13 14
α Cas
α BCAR3
α β-tubulin

B.

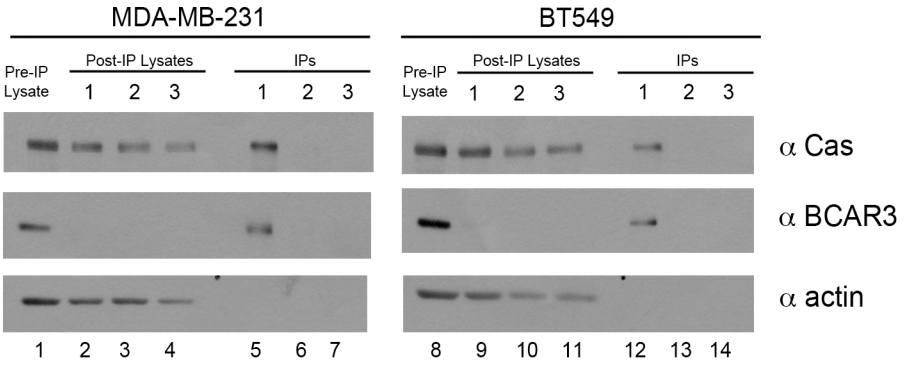

BCAR3 Immunoprecipitations (IPs)
MDA-MB-231
BT549
Post-IP Lysates
IPs
Pre-IP Lysate
1 2 3 1 2 3
1 2 3 4 5 6 7
8 9 10 11 12 13 14
α Cas
α BCAR3
α actin

Figure 4.2: Respective cellular fractions of BCAR3 and Cas found in the BCAR3/Cas complex.

A. The complete cellular population of BCAR3 is bound to Cas. MDA-MB-231 and BT549 cells were lysed and Cas was serially immunoprecipitated from 250μg of cell extracts. Lysate samples were collected before the initial immunoprecipitation and labeled as “Pre-IP Lysate.” A total of three Cas immunoprecipitations were performed. Following each pull down, an aliquot of the supernatant was collected and labeled as “Post-IP Lysate;” the remaining fraction of the supernatant was then subject to the next immunoprecipitation. **B.** A substantial amount of Cas is found free of BCAR3. The serial BCAR3 immunoprecipitations were carried out in 200μg of cell extracts from MDA-MB-231 and BT549 cells as described above.

4.3.3 – *BCAR3 Expression Is Not Lost Via Proteolytic Degradation in the Absence of Cas*

The findings that 1) BCAR3 expression is dependent on Cas and 2) that BCAR3 exists completely in a Cas-bound state led us to hypothesize that the interaction between BCAR3 and Cas acts as a molecular anchor to stabilize BCAR3. The pathway that regulates degradation of the majority of cytosolic proteins is the ubiquitin proteasome system (UPS) (Reinstein and Ciechanover 2006). To determine whether BCAR3 becomes ubiquitinated and subsequently degraded by the proteasome in response to Cas depletion, proteasome activity was inhibited by MG-132 (10μM), and ubiquitin-tagged protein was quantified using tandem ubiquitin binding entities (TUBEs). TUBEs are composed of four linked ubiquitin-binding domains that bind with high affinity to poly-ubiquitinated proteins. The TUBEs are modified with a glutathione S-transferase (GST) tag to facilitate the isolation of TUBE/poly-ubiquitinated protein complexes (Hjerpe et al., 2009). Briefly, control and Cas-depleted MDA-MB-231 and BT549 cells, cultured in the absence or presence of MG-132 (10μM), were lysed in TUBE-containing lysis buffer and poly-ubiquitinated proteins were isolated by a conventional GST pull-down. Lysates and TUBE pull-down samples were assayed by Western blot to detect total and ubiquitinated BCAR3. If BCAR3 is processed and degraded via the UPS in the absence of Cas, then a rescue of BCAR3 protein and a corresponding increase in ubiquitinated BCAR3 present in TUBE pull-downs should be seen in Cas-depleted cells following inhibition of proteasome degradation by MG-132. However, MG-132 treatment had no such

effect on BCAR3 protein levels (Fig. 4.3A; compare lanes 3 and 4, and lanes 7 and 8) or on the accumulation of ubiquitinated BCAR3 (Fig. 4.3B; compare lanes 3 and 4, and lanes 7 and 8). These results suggest that BCAR3 is not processed through the UPS in the absence of Cas.

A second major pathway of protein degradation in eukaryotic cells involves the internalization of proteins into intracellular vesicles and the routing of these vesicles to lysosomes (Reinstein and Ciechanover 2006). To determine if BCAR3 is degraded within lysosomes in the absence of Cas, lysosome-dependent proteolysis was inhibited using chloroquine (CQ) to prevent compartment acidification and by the inhibition of resident cathepsins with E-64. Cas was knocked down in BT549 cells and 48 hours post-knockdown, cells were treated with either CQ (20μM), E-64 (10μM), or in combination for 24 hours. As was the case for proteasome inhibition, BCAR3 expression was not rescued under conditions of lysosome inhibition (Fig. 4.3C).

A caveat to the experiments represented in Figure 4.3 is that controls are missing to ensure that the inhibitors worked. For the MG-132 experiments, several cyclins were blotted for but we were unsuccessful in our attempts to detect expression by Western blot. The inhibition of lysosome acidification was tested through the use of LysoTracker fluorescent probes (Invitrogen), but were similarly unsuccessful in detecting this marker.

Figure 4.3

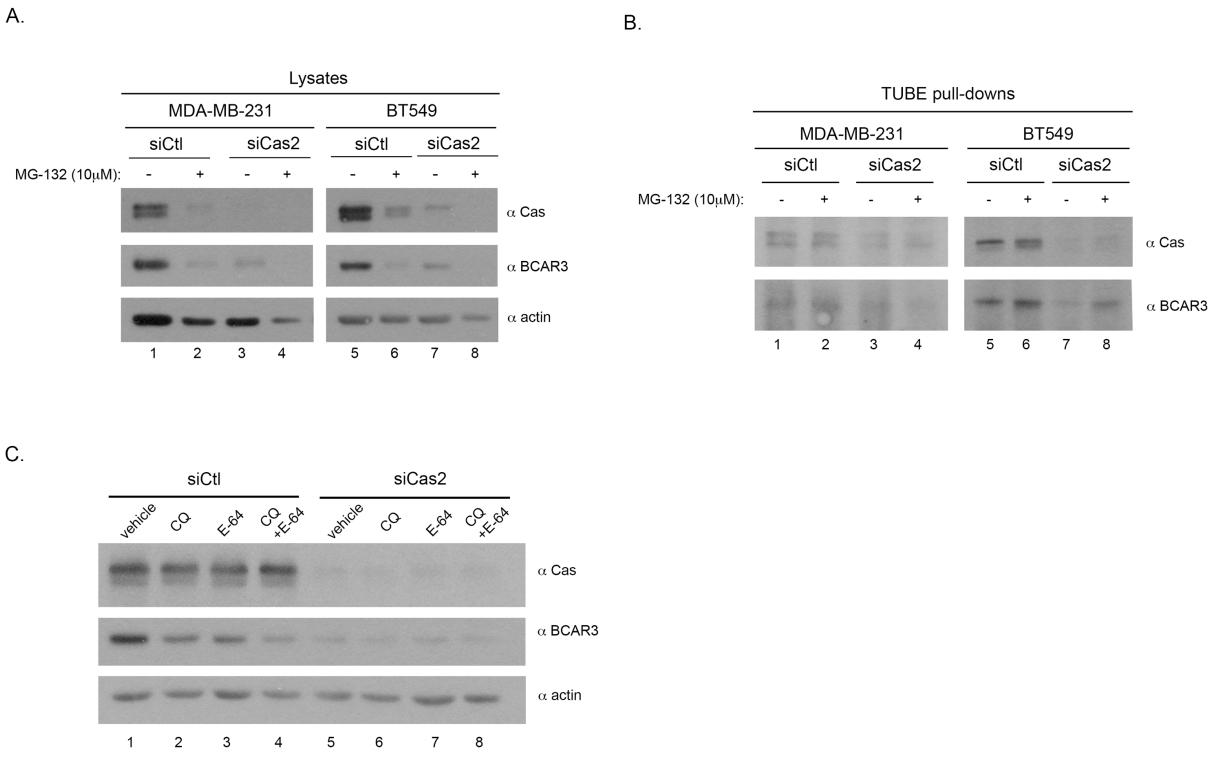

A.
Lysates
MDA-MB-231
BT549
siCtl
siCas2
siCtl
siCas2
MG-132 (10μM):
- + - + - + - +
α Cas
α BCAR3
α actin
1 2 3 4 5 6 7 8
B.
TUBE pull-downs
MDA-MB-231
BT549
siCtl
siCas2
siCtl
siCas2
MG-132 (10μM):
- + - + - + - +
α Cas
α BCAR3
1 2 3 4 5 6 7 8
C.
siCtl
siCas2
vehicle
CQ
E-64
CQ +E-64
vehicle
CQ
E-64
CQ +E-64
α Cas
α BCAR3
α actin
1 2 3 4 5 6 7 8

Figure 4.3: BCAR3 is not degraded via the proteasome or the lysosomal degradation pathway following Cas knockdown.

A. BCAR3 protein expression is not rescued by MG-132-mediated proteasome inhibition in the absence of Cas. MDA-MB-231 and BT549 cells were reverse-transfected with control or Cas-specific siRNAs. 48 hours post-knockdown, cells were cultured in the presence or absence of MG-132 (10μM). Cell lysates were subject to Western blot analysis to detect BCAR3 and Cas using antibodies that specifically recognize these proteins. **B.** BCAR3 is not ubiquitinated following Cas knockdown. MDA-MB-231 and BT549 were treated as described in A. and cells were lysed in GST-TUBE-containing lysis buffer (0.2μg/μl). TUBE lysates were incubated on ice for 15 minutes following by a 2 hour pull-down with Glutathione Sepharose 4B at 4C°. Samples were washed and protein was eluted in 1X Laemmli sample buffer. GST pull-downs were assayed by Western blot and a BCAR3 antibody was used to detect levels of ubiquitinated BCAR3 present in these samples. **C.** Inhibition of the lysosomal degradation pathway does not rescue BCAR3 in Cas-deficient cells. 48 hours post-knockdown, control and Cas-depleted BT549 cells were cultured in the presence of CQ (20μM) and E-64 (10μM) alone or in combination. Cells were lysed and proteins were analyzed by Western blot as described in A.

Surprisingly, BCAR3 and Cas are not accumulated in control cells following inhibition of either the UPS or the lysosomal degradation pathway. However, a caveat to the proteasome inhibition experiments is that MG-132 treatment alone caused a dramatic loss of BCAR3 and Cas (Fig. 4.3A; lanes 2 and 6). A previous study reported that MG-132 treatment induces apoptosis and protease-dependent Cas cleavage (Zhao and Vuori 2011). In agreement with that report, we observed an increase in cell death, proportional to the degree of BCAR3 and Cas loss, in both cell lines following MG-132 treatment. Caspase cleavage has been reported to produce a carboxyl-terminal 31kDa Cas fragment that has been implicated in pro-apoptotic signaling (Kim, Kook et al. 2004). We next examined whether this cleavage product accumulated following MG-132 treatment under the control conditions used in our studies. MG-132 treatment of BT549 cells resulted in a decrease in full length Cas (Fig. 4.4A; top panel, compare lanes 1 and 2) coincident with the appearance of a 31kDa carboxyl-terminal Cas fragment (middle panel). These effects were partially reversed by treatment with the caspase inhibitor z-VAD-fmk (compare lanes 2 and 4). As was shown in Figure 4.3, MG-132 treatment also resulted in a loss of BCAR3 expression (Fig. 4.4B; lane 2), which was similarly relieved by treatment with z-VAD-fmk (lanes 4 and 6). However, we were unable to deduce if this rescue was due to the inhibition of BCAR3-specific caspase cleavage or to the retention of full-length Cas.

Anoikis is a form of apoptosis activated when cell contact with the ECM is disrupted (Wei, Yang et al. 2002, Kim, Kook et al. 2004). Cas is an important part

of integrin-based adhesion complexes and Cas has been shown to play a role in both adhesion-mediated survival and anoikis (Cho and Klemke 2000, Wei, Yang et al. 2002, Wei, Yang et al. 2004). Based on these considerations, we hypothesized that depletion of Cas may be recognized as a form of cell stress that results in activation of stress-related caspases and/or calpains that proteolytically cleave BCAR3. To test this hypothesis, Cas-depleted BT549 cells were treated with either z-VAD-fmk or the calpain inhibitors ALLM and Calpain Inhibitor III (C.I. III) alone or in combination. None of these protease inhibitors had a stabilizing effect on BCAR3 following Cas knockdown (Fig. 4.4C & D). We also observed no change in the expression or size of the pro-apoptotic protein Bax (Fig. 4.4D, bottom panel), which is cleaved by calpains from 21kDa to a more active 18kDa form upon the initiation of anoikis (Gilmore, Metcalfe et al. 2000, Toyota, Yanase et al. 2003, Yamaguchi, Woods et al. 2008). These data argue that Cas depletion does not induce calpain or caspase activity under these conditions, and this lack of protease activation suggest that anoikis is not initiated following knockdown.

Together, the results of this section demonstrate that Cas-dependent BCAR3 loss is not due to an increase in BCAR3 protein degradation. However, there is a major caveat to these experiments: given the induction of cell death and the accompanied proteolytic cleavage of Cas following proteasome inhibition (Fig. 4.4A), we are unable to conclusively rule out the possibility that BCAR3 is lost via UPS processing following Cas depletion. This is because, in respect to BCAR3 expression, MG-132 treatment effectively promotes the same result as

knocking down Cas (Fig. 4.3A; compare lanes 1 to 2 and 5 to 6). In control cells, MG-132 treatment initiates an apoptotic signaling pathway that depletes full-length Cas, promotes the accumulation of the 31kDa pro-apoptotic Cas cleavage fragment, and ultimately leads to the loss of BCAR3, possibly through the action of some unidentified protease.

Figure 4.4

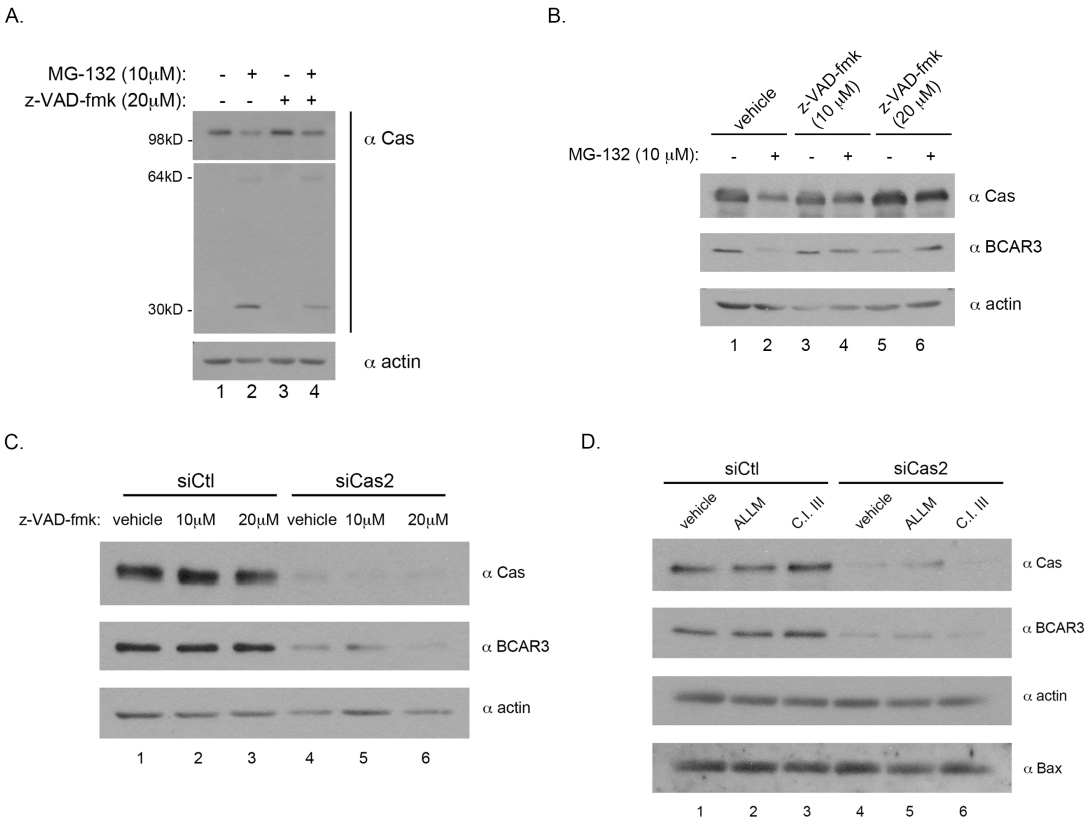

A.
MG-132 (10μM): - + - +
z-VAD-fmk (20μM): - - + +
98kD -
64kD -
30kD -
α Cas
α actin
1 2 3 4
B.
vehicle
z-VAD-fmk (10 μM)
z-VAD-fmk (20 μM)
MG-132 (10 μM): - + - + - +
α Cas
α BCAR3
α actin
1 2 3 4 5 6
C.
siCtl
siCas2
z-VAD-fmk: vehicle 10μM 20μM vehicle 10μM 20μM
α Cas
α BCAR3
α actin
1 2 3 4 5 6
D.
siCtl
siCas2
vehicle
ALLM
C.I. III
vehicle
ALLM
C.I. III
α Cas
α BCAR3
α actin
α Bax
1 2 3 4 5 6

Figure 4.4: BCAR3 is not degraded by caspase or calpain proteases following Cas knockdown.

A. Caspase inhibition partially blocks the Cas degradation observed following MG-132 treatment. BT549 cells were cultured in the presence or absence of z-VAD-fmk (20μM) for 24 hours followed by treatment with vehicle (DMSO) or MG-132 (10μM) for an additional 24 hours. Cells were maintained in z-VAD-fmk for the duration of the assay. Cas expression was assayed by Western blot using a Cas-specific antibody (Cas 6G11) that recognizes an epitope on the C-terminus of Cas to detect both full-length and the C-terminal 31kDa fragment. Actin is used as a loading control. **B.** Caspase inhibition rescues the loss of BCAR3 seen under MG-132 treatment conditions. BT549 cells were treated with two different concentrations of z-VAD-fmk for 24 hours followed by an additional 24 hours of MG-132 treatment. Cells were lysed and BCAR3 and Cas protein expression was detected by Western blot with antibodies specific to both BCAR3 and Cas. **C.** and **D.** BCAR3 is not degraded by caspases (C.) or calpains (D.) following Cas depletion. BT549 cells were transfected with control or siCas oligos and 48 hours after knockdown, were treated with vehicle (DMSO), z-VAD-fmk (C.), or with the calpain inhibitors ALLM (20μM) and C.I. III (20μM) alone or in combination for 24 hours (D.). BCAR3 and Cas expression was assed via Western blot with BCAR3- and Cas-specific antibodies. As a control for calpain activity, blots were probed with a Bax-specific antibody to detect total protein (D.).

4.3.4 – *Expression of Exogenous BCAR3 Requires the Presence of, But Not a Physical Association to, Cas*

In the preceding section we were unable to prove whether or not Cas-free BCAR3 is degraded via the proteasome, so we decided to address this question through a different approach. If BCAR3 is unstable and degraded in the absence of a physical interaction with Cas, we hypothesized that a BCAR3 construct that contains a mutation that blocks BCAR3-Cas association should be less stable than its wild-type (WT) counterpart. To specifically address this, we utilized two BCAR3 constructs: one that encodes WT protein and a second that contains a carboxyl-terminal arginine-to-alanine point mutation predicted to block its association to Cas (R748A BCAR3) (Vanden Borre, Near et al. 2011). First, the inability of the R748A mutant to bind to Cas was confirmed by immunoprecipitating Myc-tagged versions of these proteins that had been ectopically expressed in BT549 cells (Fig. 4.5A). Next, MDA-MB-231 cells were reverse-transfected with control or siCas oligos and 48 hours post-knockdown, control and Cas-depleted cells were transfected with either GFP-WT or GFP-R748A BCAR3. The GFP tag creates a fluorescent protein that is approximately 25kDa larger than endogenous BCAR3, which allows for detection of exogenous protein by flow cytometry and to differentiate between exogenous and endogenous protein by Western blot. Ectopic WT BCAR3 was expressed to a much lesser degree in Cas-depleted cells than in control cells (Fig. 4.5B, second panel from the top; compare lane 2 to lane 5), indicating that the exogenous protein displays the same pattern of Cas-dependent regulation as endogenous

BCAR3. Interestingly, R748A was readily expressed when endogenous Cas was present but, like the WT protein, accumulated to a much lesser degree in the absence of Cas (Fig. 4.5B; lanes 3 and 6). To ensure that the patterns of expression observed by Western blot were not due to differences in transfection efficiency across the constructs, samples were assayed for GFP expression by flow cytometry. Similar percentages of GFP-expressing cells were found across all samples (Fig. 4.5C). The Mean Fluorescence Intensity (MFI) of the WT GFP-expressing population is lower in Cas-depleted cells than in the control cells (Fig. 4.5C). While only one experiment, the flow cytometry data indicates that across all samples a similar percentage of cells are expressing GFP-tagged protein, but the average level of expression of GFP-tagged protein is reduced in the Cas-deficient population. Collectively, the findings of this section argue against the model of Cas as a BCAR3-stabilizing anchor, and speak to the existence and activity of a Cas-dependent mechanism that controls BCAR3 expression.

Figure 4.5

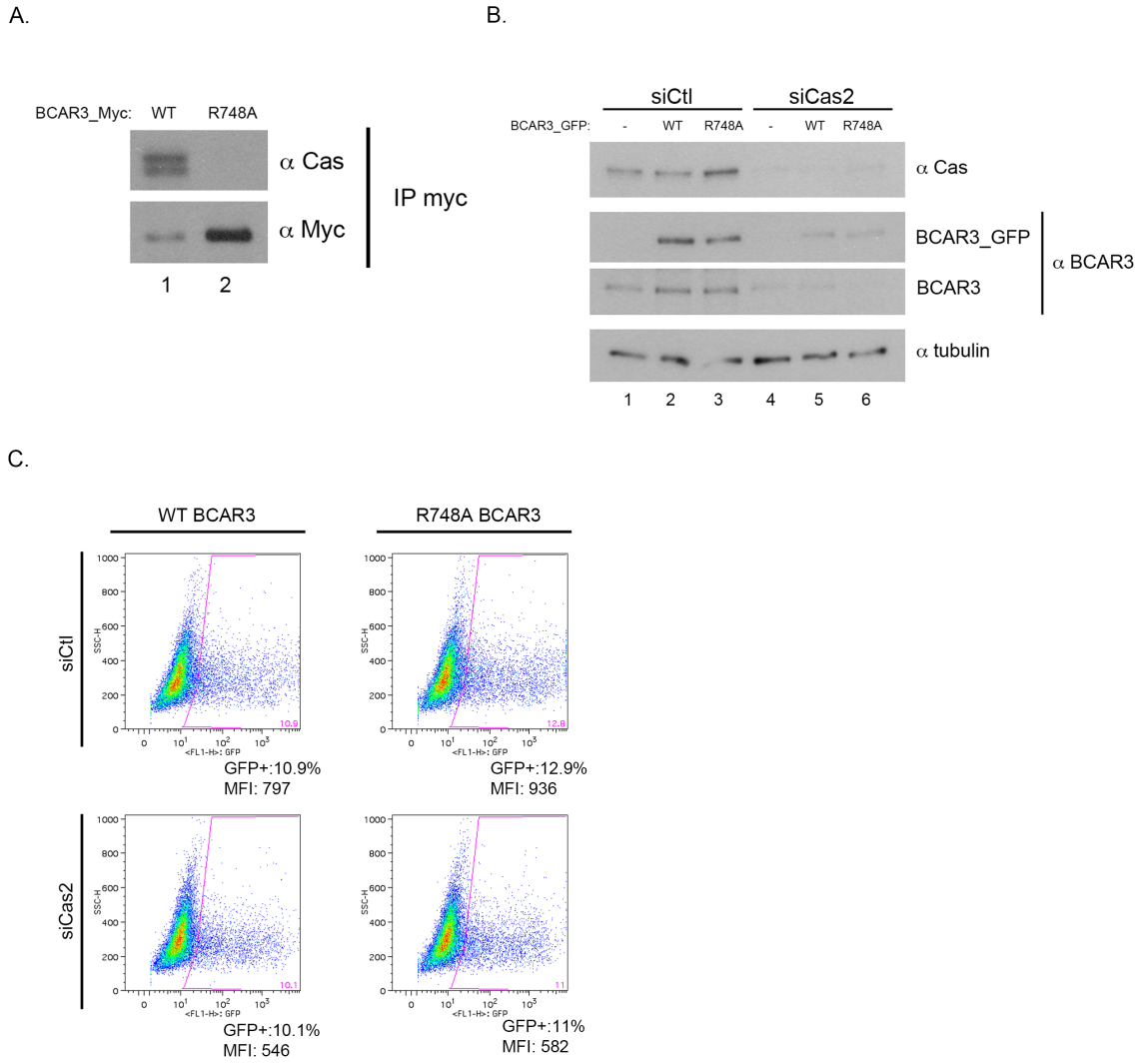

A.
BCAR3_Myc: WT R748A
α Cas
α Myc
IP myc
1 2
B.
siCtl
siCas2
BCAR3_GFP: - WT R748A - WT R748A
α Cas
BCAR3_GFP
BCAR3
α BCAR3
α tubulin
1 2 3 4 5 6
C.
WT BCAR3
R748A BCAR3
siCtl
siCas2
SSC-H
<FL1-H>: GFP
10.9
12.8
10.1
11
GFP+:10.9%
MFI: 797
GFP+:12.9%
MFI: 936
GFP+:10.1%
MFI: 546
GFP+:11%
MFI: 582

Figure 4.5: The physical association to Cas is not required for BCAR3 protein stability.

A. BCAR3 R748A does not form a complex with Cas. Confluent plates of BT549 cells were transfected with either 24μg of Myc-tagged WT or R748A BCAR3 and 48 hours later cells were lysed and exogenous protein was immunoprecipitated using a Myc antibody. Immune complexes were assayed by Western blot using Cas and Myc antibodies to detect bound Cas and total Myc, respectively. **B.** Cas is necessary for the expression of exogenous protein, but the physical association is not required for protein stability. MDA-MB-231 cells were reverse-transfected with control or Cas-specific oligos. 48 hours post-knockdown, cells were transfected with either 1.6μg of GFP-tagged WT or R748A BCAR3 and cultured for an additional 24 hours. Cells were lysed and Western blots were carried out using antibodies specific to BCAR3 and Cas. Tubulin is used as a loading control. **C.** Analysis of transfectants by flow cytometry. Knockdown of Cas and transient expression of exogenous BCAR3 was performed as described in **B.**; cells were collected and stained with Live/Dead Fixable Violet Dead Cell Stain kit (Invitrogen). Samples were then assayed on a FACSCaliber flow cytometer set to appropriate channels to detect both GFP-positive (FL1-H) and the live/dead stained (FL5-H) cells. Analysis of flow cytometry data on live cells was performed using FlowJo software.

4.3.5 – *A Similar Rate of Protein Degradation Exists for Both BCAR3 and Cas*

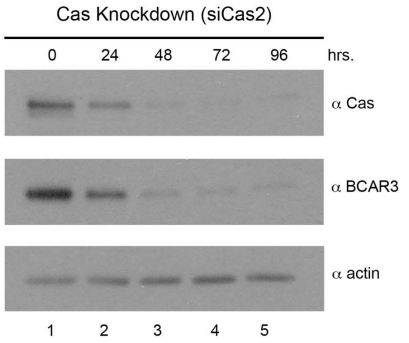
We next looked at the rate of BCAR3 and Cas degradation following Cas knockdown with the expectation that, if Cas plays an essential role in the regulation of BCAR3 expression, then we should observe a proportional decrease in BCAR3 as Cas is lost. The production of new Cas protein was inhibited through siRNA-mediated knockdown and we followed the degradation of pre-existing protein over time. The level of Cas and BCAR3 protein was analyzed at 24, 48, 72, or 96 hours post-knockdown. Upon inhibition of Cas production, BCAR3 and Cas displayed similar rates of degradation (Fig. 4.6A). This result further demonstrates that BCAR3 protein is lost as a function of Cas expression. Moreover, this result suggests that Cas actively and positively regulates a mechanism that controls BCAR3 protein levels as a function proportional to the amount of Cas protein found in the cell.

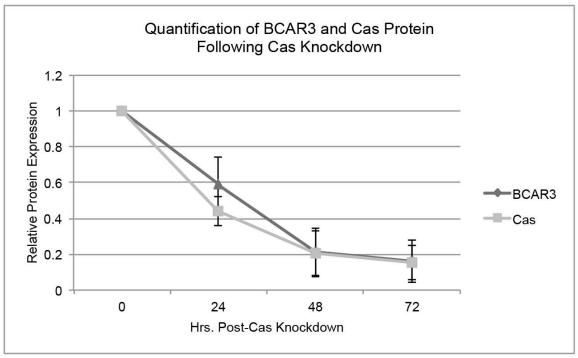
The data presented in Figure 4.6A examine BCAR3 and Cas degradation following inhibition of Cas synthesis by RNA interference. To further explore the co-regulation of Cas and BCAR3 degradation, we next looked at the rate of protein degradation following a global, cycloheximide (CHX)-induced block in translation with the same expectation: that if Cas is involved in the regulation of BCAR3 expression, then as less Cas is made, a similar and corresponding decrease in BCAR3 protein should also be observed. BT549 cells were treated with CHX for 24, 48, 72, or 96 hours. At each time point, cells were lysed and protein levels were assessed by Western blot. Again, both proteins exhibited a

similar rate of degradation and a half-life of approximately 24 hours (Fig. 4.6B). This tight correlation in the rate of BCAR3 and Cas degradation following the specific blockade of Cas protein synthesis (via Cas siRNA-mediated knockdown) or a total inhibition of protein synthesis (CHX) provides further support for the existence of a Cas-mediated mechanism regulating BCAR3 expression.

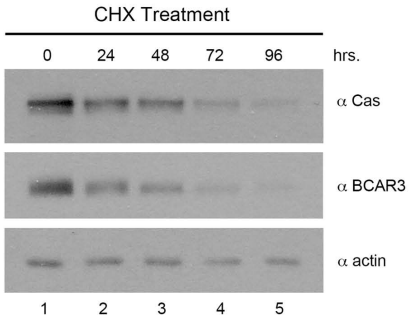
Figure 4.6

A.


Cas Knockdown (siCas2)
0
24
48
72
96
hrs.
α Cas
α BCAR3
α actin
1
2
3
4
5


Quantification of BCAR3 and Cas Protein Following Cas Knockdown
Relative Protein Expression
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
72
Hrs. Post-Cas Knockdown
BCAR3
Cas

B.


CHX Treatment
0
24
48
72
96
hrs.
α Cas
α BCAR3
α actin
1
2
3
4
5

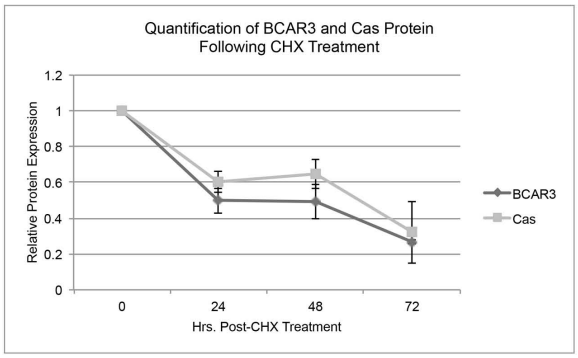

Quantification of BCAR3 and Cas Protein Following CHX Treatment
Relative Protein Expression
1.2
1
0.8
0.6
0.4
0.2
0
0
24
48
72
Hrs. Post-CHX Treatment
BCAR3
Cas

Figure 4.6: BCAR3 and Cas exhibit similar rates of protein degradation.

A. Following Cas knockdown BCAR3 and Cas display a similar rate of degradation. BT549 cells were transfected with Cas-specific oligos, and lysates were collected at 0, 24, 48, 72, and 96 hours post-transfection. BCAR3 and Cas protein levels were detected by Western blot using BCAR3 and Cas specific antibodies. Actin levels were used as a loading control. Data shown is representative of 3 experiments. Graph represents the quantification of relative protein levels analyzed by densitometry using actin as internal control. **B.** Rate of protein turnover following CHX-induced block in translation. BT549 cells were treated with CHX (25μg/ml) for 0, 24, 48, 72, and 96 hours. Cells were lysed at each time point and the level of protein remaining was detected by Western blot. Data is representative of 2 experiments. Graph represents quantitation of relative protein levels by densitometry.

4.3.6 – *BCAR3 Transcription Does Not Require Cas, but Efficient BCAR3 Protein Synthesis Is Cas-Dependent*

The data shown above are consistent with there being a block in the production of BCAR3 protein in Cas-depleted cells. To determine whether changes in Cas expression alter the steady-state levels of BCAR3 mRNA, the relative amount of BCAR3 mRNA present in control or Cas-depleted cells was determined by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). MDA-MB-231 and BT549 cells were transfected with control or one of two different Cas-targeted siRNAs. Cells were lysed and RNA was collected and prepared for RT-PCR analysis 72 hours post-knockdown. No significant difference in the level of BCAR3 transcript was observed in the absence of Cas (Fig. 4.7). These data indicate that Cas does not have a direct effect on steady-state levels of BCAR3 mRNA.

Figure 4.7

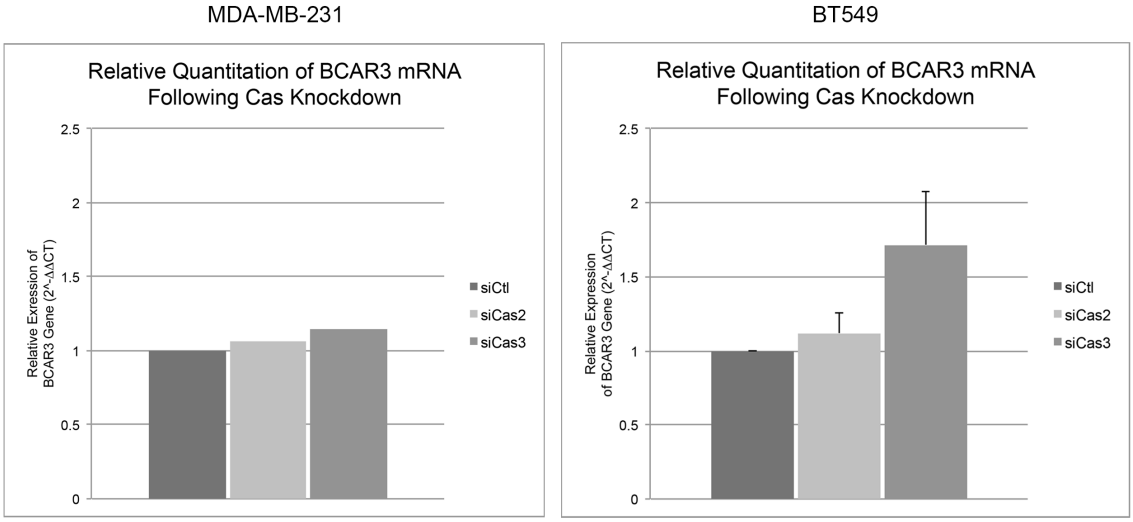


Figure 4.7: Steady-state levels of BCAR3 mRNA are unaffected by Cas loss. Knockdown of Cas has no effect on the levels of BCAR3 mRNA in MDA-MB-231 or BT549 cells. Cells were transfected with control, siCas2, or siCas3 oligos. 72 hours post-knockdown cells were lysed and total RNA was collected. BCAR3 mRNA was assayed using quantitative reverse transcriptase-PCR. 18S rRNA was used as an internal standard. Relative levels of BCAR3 mRNA were quantified by the comparative Ct method ($2^{-\Delta\Delta Ct}$). MDA-MB-231; n=1. BT549; n=3, no significant difference in total mRNA exists.

Finally, we sought to determine whether Cas regulates the synthesis of BCAR3 protein. To detect newly synthesized protein, we utilized an amino acid analog of methionine called AHA (L-azidohomoalaine), which contains an azide moiety that allows for the direct labeling of AHA-incorporated proteins with an alkyne-tagged biotin molecule. To directly assess whether Cas regulates the synthesis of BCAR3, BT549 cells were methionine-depleted, and then incubated with AHA (25μM) for various lengths of time 72 hours post-Cas knockdown. BCAR3 was then immunoprecipitated and the levels of AHA-containing proteins were assayed by Western blot using streptavidin. We first examined the presence of newly synthesized BCAR3 by AHA-incorporation through a short time course of 30 and 60 minutes (Fig. 4.8A). Results show that significantly less newly synthesized BCAR3 was present in Cas-depleted cells compared to their control counterparts at 30 and 60 minutes of synthesis (Fig. 4.8A; top panels). In a subsequent experiment, the reduction in BCAR3 synthesis was noted through 180 minutes (Fig. 4.8B). Together, these results are suggestive of a Cas-dependent mechanism of BCAR3 protein synthesis.

A pulse-chase experiment was performed next to determine whether the stability of newly synthesized BCAR3 protein is dependent on Cas. Control and Cas-depleted cells were incubated with AHA for 60 and 90 minutes, the AHA-containing media was then removed, and the cells were cultured in normal media for an additional 30 and 90 minutes. As expected, a reduction in newly synthesized BCAR3 was observed in Cas-depleted cells after 60 and 90 minutes of incubation in AHA-containing media (Fig. 4.8C; compare lanes 1 through 4).

Following AHA washout, the amount of newly synthesized BCAR3 protein was maintained over the next 90 minutes in both control and siCas-treated cells (compare lanes 3 and 4 to 5 through 8). This result demonstrates that impaired BCAR3 synthesis, and not protein degradation, is responsible for the loss of BCAR3 expression following the knockdown of Cas. Together, these results provide strong evidence supporting a critical role for Cas in the regulation of BCAR3 protein synthesis.

Figure 4.8

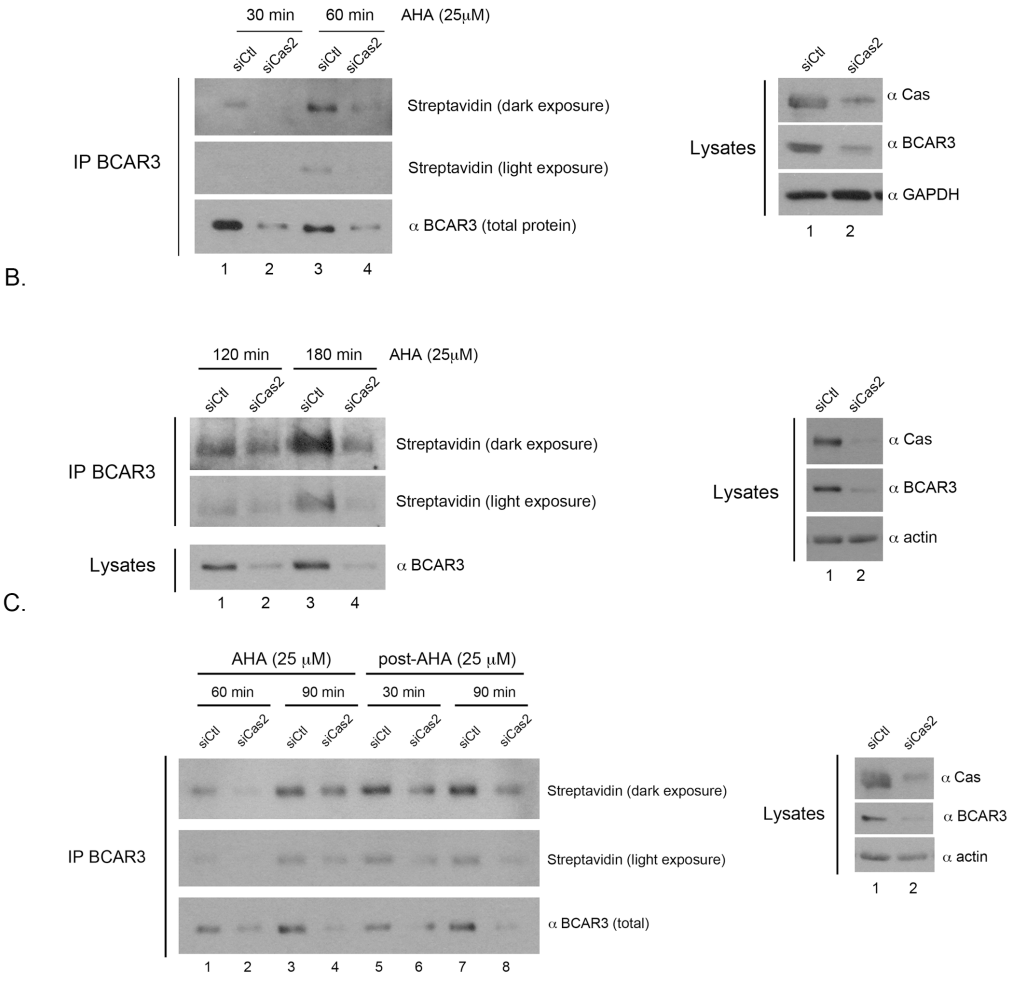

A.
30 min
60 min
AHA (25μM)
siCtl
siCas2
siCtl
siCas2
IP BCAR3
Streptavidin (dark exposure)
Streptavidin (light exposure)
α BCAR3 (total protein)
1 2 3 4
Lysates
siCtl
siCas2
α Cas
α BCAR3
α GAPDH
1 2
B.
120 min
180 min
AHA (25μM)
siCtl
siCas2
siCtl
siCas2
IP BCAR3
Streptavidin (dark exposure)
Streptavidin (light exposure)
Lysates
α BCAR3
1 2 3 4
Lysates
siCtl
siCas2
α Cas
α BCAR3
α actin
1 2
C.
AHA (25 μM)
post-AHA (25 μM)
60 min
90 min
30 min
90 min
siCtl
siCas2
siCtl
siCas2
siCtl
siCas2
siCtl
siCas2
IP BCAR3
Streptavidin (dark exposure)
Streptavidin (light exposure)
α BCAR3 (total)
1 2 3 4 5 6 7 8
Lysates
siCtl
siCas2
α Cas
α BCAR3
α actin
1 2

Figure 4.8: Cas-depleted cells exhibit reduced BCAR3 synthesis.

A. and **B.** BCAR3 synthesis is reduced in the absence of Cas. 72 hours after knockdown, control and Cas-depleted cells were depleted of methionine for 1 hour and then metabolically labeled with AHA for 30 and 60 (A), or 120 and 180 (B) minutes. Cells were lysed and BCAR3 was immunoprecipitated from 500μg (A) or 200μg (B) of total protein. Biotin labeling reactions were carried out in immune complexes to enable the detection of AHA-incorporated molecules of BCAR3 by Western blot. Blots were probed with HRP-conjugated streptavidin and a BCAR3 specific antibody to visualize the presence of nascent and total BCAR3, respectively. Lysates were assayed by Western blot using Cas and BCAR3 specific antibodies to show total levels of both proteins. **C.** Nascent protein is not lost in the absence of Cas. As before, BT549 cells were transfected with control or siCas oligos and 72 hours later were cultured in AHA-containing media for 60 and 90 minutes. Following 90 minutes, AHA-containing media was removed and cells were washed once with PBS and replenished with normal media for an additional 30 and 90 minutes. Nascent and total BCAR3 protein was detected as described above.

4.4 – Discussion

In the present study, we initially set out to investigate Cas-independent functions of BCAR3. To begin to explore such potential roles, Cas was knocked down in two breast cancer cell lines and, surprisingly, BCAR3 expression was also lost. The subsequent finding that Cas is required for BCAR3 synthesis was unexpected, as a functional link between Cas and translational regulation has, to our knowledge, never been reported. However, a number of known Cas-associated proteins have been implicated in the regulation of proteins that mediate cap-dependent translation.

4.4.1 – *Hypothetical Mechanism of Cas-mediated Translation*

We propose a model (Fig. 4.9) in which Cas and Src cooperatively promote protein synthesis through the negative regulation of protein phosphatase 2A (PP2A) and 4EBP-1, a protein that binds to eukaryotic translation initiation factor 4E (eIF4E). Briefly, the initiation of cap-dependent translation is dependent on eIF4E binding to eIF4G, the final and rate-limiting step in the formation of the eIF4F translation initiation complex (Li, Yue et al. 2010, Kong and Lasko 2012). Other proteins that bind directly to eIF4E negatively regulate the initiation of translation by blocking the association between eIF4E and eIF4G; these proteins are known as eIF4E-binding proteins (4EBPs) (Kong and Lasko 2012). The expression of one such protein, 4EBP-1, and its ability to bind to eIF4E are positively regulated by the expression and activity of the serine/threonine phosphatase PP2A (Li, Yue et al. 2010). PP2A also negatively regulates eIF4E

serine phosphorylation, as well as that of Mnk1, the kinase responsible for the addition of this activating phosphate group to eIF4E (Li, Yue et al. 2010). Intriguingly, Src inactivates PP2A through phosphorylation at tyrosine 307 and this inactive form of PP2A forms a complex with Cas (Chen, Parsons et al. 1994, Yokoyama and Miller 2001, Barisic, Schmidt et al. 2010). As discussed in Chapters 1 and 3 of this thesis, increased Cas expression augments Src kinase activity [(Burnham, Bruce-Staskal et al. 2000, Brabek, Constancio et al. 2004); Fig. 1.4B]. Furthermore, increased 4EBP-1 phosphorylation is observed in v-*Src*-transformed cells, a modification known to inhibit its interaction with eIF4E (Tuhackova, Sovova et al. 1999). Viewing these findings collectively, we suggest that Cas and Src function cooperatively to promote BCAR3 expression by negatively regulating PP2A and 4EBP-1 (in effect, activating eIF4E) (Fig. 4.9A). The breakdown of such a signaling pathway, mediated by the depletion of Cas in the current study (Fig. 4.9B), would lead to a reduction in the rate of eIF4E-dependent protein synthesis through the increased activity of PP2A and 4EBP-1. The existence of such a signaling pathway remains to be tested. However, it should be noted that this is just one mechanism through which Cas may be controlling BCAR3 synthesis. These potential mechanisms, and their biological significance, are discussed in detail in Chapter 5.

Figure 4.9.

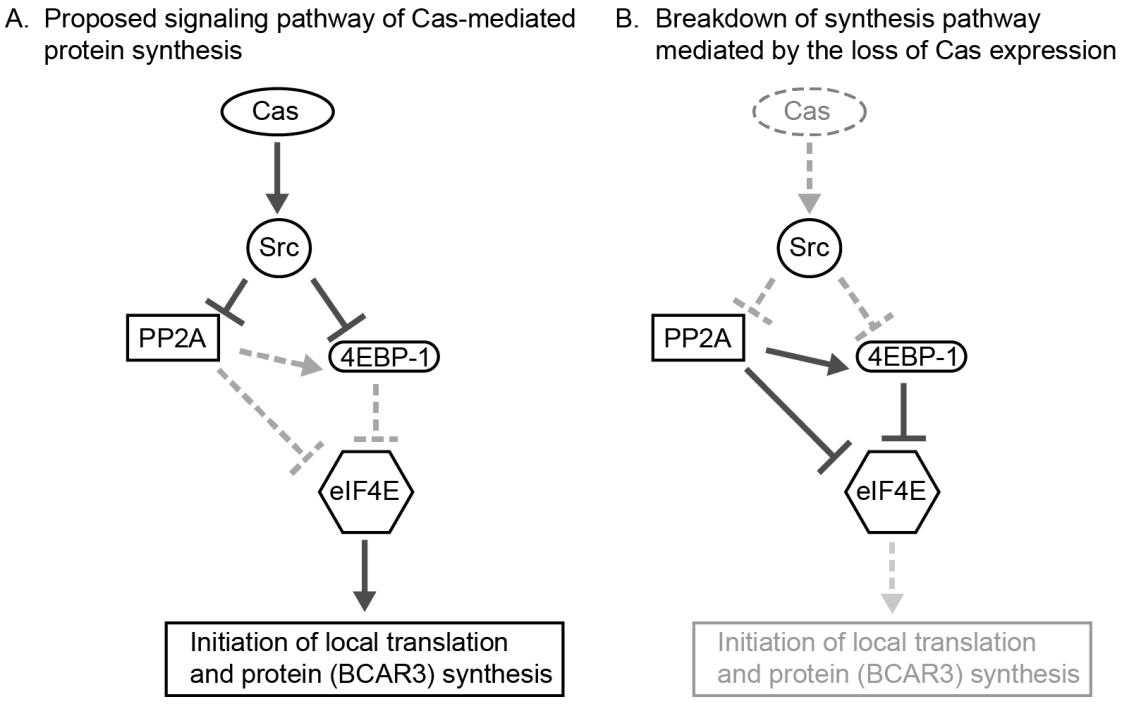


Figure 4.9: Schematic model of the Cas-mediated translation pathway.

A. Cas influences pro-translational signaling by binding to and activating Src. Src activity promotes the addition of inhibitory phosphate groups (directly or indirectly) to PP2A and/or 4EBP-1. The inhibition of these two proteins leads to increased eIF4E-mediated translation. **B.** In the absence of Cas, Src kinase activity is decreased and results in a corresponding reduction in translation and protein synthesis. A loss of Src activity leads to heightened PP2A and 4EBP-1 activities. PP2A and 4EBP-1 cooperate to prevent the initiation of translation through the negative regulation of eIF4E.

4.4.2 – *Cas-dependent BCAR3 Expression in the Context of Temporal and Spatial Protein Synthesis*

Translation machinery and mRNA are localized at focal adhesions and at the protrusive fronts of migrating cells (Chicurel, Singer et al. 1998, Mili, Moissoglu et al. 2008). This spatial positioning is thought to allow for the rapid synthesis of specific proteins to mediate distinct responses to extracellular cues. Interestingly, in response to adhesion and growth factor stimulation, both BCAR3 and Cas are localized to these regions (Ballestrem, Erez et al. 2006, Schrecengost, Riggins et al. 2007, Sun, Cheng et al. 2012). Based on the findings presented in this chapter, we speculate that Cas plays a role in the spatial and temporal translation of BCAR3 to promote migratory and invasive phenotypes in breast cancer cells. This, along with hypothetical Cas-dependent protein synthesis pathways, is further discussed in Chapter 5.

4.4.3 – *Potential Relevance of Cas-dependent BCAR3 Expression in Normal and Cancer Cell Biology*

Figure 4.2 shows that the entire cellular population of BCAR3 is in complex with Cas, which suggests that Cas plays an important role in BCAR3 signaling and function. The subsequent finding that Cas is required for the synthesis of BCAR3 demonstrates a complete functional reliance on Cas. Recently, it was reported that the physical association between BCAR3 and Cas is required for the promotion of Cas/Src binding and the BCAR3-dependent, Src-mediated increase in Cas phosphorylation (Makkinje, Near et al. 2009), but is

dispensable for the induction of antiestrogen resistance and Rac activation (Vanden Borre, Near et al. 2011). These studies were performed in the minimally invasive MCF7 breast cancer cell line, which expresses relatively low levels of BCAR3 protein (Near, Zhang et al. 2007, Schrecengost, Riggins et al. 2007). The requirement for Cas association was therefore tested by overexpression of a BCAR3 mutant similar to the R748A construct used here. While these findings do provide evidence for Cas-independent BCAR3 functions, they do so only under conditions of ectopic protein expression and do not directly address a functional independence from Cas within the population of endogenous protein. Taken together with the data presented here, it is clear that Cas is critically involved in the regulation of BCAR3 function as Cas controls its expression, but BCAR3 does appear to mediate signaling independent of its association with Cas.

Regardless of the exact mechanism of how Cas controls BCAR3 synthesis, an interesting question that remains is whether Cas-dependent BCAR3 synthesis occurs in other cells and tissues. One possibility is that this is unique to breast cancer cells representative of a certain advanced disease stage. Recently, we have found that the breast cancer cell lines utilized in this report appear to exhibit a form of “BCAR3 addiction,” in that the cells appear to be dependent upon BCAR3 for their ability to effectively migrate, invade, and undergo proper cytoskeletal responses to growth factors and adhesion to substrate [(Schrecengost, Riggins et al. 2007) Wilson et al., 2013, *manuscript in progress*]. In the absence of BCAR3, MDA-MB-231 and BT549 cells adopt a contractile phenotype and are severely limited in their ability to disassemble

stress fibers, form peripheral membrane protrusions, turnover adhesions, and efficiently perform haptotactic migration and invasion through matrigel [(Schrecengost, Riggins et al. 2007) Wilson et al., 2013, *manuscript in progress*]. The apparent dependency on BCAR3 for motility in these cells is both surprising and interesting given that BCAR3 knockout mice, with the exception of cataracts and ruptures of the ocular lens, display no major developmental or postnatal defects (Near, Smith et al. 2009). The overall fitness of these mice, compared with the crippling defects of BCAR3 depletion in breast cancer cells, led us to hypothesize that cancer cells develop a pathway addiction during tumorigenesis that results in a reliance on BCAR3 signaling not exhibited by normal cells. Is it possible that “BCAR3 addiction” and Cas-dependent BCAR3 synthesis are both markers of an aggressive disease state? Can constitutive Cas signaling augment BCAR3 expression, and does this increase the metastatic potential of tumor cells? It will be interesting to directly address these questions in both transformed and non-transformed mammary cell lines, as well as in cells of other tissues that express these two proteins.

The NSP protein Shep1 associates with Cas in human and mouse brains (Dodelet, Pazzagli et al. 1999, Sakakibara and Hattori 2000, Vervoort, Roselli et al. 2007, Roselli, Wallez et al. 2010). Genetic deletion of Shep1 in mice leads to a high incidence of perinatal lethality, and analysis of brain tissue from Shep1 knockout animals revealed that Shep1 regulates Cas/Src signaling in the same manner as has been shown for BCAR3 in human breast cancer cells (Roselli, Wallez et al. 2010, Schuh, Guerrero et al. 2010). This animal study identifies a

critical biological role for Shep1 and highlights the importance of the Shep1/Cas/Src signaling node in the brain. It appears that an NSP/Cas/Src signaling pathway is conserved in both non-transformed and cancer cells. BCAR3 and Shep1 can both bind to the Cas family member NEDD9, and these interactions augment its phosphorylation (Near, Zhang et al. 2007, Wallez, Mace et al. 2012). High Cas and NEDD9 expression is associated with development and progression of many human cancers (Tikhmyanova, Little et al. 2010). If Cas family members regulate the synthesis of BCAR3 and/or Shep1 in other tissues, then heightened Cas/NEDD9 expression could hyper-activate NSP/Cas/Src signaling. The results presented here may provide important insights into how Cas and NEDD9 may act to perpetuate their activity to establish, sustain, and promote malignant states.

Chapter 5. Discussion

5.1 – Summary of Thesis Background, Goals, and Findings

Prior to this study, it was known that overexpression of BCAR3 or Cas enabled the proliferation of breast cancer cell lines in the presence of antiestrogens (van Agthoven, van Agthoven et al. 1998, Cai, Iyer et al. 2003, Riggins, Thomas et al. 2006). Transient overexpression studies had also shown that BCAR3 acts cooperatively with Cas to induce the migration of murine fibroblasts (Riggins, Quilliam et al. 2003). Furthermore, the physical association between BCAR3 and Cas had been demonstrated (Cai, Clayton et al. 1999, Gotoh, Cai et al. 2000), as had the ability of BCAR3 to synergistically enhance Cas-mediated Src activation in the non-human primate COS-1 cell line (Riggins, Quilliam et al. 2003).

Within the context the above findings provided, the original goal of this thesis was to elucidate the molecular mechanism(s) of BCAR3-mediated antiestrogen resistance, with the specific aim of determining whether Cas and Src signaling are essential components of this process. Unfortunately, the ability of BCAR3 to establish a drug resistant phenotype could not be replicated or corroborated here (see Appendix). However, we did discover that BCAR3 is a regulator of the Cas/Src signaling pathway in breast cancer cell lines. The data presented in Chapter 3 demonstrate that BCAR3 expression is positively correlated with Cas/Src interactions, Src kinase activity, Src-dependent Cas tyrosine phosphorylation, and Cas/Crk coupling. These findings, along with other

data showing that BCAR3 regulates cell adhesion and spreading, serve to better define the molecular framework through which BCAR3 signals. Further investigation into the relationship between BCAR3 and Cas revealed the novel finding that Cas is an essential regulator of BCAR3 synthesis (Chapter 4). While much work remains to be done to identify the accessory proteins through which Cas mediates BCAR3 expression, this finding represents an intriguing and exciting new role for Cas in the promotion of cellular and biological processes.

Collectively, the research performed during this thesis project shows that BCAR3 functions to potentiate Cas signaling, and that its expression is at the same time regulated by signals produced downstream of Cas. Taken together, these two points suggest that Cas controls its signaling capacity through the regulated expression of its own modifier. As aberrant Cas expression and signaling have been linked to many human cancers (Tikhmyanova, Little et al. 2010, Guerrero, Parsons et al. 2012), these data contribute to our understanding of how Cas may function to sustain its own signaling in the course pathological processes.

5.2 – Perspectives on BCAR3 Signaling Networks

Through the data presented here, and together with additional work from our lab and others, the signaling networks in which BCAR3 functions have become much more clear. In Chapter 4, we show that the complete cellular pool of BCAR3 is found in complex with Cas (Fig. 4.2A), which indicates that BCAR3 is positioned to work through or within Cas signaling pathways. This is in

agreement with the findings of Chapter 3 and other published reports establishing that BCAR3 plays a prominent role in the Src/Cas/Crk signaling pathway, which when activated, has pleiotropic effects on cell behavior [(Schrecengost, Riggins et al. 2007, Makkinje, Near et al. 2009, Sriram and Birge 2010, Makkinje, Vanden Borre et al. 2012, Tsuda and Tanaka 2012) Wilson et al., 2013, *manuscript in progress*]. Moreover, the ability of BCAR3 to promote PI3K signaling (Felekkis, Quilliam et al. 2005), activate Rac [(Cai, Iyer et al. 2003) Wilson et al., 2013, *manuscript in progress*], and induce cyclin D1 protein expression (Cai, Iyer et al. 2003, Near, Zhang et al. 2007) can all be explained by links to activate Src/Cas/Crk signaling (Riggins, DeBerry et al. 2003, Cabodi, Tinnirello et al. 2006, Defilippi, Di Stefano et al. 2006, Cabodi, del Pilar Camacho-Leal et al. 2010). Research showing that the SH2 domain of BCAR3 binds to EGFR following EGF stimulation (Oh, van Agthoven et al. 2008) and to PTPα in response to adhesion (Sun, Cheng et al. 2012) serves to further improve our understanding of how BCAR3 signaling complexes are spatially positioned to membrane-proximal sites to influence cell behavior in response to chemical and physical external cues.

Although a complete enumeration of BCAR3-interacting partners is needed to comprehend the full scope of BCAR3 function, and while important questions remain regarding the mechanism(s) by which BCAR3 promotes antiestrogen resistance (Near, Zhang et al. 2007) and breast cancer cell motility (Schrecengost, Riggins et al. 2007), much of the research discussed above places BCAR3 within a well-established signaling network that regulates cell

proliferation, survival, and migration (Fig. 5.1). Therefore, continued work into defining the specifics of these mechanisms may prove to be derivative. Given that the overexpression of BCAR3 alone is capable of inducing such signaling and cell behaviors, two compelling questions are 1) how is the expression of BCAR3 regulated? And 2) how does this affect the BCAR3/Cas/Src signaling axis? In Chapter 4, we uncover a novel role for Cas in the regulation of BCAR3 protein synthesis in highly migratory and invasive breast cancer cell lines. This finding adds an intriguing layer of complexity to the functional relationship between BCAR3 and Cas, and provides one potential mechanism in which BCAR3 expression becomes elevated to promote the activity of the BCAR3/Cas/Src signaling axis.

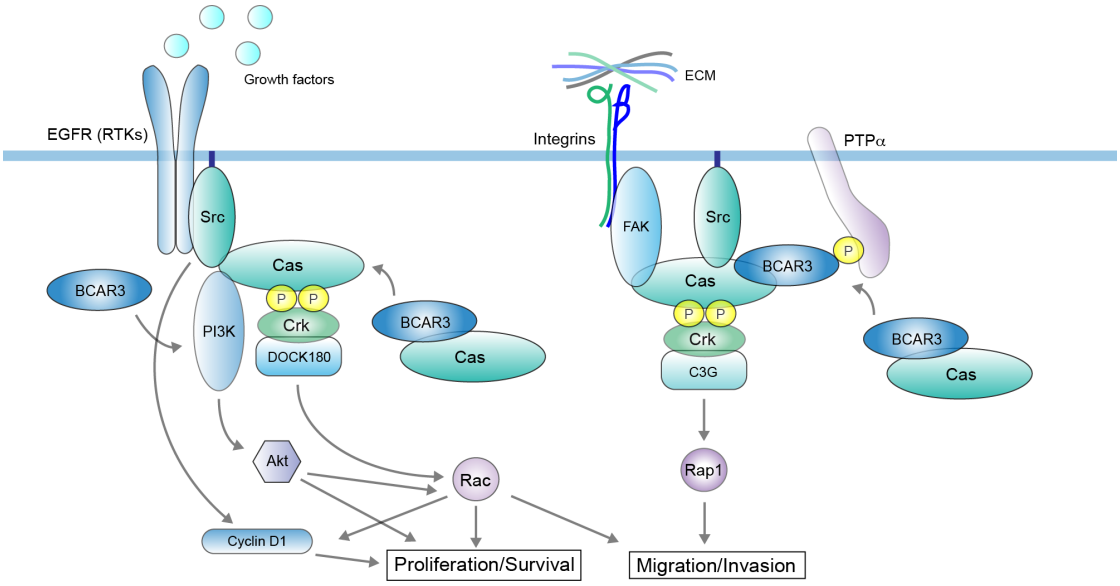


Figure 5.1: BCAR3 signaling networks.

BCAR3 transduces proliferative, survival, and migratory signaling downstream of integrin engagement and growth factor receptor stimulation. The SH2 domain of BCAR3 binds to phosphorylated tyrosine residues of transmembrane proteins like PTPα, and this interaction is necessary for the full transduction of focal adhesion signals downstream of integrin/ECM engagement. BCAR3 has also been shown to transduce proliferative signals downstream of EGF stimulation. The kinase activity of Src and the tyrosine phosphorylation of Cas are major effectors of BCAR3-mediated signaling. The SH2 domain of Crk directly interacts with the tyrosine phosphorylated substrate domain of Cas, and this interaction activates the small GTPases Rac and Rap1 through recruitment of their respective GEFs, DOCK180 and C3G. BCAR3 also mediates signals to PI3K/Akt, Rac, and cyclin D1 to induce resistance to antiestrogens.

5.3 – Perspectives on the BCAR3/Cas/Src Signaling Axis

While much is known about the signal transduction pathways downstream of Src-dependent Cas tyrosine phosphorylation, several important questions regarding the BCAR3/Cas/Src signaling axis remain to be addressed. One major unresolved issue is how BCAR3 acts to promote Cas/Src complex formation independent of a direct or indirect association with Src. In Chapter 3, we show that BCAR3 is a positive regulator of Cas/Src association (Figs. 3.3C and 3.6D), and hypothesize that this ability to mediate complex formation is the mechanism by which BCAR3 enhances Src kinase activity (3.6B) and Src-dependent Cas tyrosine phosphorylation (3.6C). Given this functional association, and that the binding sites for both BCAR3 and Src are spatially distinct within the carboxyl-terminus of Cas, the existence of a regulatory, tripartite complex consisting of BCAR3, Cas, and Src was initially theorized. However, no such complex was detected. In contrast, two distinct pools of Cas were present in the cell, a BCAR3-bound population and a population associated with Src (Fig. 3.2). We also show in Chapter 4 that a substantial fraction of Cas is free of BCAR3 (Fig. 4.2B), which likely contains the Src-bound pool. Recently, it was demonstrated that BCAR3 overexpression augments the binding of a recombinant Src SH3 domain to Cas, and that this requires the physical association between BCAR3 and Cas (Makkinje, Vanden Borre et al. 2012). This same study goes on to show that this physical interaction is also necessary for BCAR3-mediated Cas tyrosine phosphorylation.

Collectively, these studies are consistent with a dynamic process wherein the binding of BCAR3 to Cas increases the affinity of the Src SH3 domain for the poly-proline region (RPLPSPP) of Cas. One possible explanation is that the association with BCAR3 causes a conformational change within the carboxyl-terminus of Cas that makes the poly-proline region more accessible to the SH3 domain of Src. BCAR3 binds to a four-helix bundle on Cas approximately 100 amino acids carboxyl-terminal to the bipartite Src binding site. It is possible that the association of BCAR3 physically arranges or positions this adjacent amino-terminal region of Cas in such a way that promotes Src SH3 domain/Cas interactions. Recently, the crystal structure of the Shep1-Cas complex was obtained (Mace, Wallez et al. 2011). The structure of Cas-bound Shep1, which shares a very close structural similarity to BCAR3 (r.m.s. deviation of 0.97Å), was solved using partial carboxyl-terminal regions of both proteins. This fragment of Cas does not contain the bipartite Src binding site, but future crystallization and *in vitro* work using a larger region of Cas could predict whether BCAR3 binding structurally alters Cas in such a way as to make the Src binding sites more available and thus the interaction more preferable.

Given the absence of a tripartite complex (Fig. 3.2), the association between BCAR3 and Cas is likely to be quickly lost once Src binds to Cas, perhaps as a result of an additional conformational change in Cas. Following the Src SH3 domain/Cas interaction, which serves as the initial anchor for the association between Cas and Src (Pellicena and Miller 2001), Src phosphorylates nearby tyrosine residues of Cas. These phosphorylated residues

then serve as the binding site for the Src SH2 domain (Pellicena and Miller 2001). While this bipartite association is proposed to fully activate the kinase (see Fig. 1.4) and promote the processive phosphorylation of YxxP sites within the Cas substrate domain (Burnham, Bruce-Staskal et al. 2000, Patwardhan and Miller 2007), it may also serve to physically disassociate BCAR3 through steric hindrance, or to foster structural alterations in the carboxyl-terminus of Cas that reduce the affinity for BCAR3. Interestingly, large-scale proteomics studies designed to identify novel Src substrates have shown that BCAR3 is tyrosine phosphorylated by Src (Luo, Slebos et al. 2008, Heckel, Czupalla et al. 2009). The functional and biochemical consequence of this phosphorylation is unknown, and perhaps it may serve to further dissociate BCAR3 from Cas. Much *in vitro* and *in vivo* work is needed to answer these issues, but their resolution will provide a much greater understanding of the molecular regulation of the BCAR3/Cas/Src signaling axis.

We propose in Chapter 3 that BCAR3 mediates cell adhesion and spreading through a relocalization or repositioning of Cas at the membrane, thereby increasing its association to Src (Fig. 3.10). This is based on previous data from our lab showing that BCAR3 overexpression induces a polarized cell phenotype and Cas localization to lamellipodia (Riggins, Quilliam et al. 2003). Conversely, Cas localization at the cell periphery is lost following BCAR3 depletion (Schrecengost, Riggins et al. 2007). More recently, Makkinje et al. demonstrated enhanced BCAR3, Cas, and Src colocalization at peripheral regions of MCF7 cells that stably overexpress BCAR3 and transiently

overexpress Src (Makkinje, Vanden Borre et al. 2012). How BCAR3 protein gets to these membrane-proximal regions to so reposition Cas is a question that is yet to be completely answered. Recent work by Sun et al. provides one possible mechanism for how BCAR3 becomes anchored at the membrane. In response to adhesion to fibronectin, the SH2 domain of BCAR3 binds to a phosphorylated tyrosine residue (Y789) of the transmembrane receptor-like phosphatase PTPα (Sun, Cheng et al. 2012). This binding was shown to be direct by Far-Western analysis using the SH2 domain of BCAR3 as a probe for proteins isolated from cells expressing PTPα (Sun, Cheng et al. 2012). In addition, multi-protein complexes containing BCAR3, Cas, and Src were present in PTPα immune complexes, and the disruption of the SH2/Y789 interaction between BCAR3 and PTPα abolished these complexes. This study provides a model for how, through its SH2 domain, BCAR3 binding to transmembrane proteins may function to redistribute Cas to these regions.

The finding in Chapter 4 that Cas expression is required for BCAR3 protein synthesis (Fig. 4.8) seems, at first, to be at odds with a model in which BCAR3 functions to redistribute Cas at the cell membrane (Fig. 3.10). However, this can be reconciled by considering the possibility that Cas mediates localized BCAR3 synthesis at membrane-proximal regions. In the discussion of Chapter 4, we also hypothesize that BCAR3 synthesis is initiated as part of the cellular response to adhesion and growth factor stimulation. When BCAR3 expression is blocked, as is the case in siRNA-mediated BCAR3 depletion, then the cellular response to such environmental stimuli is dramatically impaired, including the

relocalization of Cas. It is important to note, however, that upon BCAR3 depletion, Cas protein is not lost at peripheral membrane ruffles per se, but rather it is the membrane ruffles themselves that are lost (Wilson et al., 2012, *manuscript in progress*). Thus, it is possible that Cas is properly positioned to augment the synthesis of BCAR3 at these membrane-proximal sites, but that these regions are abolished under conditions of BCAR3 knockdown.

The potential mechanisms of how Cas may be regulating BCAR3 synthesis, and the potential effects of such regulatory mechanisms on normal and tumorigenic cell behavior, are discussed in detail in the following section.

5.4 – Potential Mechanisms and Implications of Cas-dependent BCAR3 Synthesis

Based on published data that provide a mechanistic link between Cas and the Src-dependent regulation of PP2A and 4EBP-1, a model is presented in Chapter 4 (Fig. 4.9) to explain how Cas may regulate BCAR3 synthesis. However, there are a number of other potential mechanisms by which Cas could control the translation of BCAR3. Several key pieces of data must be considered in the development of any model: First, loss of Cas protein results in the impaired synthesis of BCAR3 (Fig. 4.8). This indicates that Cas is a necessary factor in a signaling pathway that ultimately regulates BCAR3 translation. Second, the steady-state levels of BCAR3 mRNA are unaffected by the loss of Cas (Fig. 4.7), indicating that translation is not inhibited due to a reduction in message. Third, the protein levels of FAK (Fig. 4.1C), actin (Fig. 4.1), and tubulin (Fig. 4.5B) are

unchanged in the absence of Cas. This demonstrates that Cas does not affect translation on a global level, and that the specificity for control of BCAR3 synthesis must derived from an intrinsic feature of BCAR3 RNA or protein. Fourth, similar to endogenous protein, the expression of ectopic BCAR3 protein is reduced in Cas depleted cells (Fig. 4.5). This indicates that the specificity for this control is not derived from the 5' or 3' untranslated region (UTR) of BCAR3 mRNA. Finally, the siRNA-mediated knockdown of BCAR3 appears to have no effect on overall Cas protein levels (Fig. 3.3B), implying that the synthesis of these two proteins is not mediated through a co-dependent translational mechanism.

5.4.1 – *Potential Mechanisms of Cas-dependent BCAR3 Synthesis During Translation Initiation*

Translation is predominately regulated at the initiation step (Jackson, Hellen et al. 2010, Liu, Han et al. 2013). We hypothesize that Cas may be a key participant in a signaling mechanism that controls one or more steps during the initiation of translation. The model proposed in Chapter 4 (Fig. 4.9) describes a Cas-mediated, Src-dependent promotion of initiation through the negative regulation of PP2A and 4EBP-1. However, it is formally possible that Cas may also influence translation initiation via other signaling proteins. For example, Cas may influence the activity of mTOR, a well-established regulator of initiation, through activation of the Erk1/2 MAPK (ERK) and/or PI3K/Akt signaling pathways (Pham, Sugden et al. 2000, Kong and Lasko 2012). Cas overexpression leads to

hyperphosphorylated ERK and PI3K in three-dimensional cultures of mouse mammary epithelial cells in response to estrogen or EGF (Camacho Leal Mdel, Pincini et al. 2012). This finding supports our hypothesis that Cas functions to mediate the synthesis of BCAR3 in response to growth factor stimulation. Similarly, Cas overexpression in ErbB2-transformed breast epithelial cells leads to increased ERK and Akt phosphorylation and, moreover, an ERK-dependent activation of the mTOR/p70S6-kinase signaling pathway (Tornillo, Bisaro et al. 2011). These data provide alternate signaling pathways by which Cas could influence the initiation of protein synthesis; however, it is important to note that the activities of these pathways are not necessarily mutually exclusive. For example, the activity of mTOR and its associated protein complex controls the initiation of protein synthesis, in part, through the phosphorylation of 4EBP-1 (Kong and Lasko 2012), the same molecule that can be regulated by Src as described in Figure 4.9. Second, estrogen treatment of breast cancer cells induces a complex containing Cas, the estrogen receptor, Src, and the p85 regulatory subunit of PI3K (Cabodi, Moro et al. 2004). This same study showed that Cas overexpression augments estrogen-mediated ERK phosphorylation four-fold through a pathway that is dependent on Src kinase activity (Cabodi, Moro et al. 2004). Together, these studies demonstrate that Cas, in response to extracellular signals, functions in a network of signaling molecules that are capable of inducing protein synthesis (Fig. 5.2A).

5.4.2 – *Potential Mechanisms that Mediate the Specific Control of BCAR3 Synthesis*

While the mechanisms described above account for how Cas may control protein synthesis, they fail to address the issue of BCAR3 specificity. One possible explanation for this specificity is that BCAR3 mRNA is transported to sites in the cell where Cas is located, and that Cas then promotes the translation of these transcripts through the regulation of nearby translational machinery. Given that endogenous and ectopic BCAR3 proteins are both similarly reduced in the absence of Cas (Fig. 4.5), this model would require that the coding sequence (CDS) of BCAR3 mRNA contain a "localization element" or "zip code." RNA-binding proteins recognize these localization signals and facilitate mRNA transport to specific subcellular regions of the cell. While the zip codes of many mRNAs are present in the 3' UTR, the existence of such elements within the CDS is not unprecedented (Martin and Ephrussi 2009). For example, *ASH1* mRNA is localized to the budding tips of yeast, and three of its four localization elements are located entirely within the CDS (Gonzalez, Buonomo et al. 1999, Chartrand, Meng et al. 2002). Additionally, during *Drosophila* oogenesis, the sequence necessary for the transport and localization of *yemanuclein-alpha* mRNA to specific regions of developing oocytes is also present in its coding region (Capri, Santoni et al. 1997). A related model involving BCAR3 mRNA localization (and thereby synthesis) is one wherein Cas regulates the activity of a specific RNA-binding protein that is necessary for the synthesis of BCAR3 (see the discussions on FMRP below).

In the discussion of Chapter 4, we hypothesize that, as cells form protrusions and turn over adhesions during migration, Cas mediates the expression of pro-migratory proteins like BCAR3 at these dynamic sites through the regulation of nearby translational machinery (Fig. 5.2A). The ability to establish and maintain cell polarity through localized, translational control plays an important role during development and in the function of many somatic cells (Mili and Macara 2009, Kong and Lasko 2012). Components of translation and the accumulation of mRNA have been observed at the leading edge of migrating cells (Mili, Moissoglu et al. 2008, Mili and Macara 2009). Moreover, Chicurel et al. showed that, as cells interacted with fibronectin (FN)-coated beads, mRNA and ribosomes are detected at focal adhesion complexes at the cell-bead interface beginning at 5 minutes and reaching maximum levels at approximately 20 minutes (Chicurel, Singer et al. 1998). Interestingly, Cas and BCAR3 are also found at the cell periphery following growth factor-stimulation (Schrecengost, Riggins et al. 2007) and at focal adhesions following integrin engagement (Ballestrem, Erez et al. 2006, Sun, Cheng et al. 2012). Sun et al. demonstrated that within 15 minutes of cell adhesion to FN, BCAR3 is detected in focal adhesion-enriched cell fractions (Sun, Cheng et al. 2012). While the authors state that this is due to a relocation of BCAR3 from the cytosol to focal adhesions, there is no apparent decrease in the cytosolic pool of BCAR3 following FN stimulation (Sun, Cheng et al. 2012). Thus, this focal adhesion-associated pool may in fact represent newly synthesized protein. Moreover, we would argue that a pre-existing pool of steady-state BCAR3 protein is necessary

to mediate the expression of additional protein at focal adhesions, since siRNA-mediated BCAR3 depletion results in a dramatic loss of adhesion-dependent Cas signaling, as measured by total tyrosine phosphorylation (Sun, Cheng et al. 2012).

Tyrosine phosphorylated Cas is a major component of integrin-mediated focal adhesion complexes (Vuori and Ruoslahti 1995, Ballestrem, Erez et al. 2006). A recent study showed that along with Cas, a protein called Fragile X Mental Retardation Syndrome-related protein 2 (FXR2) is recruited to clustered $\alpha\beta1$ integrin receptors (the specific α subunit was not reported) (Sansing, Sarkeshik et al. 2011). FXR2 and its paralog, Fragile X Mental Retardation Protein (FMRP), are RNA-binding proteins, and known regulators of mRNA localization and translation initiation (Napoli, Mercaldo et al. 2008, Adams-Cioaba, Guo et al. 2010, Kong and Lasko 2012). FMRP has also been observed at the leading edge of migrating fibroblasts as part of a complex that regulates the accumulation of mRNA at migratory protrusions (Mili, Moissoglu et al. 2008). Interestingly, PP2A directly regulates FMRP function in neurons (Niere, Wilkerson et al. 2012). This finding provides an additional component through which the proposed Cas/Src/PP2A (Fig. 4.9) signaling network could influence translation.

Is the rapid and/or localized synthesis of BCAR3 one way in which quiescent somatic cells quickly respond to changes in environmental cues to facilitate migration during processes such as wound repair, inflammation, or to maintain tissue homeostasis? Compelling connections can be made between our

current understanding of localized translational regulation and the data presented in this thesis. However, we can so far only speculate that Cas functions at these sites to regulate machinery that controls the translation of migratory proteins like BCAR3. Work remains to be done to prove whether such mechanisms exist.

A second mechanism by which BCAR3 translation could be specifically regulated by Cas is through a microRNA (miRNA). Thus, one could envision a miRNA whose expression is regulated by Cas that functions to suppress BCAR3 synthesis. While the first and best-characterized miRNAs were shown to bind to sequences present in the 3' UTR of their targets (similar to many of the localization elements that control mRNA transport), there are many examples of miRNAs that bind to the CDS of their target mRNAs, including, but not limited to, miR-296, -470, and -134 that target multiple CDS sites within *Nanog*, *Oct4*, and *Sox2* during mouse embryonic stem cell differentiation (Rigoutsos 2009, Pasquinelli 2012). This precedent of CDS recognition by miRNAs reconciles the absence of the endogenous 3' UTR in ectopic BCAR3 transcript, therefore allowing for the potential regulation of both endogenous and ectopic BCAR3 protein synthesis via miRNA repression. Once bound to their target sites, miRNAs appear to have two mechanisms through which protein synthesis can be repressed: a true inhibition of translation (possibly by the displacement of mRNA from polysomes) or induction of mRNA degradation (Jackson, Hellen et al. 2010). Given that the steady-state levels of BCAR3 mRNA are unchanged in the

absence of Cas (Fig. 4.7), it would appear that the Cas-regulated miRNA would have to prevent BCAR3 translation rather than induce mRNA degradation.

5.4.3 – *Potential Mechanisms of Cas-dependent Regulation of Translational Elongation*

Recent studies demonstrate that, in addition to initiation, protein synthesis can also be regulated at the elongation stage of translation. Interestingly, the action of miRNAs and FMRP are implicated in the regulation of translation elongation as well as initiation. For example, some miRNAs appear to have the ability to inhibit translation at post-initiation stages, as miRNA-repressed mRNAs have been found in translation-competent polysomes (Jackson, Hellen et al. 2010). The rate of translation can be regulated by ribosome stalling or pausing during elongation (Kong and Lasko 2012). FMRP, the regulator of mRNA localization and translation initiation discussed above, is also implicated in ribosomal stalling (Darnell, Van Driesche et al. 2011). FMRP binds to the coding region of mRNAs and acts to reversibly stall ribosomes on these transcripts (Darnell, Van Driesche et al. 2011). As Cas is present in FMRP-associated cell fractions, and PP2A has been shown to influence FMRP function (see above), it is possible that Cas specifically regulates BCAR3 synthesis by influencing FMRP association to BCAR3 transcript.

The two mechanisms described above satisfy the caveats of specificity and ectopic protein expression through the involvement of regulatory factors that bind to the CDS of BCAR3. The activity of other regulatory proteins, such as

elongation factors and chaperones, that could potentially mediate BCAR3 elongation would have to rely on BCAR3-specific localization patterns, as described in the preceding section, to comply with the restrictions set by our experimental findings. The possibility that Cas signaling regulates the activity of elongation factors is intriguing, as Src and PI3K are components of signaling pathways that affect the phosphorylation of the elongation factors eEF1A and eEF2 and influence translational control (Barrera, Flores-Mendez et al. 2010). Additionally, the elongation stage of BCAR3 synthesis could be controlled through the Cas-dependent regulation of chaperone proteins. Two recently published papers describe chaperone-mediated elongation pausing in response to the accumulation of misfolded proteins (Liu, Han et al. 2013, Shalgi, Hurt et al. 2013). These studies demonstrate that the association of chaperones (specifically Hsp70 proteins) with ribosomes is lost after proteotoxic stress, and that this facilitates translational repression by halting ribosomes on transcripts. Furthermore, an accelerated rate of elongation (and protein synthesis) was observed in a system designed to increase chaperone interactions with nascent peptide chains (Liu, Han et al. 2013). While these studies focused on a global, co-translational repression of elongation via altered chaperone activity, the loss of Cas may specifically inhibit BCAR3 synthesis by causing a reduction in the binding capacity of essential chaperones to localized ribosomes or to the amino-terminal end of the nascent BCAR3 peptide as it emerges from the ribosomal exit tunnel. Alternately, Cas may be required, directly or indirectly, for the correct post-translational folding of BCAR3. Under this circumstance, the depletion of

Cas would result in the sequestration of chaperones to a misfolded population of BCAR3 and away from nearby polysomes, thus halting nascent chain elongation. Again, to reconcile the issue of specificity, this Cas-mediated activity must occur within subcellular regions where BCAR3 transcripts (and possibly other protein transcripts) are co-localized along with translational machinery. Irrespective of the exact mechanism, the control of BCAR3 synthesis by Cas has intriguing implications for both normal and tumor cell biology.

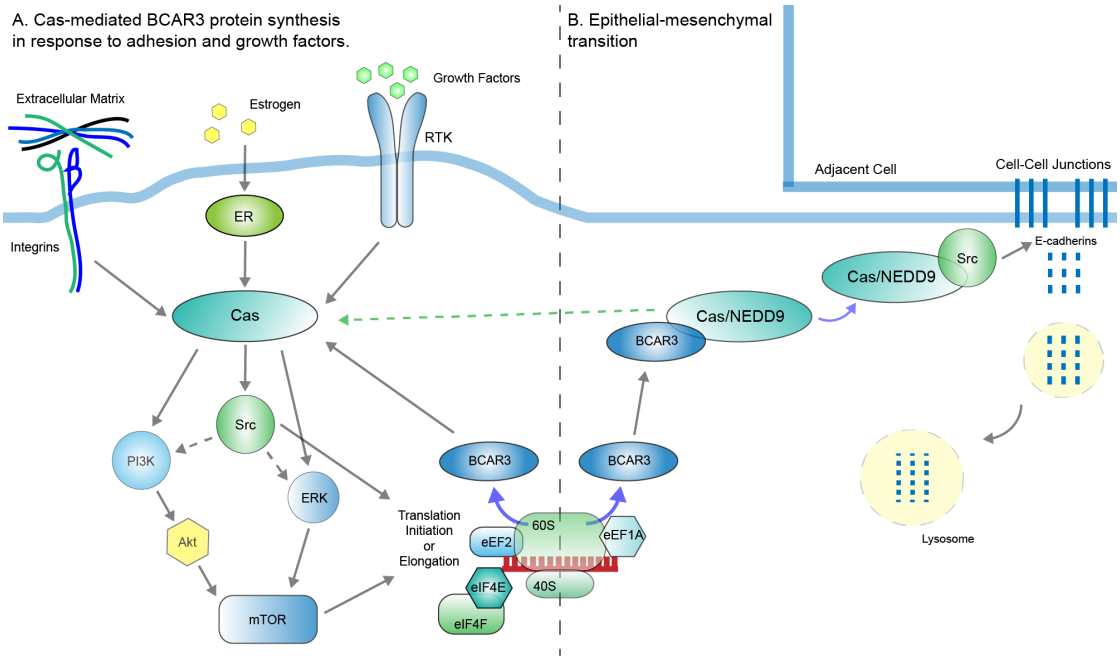


Figure 5.2: Biological roles of proposed Cas-mediated BCAR3 synthesis.

A. Increased BCAR3 expression in migratory responses to adhesions and growth factor receptor stimulation. Cells polarize in response to external cues and Cas signaling networks are activated, possibly leading to the activity of the Src-dependent mechanism in Figure 4.9 and/or to Src-dependent or -independent PI3K or ERK-mediated protein synthesis through mTOR. This potentially leads to the activation of membrane-proximal translation complexes and the localized synthesis of BCAR3. The expression of BCAR3 is necessary for appropriate cell migration in response to the environmental signals. **B.** Amplification of EMT through BCAR3/Cas/Src. Deregulated Cas expression leads to increased Src activity and the simultaneous degradation of E-cadherin at cell-cell junctions and augmented BCAR3 expression. Through the continued expression of BCAR3 the system perpetuates, causing malignant progression of epithelial cells.

5.4.4 – *Potential Implications of Aberrant Cas-dependent BCAR3 Synthesis in Tumor Cell Functions*

A growing number of studies, using different mouse models and evaluating human tumor tissue, provide evidence supporting a role for Cas and its family member NEDD9 in the initiation and progression of cancer, particularly the development of metastatic disease (Tikhmyanova, Little et al. 2010, Guerrero, Parsons et al. 2012). The data in Chapter 3, along with findings presented in other published reports, describe the ability of BCAR3 to both bind to, and activate, Cas and NEDD9 (Cai, Iyer et al. 2003, Riggins, Quilliam et al. 2003, Schrecengost, Riggins et al. 2007, Makkinje, Near et al. 2009, Schuh, Guerrero et al. 2010, Makkinje, Vanden Borre et al. 2012). Given these data, BCAR3 is well positioned to potentiate Cas and/or NEDD9-driven signaling during disease development. Therefore, it is important to expand upon the findings reported in this thesis to more fully understand the Cas-dependent regulation of BCAR3 expression.

Several questions are posed in the discussion of Chapter 4, including: is Cas-dependent BCAR3 expression a marker of aggressive disease? Does the constitutive overexpression of Cas cause a concomitant rise in BCAR3 expression in tumor cells of different tissues? And if so, does this increase the metastatic potential of these cells? While experiments designed to directly address these questions need to be performed, there are several intriguing pieces of data that would support the existence of such a Cas/BCAR3-driven model of tumor dissemination.

Cas and NEDD9 have been implicated in the pre-metastatic process of “epithelial-mesenchymal transition” (EMT) (Guerrero, Parsons et al. 2012). The overexpression of either Cas or NEDD9 in the epithelial-like MCF7 breast cancer cell line leads to Src-dependent lysosomal degradation of the epithelial marker E-cadherin, the major component of epithelial adherens junctions, and their co-overexpression accelerates this process (Yilmaz and Christofori 2009, Tikhmyanova and Golemis 2011). Both BCAR3 protein levels and BCAR3/Cas complexes are higher in mesenchymal (MDA-MB-231, BT549, MDA-MB-435S)- than epithelial-like breast cancer cell lines (MCF7, T47D, ZR-75-1)(Near, Zhang et al. 2007, Schrecengost, Riggins et al. 2007). Furthermore, the overexpression of BCAR3 in MCF7 cells results in a reduction of cell-cell junction formation (and E-cadherin staining) and the increased production of fibronectin, two features associated with mesenchymal cells (Near, Zhang et al. 2007). Based on these data, we have developed the working hypothesis that BCAR3 expression is negatively co-regulated with the formation of cell-cell junctions. In this way, BCAR3 expression increases as cell junctions are dissolved and as cells convert to individual (mesenchymal), instead of collective (epithelial), bodies. The finding that Cas regulates BCAR3 synthesis fits well into this hypothesis. Within a hypothetical model of dysregulated (or stimulus-independent) Cas signaling (Fig. 5.2B), Cas-mediated Src activation leads to E-cadherin degradation, a concomitant increase in BCAR3 expression, and augmented Cas signaling. In turn, this leads to increased Src activity, further E-cadherin degradation, and higher BCAR3 expression, thus exacerbating this process. This cyclic increase in

the amplitude of Cas/Src signaling not only abolishes cell-cell junctions, but also increases migratory/invasive signals downstream of the BCAR3/Cas/Src signaling axis. This impaired mechanism could explain the relative abundance of BCAR3 protein in cell lines such as MDA-MB-231 and BT549, and the pernicious way Cas can function to enhance its own downstream signaling to drive the metastatic process.

While much of the work testing this hypothesis can be performed in two- and three-dimensional tissue culture systems, combining these studies with mouse models will be important to better understand the true physiological role of BCAR3. At the time of this writing, only two studies have been published that specifically address the role of BCAR3, and its family member Shep1, in mouse development. The following section reviews this research and proposes additional studies to further characterize the biological roles that BCAR3 and NSP proteins play in both normal and pathological processes.

5.5 – Animal Models of BCAR3 Function

The depletion of BCAR3 from MDA-MB-231 and BT549 cells results in a static, contractile phenotype, marked by decreased migratory potential, and an inability to dissolve stress fibers and turnover adhesions in response to EGF and FN [see Discussion, Chapter 4; (Schrecengost, Riggins et al. 2007); Wilson et al., 2013, *manuscript in progress*]. Is the phenotype exhibited by these cells a genuine representation of BCAR3 function or is it an artifact of immortalization and/or an adaptation that occurred through the continuous passage in a tissue

culture system? Answering this question is important, as the BCAR3 global knockout mouse demonstrates that the AND-34/BCAR3 gene and its protein product are inessential for neo- and postnatal development (Near, Smith et al. 2009). This does not rule out a role during development, however, as there are many examples of genetic redundancy and functional compensation across protein families in multicellular eukaryotic organisms (Lowell and Soriano 1996, Nowak, Boerlijst et al. 1997, Vavouri, Semple et al. 2008). Importantly, despite normal development, the overall fitness of the knockout mouse is not equal to its WT counterpart. The appearance of cataracts and the 100-percent penetrant phenotype of lens ruptures establish a distinct biological function of AND-34/BCAR3 in the maintenance of ocular tissue homeostasis. These mice can therefore prove to be very useful for establishing additional biological roles for BCAR3. How do these mice perform in assays set up to test tissue regeneration and repair? How do these mice respond to induced inflammation and how susceptible are they to certain infectious diseases? Lastly, does the loss of BCAR3 in these mice condition them to improve or reduce tumor “take” and formation in xenograft models; does it have an effect on the occurrence or rate of metastasis? Determining the answers to these questions is necessary for the full characterization of BCAR3 function.

The majority of Shep1 knockout mice die shortly after birth due to an undiscovered physiological defect, but similar to their AND-34/BCAR3 counterparts, the surviving pups that reach adulthood exhibit no widespread phenotypic defects (Roselli, Wallez et al. 2010). As AND-34/BCAR3 and Shep1

proteins are expressed across many of the same murine organs (Sakakibara and Hattori 2000, Near, Smith et al. 2009), the fitness of these animals may result from functional redundancy between these two genes. The phenotype of global or tissue-specific double knockout mice will directly address the question of redundancy. These animals may therefore be more valuable in determining how and when these two proteins function.

To test the oncogenic potential of BCAR3, genetic gain-of-function and/or xenograft mouse models are needed. Cas overexpression in the mammary glands of mice induces hyperplastic growth but is not sufficient to promote tumor formation. However, double transgenic mice that overexpress both Cas and HER2/Neu under the control of the MMTV promoter exhibit accelerated tumor formation compared to animals that express HER2/Neu alone (Cabodi, Tinnirello et al. 2006). Can BCAR3 similarly augment the transformation capacity of HER2/Neu? What is the consequence of BCAR3 and Cas co-overexpression in mammary tissue? Important information regarding the normal functional role of BCAR3 as well as its tumorigenic potential remain to be gathered by asking new questions with existing mouse models, and through the generation of double transgenic lines engineered to knockout BCAR3 and Shep1 or to co-overexpress BCAR3 and Cas.

5.6 – Perspectives on BCAR3 and Human Cancer

To validate current and future research performed in culture or in mice, studies using patient samples are needed to determine whether BCAR3 plays a pathophysiological role in human cancer. For example, it is critical to determine whether a correlation exists between BCAR3 protein levels and tumor grade, stage, disease recurrence, and/or overall patient survival. To date, no such studies have been published. And while a full qualitative assessment of the biological significance of BCAR3 may be better served by animal models (see previous section), the evaluation of BCAR3 protein from clinical samples of primary and metastatic tumors is currently one of the most important areas in which to focus.

While there are no published studies that examine the relationship between BCAR3 protein levels and metrics used to define disease/cancer severity, one study with specific clinical endpoints does examine the mRNA levels of BCAR3, Cas, and Src (van Agthoven, Sieuwerts et al. 2009). In two different cohorts of ER-positive tumor samples, the expression of BCAR3, Cas, and Src was independently evaluated for an association with 1) progression-free survival (PFS) in patients that received first-line tamoxifen therapy, and 2) metastasis-free survival (MFS) in patients that did not receive systemic adjuvant therapy. The report reveals that BCAR3 mRNA tracks with longer PFS, and that no association between BCAR3 and MFS exists. Additionally, no statistical association between Cas or Src mRNA levels and PFS or MFS was observed (van Agthoven, Sieuwerts et al. 2009). As was stated in the study, the finding that

Cas and Src mRNA expression is not associated with reduced PFS or MFS is surprising. High Cas protein levels are associated with advanced stage disease and/or are predictive of poor outcome in ovarian, breast, prostate, and non-small-cell lung cancer (Dorssers, Grebenchtchikov et al. 2004, Nick, Stone et al. 2011, Fromont, Rozet et al. 2012, Huang, Deng et al. 2012). Moreover, elevated levels of Src protein and/or kinase activity correlate with tumor progression in many human cancers (Irby and Yeatman 2000, Kim, Song et al. 2009). The results of this study demonstrate that mRNA levels may not be the most relevant factors in evaluating the potential contributions of these proteins during cancer progression. Furthermore, as the authors go on to hypothesize, the inconsistency between conclusions drawn from mRNA and protein data may be due to differences in the regulation of translation (van Agthoven, Sieuwerts et al. 2009), highlighting the need to examine BCAR3 protein levels in clinical samples. Given the findings of this study and the Cas-dependent BCAR3 synthesis reported in Chapter 4, the investigation of BCAR3 protein levels is essential for evaluating the potential contributions of BCAR3 to human cancer.

As part of the Swedish Human Protein Atlas project, a panel of different primary human tumors has been analyzed by immunohistochemistry (IHC) for BCAR3 protein levels (proteinatlas.org). Weak to moderate staining is observed across the panel. Furthermore, the pattern of staining observed in the series of malignant tissue is similar to what is seen in an unmatched series of normal tissue (proteinatlas.org). No clinical data, such as grade or stage is provided, which makes it difficult to glean any significance between BCAR3 levels and any

malignant parameter. Studies using discovery-mode mass spectrometry show many sites of tyrosine, serine, and threonine phosphorylation within BCAR3 (phosphosite.org). While these mass spectrometry-based proteomic approaches reveal that BCAR3 is subject to phosphorylation in both normal and malignant tissues (Wolf-Yadlin, Kumar et al. 2006, Luo, Slebos et al. 2008, Heckel, Czupalla et al. 2009, Wang, Tsai et al. 2010), the studies were not performed to determine functional consequences of these modifications. Perhaps phosphorylation status is a more valuable histological marker for disease progression than total protein levels. The generation of non-phosphorylatable and phosphomimetic mutants for use in loss- and gain-of-function studies will enable the elucidation of specific functions of these phosphorylation events.

Ultimately, more work needs to be done in order to make specific conclusions about BCAR3 with respect to cancer and to determine the value of current and future work into this protein. The reported “weak to moderate” IHC staining may not in and of itself indicate that BCAR3 is unimportant as a regulator of cancer progression, as small perturbations at or near the initiation of signaling cascades can have dramatic effects downstream. Therefore, the execution of studies designed to determine whether specific features of BCAR3 protein (such as overall levels, co-expression with Cas and Src, or its phosphorylation status) correlate with the grade, stage, and outcome of different cancers are of the utmost importance going forward. Not only will this work discern the significance of BCAR3 in tumorigenesis, but if an association is found, then the ability to

attribute distinct functions to BCAR3 during the promotion of specific stages of tumor development will be useful in assessing its worth as a histological marker.

5.7 – Thoughts on "Breast Cancer Antiestrogen Resistance-3"

Is "Breast Cancer Antiestrogen Resistance-3" a fitting name for this protein? Based on our inability to recapitulate a resistant phenotype and the lack of evidence in human breast cancer that points to a role in the development of antiestrogen resistance, this nomenclature both poorly describes and perhaps misrepresents the function of this protein. Indeed, the name BCAR3 is incongruous with data collected by our group and others that indicate a significant role for this protein in cell motility. For example, while BCAR3-overexpressing clones failed to exhibit antiestrogen resistance, they did exhibit an increased migratory phenotype (Wilson et al., 2013, *manuscript in progress*). Loss-of-function experiments similarly demonstrate that BCAR3 modulates the migratory and invasive capacities of breast cancer cells (Schrecengost, Riggins et al. 2007). Finally, an increasing body of research, including the work presented in this thesis, shows that BCAR3 and its family members act as modifiers of Cas family proteins, whose functions influence a wide range of cellular processes across a variety of cells. Therefore, in describing only one *in vitro* finding, the name "BCAR3" only narrowly defines the function of this protein. More research into its function *in vivo* and the determination of whether it has a role in human

cancer will be helpful in deciding whether there may be a more appropriate name.

5.8 – Summary and Final Perspectives

The data presented in Chapters 3 and 4 of this thesis contribute novel information to the field of BCAR3 and Cas research. Chapter 3 demonstrates that BCAR3 is a positive regulator of Cas signaling through modifying Cas/Src and Cas/Crk associations. These changes correlated with heightened Src kinase activity, augmented Src-dependent Cas tyrosine phosphorylation, and increased Cas/Crk coupling. This Src/Cas/Crk signaling network is a known regulator of cell motility, proliferation, and survival. Furthermore, Chapter 3 reveals that BCAR3 regulates cell adhesion and spreading in response to integrin-ECM engagement, two processes essential to intracellular signaling. Chapter 4 further describes the binding dynamics between BCAR3 and Cas by showing that the total cellular population of BCAR3 is in complex with Cas, while a substantial pool of Cas is free of BCAR3. This finding suggests that Cas plays an essential role in BCAR3 function, as does the tight correlation in the rate of BCAR3 and Cas protein degradation. Most interesting is the discovery of an absolute dependence on Cas for BCAR3 protein synthesis. Collectively, the work of this thesis better places BCAR3 within a molecular and cellular context, and attributes a previously unknown function to Cas.

The finding that Cas functions to regulate protein synthesis is novel unto itself. Determining whether Cas is coupled to mechanisms that control mRNA

localization and/or spatial translation as a means to regulate specific cellular processes is an intriguing area of potential work. First, the hypothetical signaling pathways discussed here need to be tested in order to establish Cas as a true mediator of translation. Second, additional proteins whose translation may be regulated by Cas in response to extracellular signals need to be identified, as do the functional consequences of such regulation. Lastly, once established, the other essential components of this Cas-dependent signaling pathway need to be identified. These open questions represent the beginnings of several new projects in the lab, independent of BCAR3. Continued research into the molecular composition and biological significance of these proposed mechanisms will better define Cas function within the context of normal processes such as development and tissue homeostasis.

As both BCAR3 and Cas have been implicated in the promotion of aggressive tumor cell behavior, the insights provided in this thesis may prove useful in developing a better understanding of cancer biology. This work better describes BCAR3 as a potent modulator of Cas and Src signaling, reveals a new functional complexity of Cas protein biology, and widens the scope of our understanding of the signaling potential of adaptor proteins. Future work, based on the foundations of this thesis and on questions proposed within, will continue to elucidate the physiological roles of BCAR3 and Cas, and will help determine whether either of these proteins are viable candidates for use as biomarkers and therapeutic targets.

Appendix. Doxycycline-induced False Positives in BCAR3-mediated Antiestrogen Resistance Assays

A.1 – Project Overview

The ectopic overexpression of BCAR3 confers an antiestrogen resistant phenotype in previously sensitive, ER-positive breast cancer cell lines (van Agthoven, van Agthoven et al. 1998, Cai, Iyer et al. 2003, Felekkis, Narsimhan et al. 2005). As stated in both the Introduction and Discussion chapters, the original goal of this thesis project was to further elucidate the molecular mechanism(s) of BCAR3-mediated antiestrogen resistance. Work published prior to the start of this project had shown that PI3K-dependent Rac activation is required for the generation of resistance. While necessary, this signaling pathway was shown to be insufficient during the establishment of the resistant phenotype, as cells overexpressing the other NSP family members, NSP1 and Shep1, displayed a similar induction in both PI3K and Rac activity but remained sensitive to antiestrogen treatment (Near, Zhang et al. 2007). Therefore, the first aim of this project was to identify additional signaling proteins whose activities contribute to the induction of BCAR3-mediated antiestrogen resistance.

To begin work on this aim, we generated a clonal population of the ER-positive, estrogen (E2)-dependent MCF7 cell line was that stably transduced with a BCAR3 expression vector under the control of a tetracycline (Tet)-regulated promoter. In this system, the addition of 1-to-2μg/ml of the tetracycline Doxycycline (Dox) effectively turns off BCAR3 expression (Tet-off; see Fig. 3.6A).

Following the successful generation of these Tet-off clones, we then set out to determine whether these cells, as expected, displayed resistance to the growth inhibitory effects of antiestrogen treatment. While initial studies, using the percentage of cells in S phase as a marker for cell proliferation, indicated that we had successfully generated an antiestrogen resistant clone. Later analysis using two MCF7 clones stably expressing empty vector (V3S2 and V3S3) showed that Dox treatment alone causes a dramatic reduction in the percentage of cells undergoing DNA replication. The remainder of this appendix describes both the initial resistance assays and the subsequent Dox experiments in more detail.

A.2 – Materials and Methods

Generation of MCF7 clones – For a description of how MCF7 cell clones were generated and isolated see Chapter 2 Materials and Methods. V3S3 and V3S2 were transfected with empty Myc-pTre2-Puro vector and stable clones were isolated as described in Chapter 2.

72-hour growth assays – BCAR3 overexpressing MCF7 cells were seeded in the presence or absence of 1μg/ml Dox for 48 hrs in appropriate maintenance media. Following 48 hr. incubation, cells were split and seeded in the required number of 10-cm dishes at a 10^6 cells/dish in maintenance media. Cells were then serum-starved cells for 12 hr. by the addition of serum-free phenol red-free DMEM. Cells are then treated cells with phenol-red free DMEM + 5% CSS supplemented

with vehicle (0.1% EtOH), estrogen (10^{-8} M E_2), or estrogen + tamoxifen (10^{-8} M E_2 + 10^{-6} M 4-OHT).

Src inhibitor experiments – Assays were set up as described above, but sister 10-cm dishes were added for each condition and supplemented with 10μM SU6656. At the end of the assay, cells were trypsinized and counted using 4 $1mm^2$ grids of a hemocytometer.

p27 localization assay – Growth assay was set up as described above. A microscope coverslip was placed in the bottom of each 10-cm dish, and following 72-hour treatments coverslips were collected. Cells were fixed in 3% paraformaldehyde for 20 minutes and permeabilized in 0.4% Triton X-100 for 6 minutes at room temperature. Cells were then incubated in 10% BSA/PBS for 30 minutes, anti-p27 (BD Transduction, Cat. No. 610242) at 1:500 in 2%BSA/PBS for 1 hour at room temperature followed by 1 hour of FITC-conjugated goat anti-mouse secondary (1:500). Cells were mounted onto microscope slides with Prolong Gold plus DAPI (Molecular Probes). Cells were imaged on a Nikon TE2000-E Eclipse fluorescence microscope. Pictures were taken with an ORCA cooled charged-coupled device camera controlled by Openlab software (Improvision, Inc.). Per field, nuclei positive for p27 staining were counted, and the percentage of nuclear p27 was calculated by # of cells with nuclear p27/total # cells (DAPI).

Cell cycle profile analysis – FITC BrdU/7-AAD flow kits were purchased from BD Pharmigen (Cat. No. 559619). Click-iT Pacific Blue EdU/CellCycle 633-red was purchased from Invitrogen Molecular Probes (Cat. No. A10034). Thymidine analogs were incubated with cells for 3 hours. Following incubation 10^6 were collected and stained according to manufacturer's instructions. Cell cycle analysis of BrdU-incorporated cells was performed using a FACSCalibur flow cytometer (BD Biosciences). EdU-incorporated cells were analyzed using a CyAn flow cytometer (Beckman Coulter). All acquired flow cytometry data was analyzed using FlowJo software.

A.3 – Results

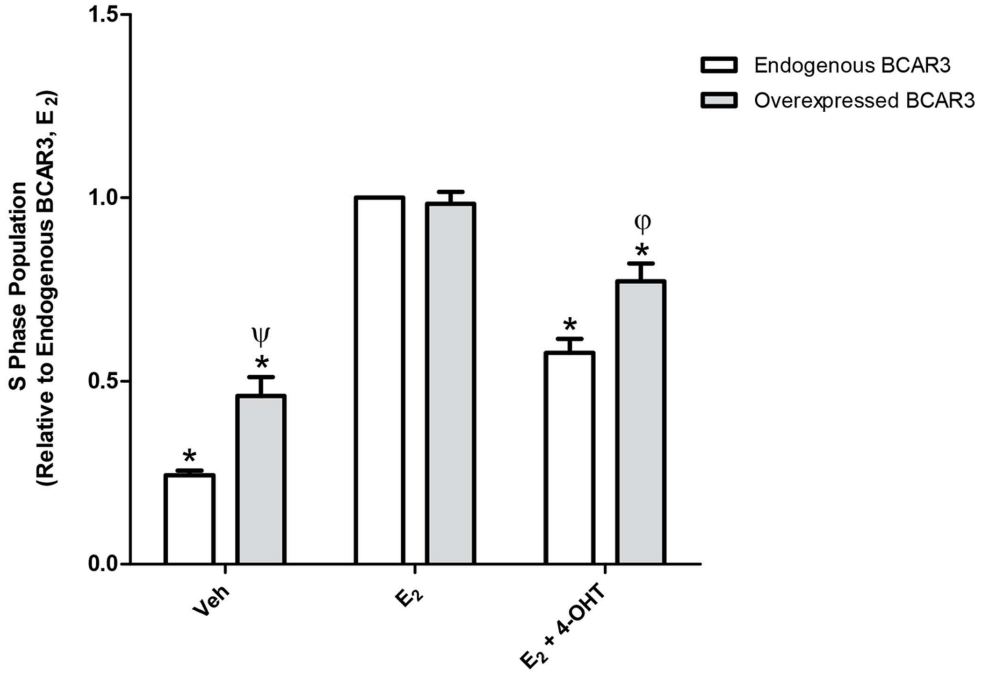
A.3.1 – *Initial Experimental Findings Using BCAR3 Overexpressing Clones*

The initial assays were designed to address whether BCAR3 overexpression confers both E2-independent growth and resistance to growth arrest by 4-hydroxytamoxifen (4-OHT). Cells were seeded and cultured for 48 hours in the presence (endogenous BCAR3) or the absence (overexpressed BCAR3) of 1μg/ml Dox to ensure that BCAR3 expression was completely shut off in the Dox treated cells. Following an overnight serum-starvation in phenol red-free DMEM, cells were re-seeded and cultured for 72 hours in phenol-red free DMEM supplemented with 5% Charcoal Stripped Serum (CSS) in the absence of estrogen (Veh; vehicle, 0.1% ethanol) or conditioned with E2 (10^{-8} M) or E2 plus 4-OHT (10^{-6} M). During the final 3 hours of the assay cells were incubated with 10 μM of bromodeoxyuridine (BrdU), an analog of thymidine, to allow for the detection of cells actively replicating DNA (S phase). Cells were then collected and flow cytometric cell cycle analysis was performed after incorporated BrdU was stained with a FITC-conjugated anti-BrdU antibody and DNA was dyed with 7-amino-actinomycin (7-AAD). Although dramatic differences were not detected, the cells overexpressing BCAR3 displayed a statistically significant increase in the percentage of proliferating cells under conditions of estrogen deprivation (Fig. A.1A; Veh; first group, gray bar) and tamoxifen treatment (Fig. A.1A; E2 + 4-OHT; third group, gray bar). However, these BCAR3 overexpressing cells did still respond to estrogen, as there was a statistical difference between estrogen-

treated cells (white bar, second group) and both estrogen-deprived and tamoxifen-treated samples. Based on these findings, it appeared that our BCAR3-overexpressing clones were both less reliant on estrogen for growth and less sensitive to the growth inhibitory effects of tamoxifen.

Figure A.1

A.


S Phase Population
(Relative to Endogenous BCAR3, E2)
1.5
1.0
0.5
0.0
Endogenous BCAR3
Overexpressed BCAR3
*
ψ
*
*
φ
*
Veh
E2
E2 + 4-OHT

B.

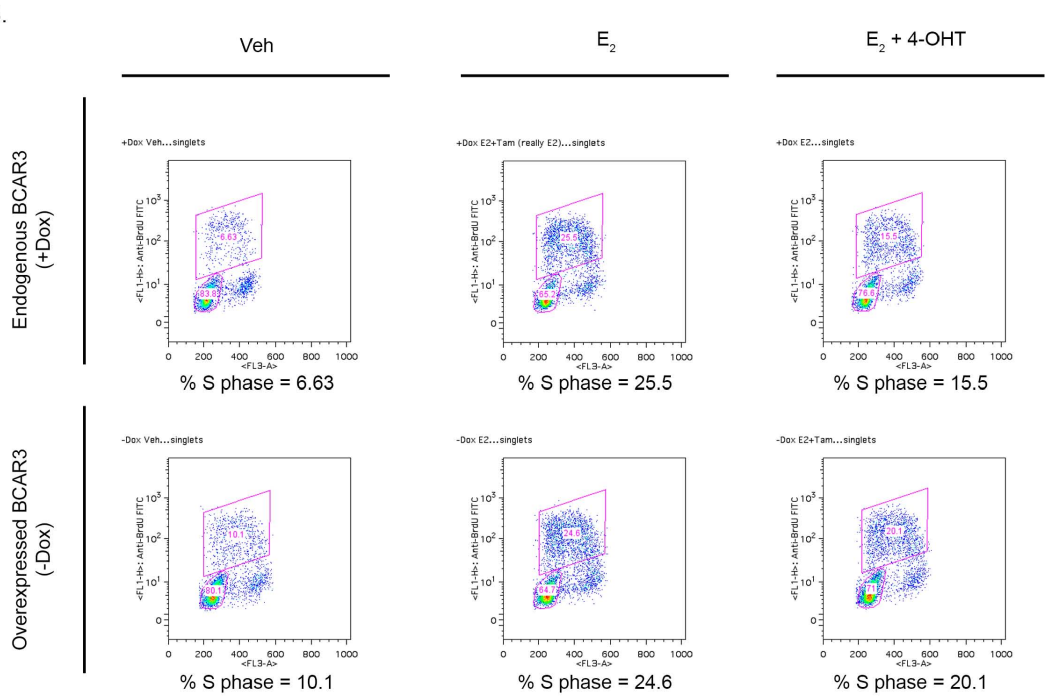

Veh
E2
E2 + 4-OHT
Endogenous BCAR3
(+Dox)
Overexpressed BCAR3
(-Dox)
+Dox Veh...singlets
+Dox E2+Tam (really E2)...singlets
+Dox E2...singlets
-Dox Veh...singlets
-Dox E2...singlets
-Dox E2+Tam...singlets
<FL1-H>: Anti-BrdU FITC
<FL3-A>
% S phase = 6.63
% S phase = 25.5
% S phase = 15.5
% S phase = 10.1
% S phase = 24.6
% S phase = 20.1

Figure A.1: BCAR3 overexpression induces a growth advantage under estrogen-deprived and tamoxifen-treated conditions.

A. MCF7 cells were cultured in the presence or absence of Dox (1μg/ml) for 48 hours followed by a 12-hour serum-starvation in phenol red-free DMEM. Cells were then split and re-seeded in hormone-starved conditions (phenol red-free DMEM + 5% CSS) supplemented with no estrogen (Veh; 0.1% ethanol), estrogen (E2, 10^{-8}M), or estrogen plus tamoxifen (E2 + 4-OHT, 10^{-6}M) for 72 hours. Cell cycle profiles were analyzed by flow cytometry. Data quantified from a total of five experiments. **B.** Representative experiment of the data quantified in A. * - Represents significance against End. BCAR3 E2; $^{\Psi}$ – difference between - E2 is significant, p-value = 0.0148; $^{\varphi}$ – difference between E2 + 4-OHT is significant, p-value = 0.0138.

As the overexpression of BCAR3 had been shown to cooperate with Cas in the promotion of Src kinase activity (Riggins et al., 2003), we next hypothesized that BCAR3 mediates tamoxifen resistance through the increased activation of Src. To determine if this was the case, we utilized the Src kinase inhibitor SU6656, and a growth assay was performed as described above with the inclusion of sister dishes of each condition treated with 10μM SU6656. Following the 72-hour treatment cells were trypsinized and population growth was determined by counting on a hemocytometer. As expected, BCAR3 overexpressing cells significantly outgrew their estrogen-deprived and tamoxifen-treated control counterparts, and this growth advantage was abolished in the presence of Src inhibitor (Fig. A.2). At the time, this finding indicated that the kinase activity of Src is a necessary component of BCAR3-mediated antiestrogen resistance.

Part of the cellular response that induces growth arrest following tamoxifen treatment is the nuclear accumulation of the cyclin-dependent kinase (CDK) inhibitor p27. When localized in the nucleus, this protein facilitates cell cycle arrest, and phosphorylation by Src promotes its proteolysis and thereby cell cycle progression (Chen, Alvarez et al. 2011). Based on these data, we hypothesized that BCAR3 promotes cell growth in the absence of estrogen and in the presence of tamoxifen through a Src-dependent reduction in p27 nuclear localization. Cells were again pre-conditioned with or without Dox, and a growth assay was performed as above. To analyze the subcellular localization of p27, a microscope coverslip was placed in the bottom of dishes of each condition. At the

end of the assay, coverslips were collected and prepared for immunofluorescence analysis by fluorescently labeling p27. Following estrogen deprivation and tamoxifen treatment, the percentage of cells with nuclear p27 was significantly lower in BCAR3 overexpressers compared to their control counterparts (Fig. A.3). This reduction in the nuclear localization of p27 suggests that BCAR3 overexpression causes cells to be less responsive to tamoxifen-induced cell cycle arrest by spatially altering the expression of this CDK inhibitor.

Based on the results collected so far, we hypothesized that BCAR3 mediates antiestrogen resistance through Src-dependent regulation of p27. Furthermore, work performed in parallel with the above experiments had shown that BCAR3 overexpression leads to increased Src activity and Src-dependent Cas tyrosine phosphorylation (Chapter 3, Fig. 3.6). Therefore, experiments were set up to examine the requirement of Cas during the establishment of BCAR3-mediated resistance. The potential involvement of Cas was tested using the siRNA-mediated knockdown of Cas, and the subsequent finding that Cas-depletion results in BCAR3 loss in these experiments, in part, led to the work of Chapter 4. However, concurrent work done by another graduate student in our lab had revealed that Dox treatment alone had dramatic effects on cell proliferation. The following section reviews that work.

Figure A.2

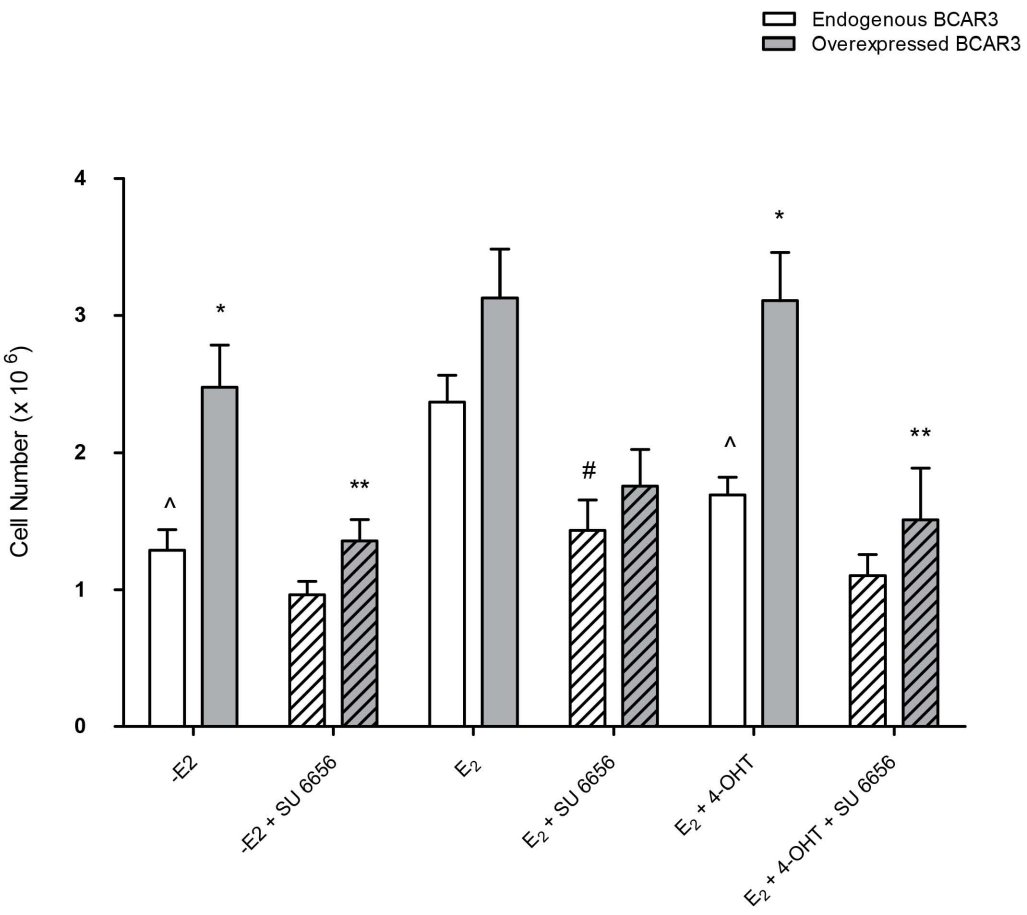

Endogenous BCAR3
Overexpressed BCAR3
Cell Number (x 10 6)
4
3
2
1
0
^
*
**
#
^
*
**
-E2
-E2 + SU 6656
E2
E2 + SU 6656
E2 + 4-OHT
E2 + 4-OHT + SU 6656

Figure A.2: Src inhibition abolishes BCAR3-mediated estrogen independence and tamoxifen resistance.

BCAR3-mediated estrogen independence and tamoxifen resistance requires Src kinase activity. Growth assay was performed as described in Figure A.2. Sister SU (10mM)-treated conditions were added to address the requirement of Src activity. For each condition, cells were counted in 4 1mm^2 grids of a hemocytometer. Data were quantitated from five experiments. * Difference is significant between overexpressed and endogenous samples of the same treatment condition. ^ Difference is significant from Endogenous BCAR3, E_2. # Significant difference between Endogenous BCAR3, E2 and Endogenous BCAR3, E2 + SU 6656. ** Difference is significant between overexpressed (gray bars) samples and the corresponding treatment condition plus SU 6656 (gray, striped bars).

Figure A.3

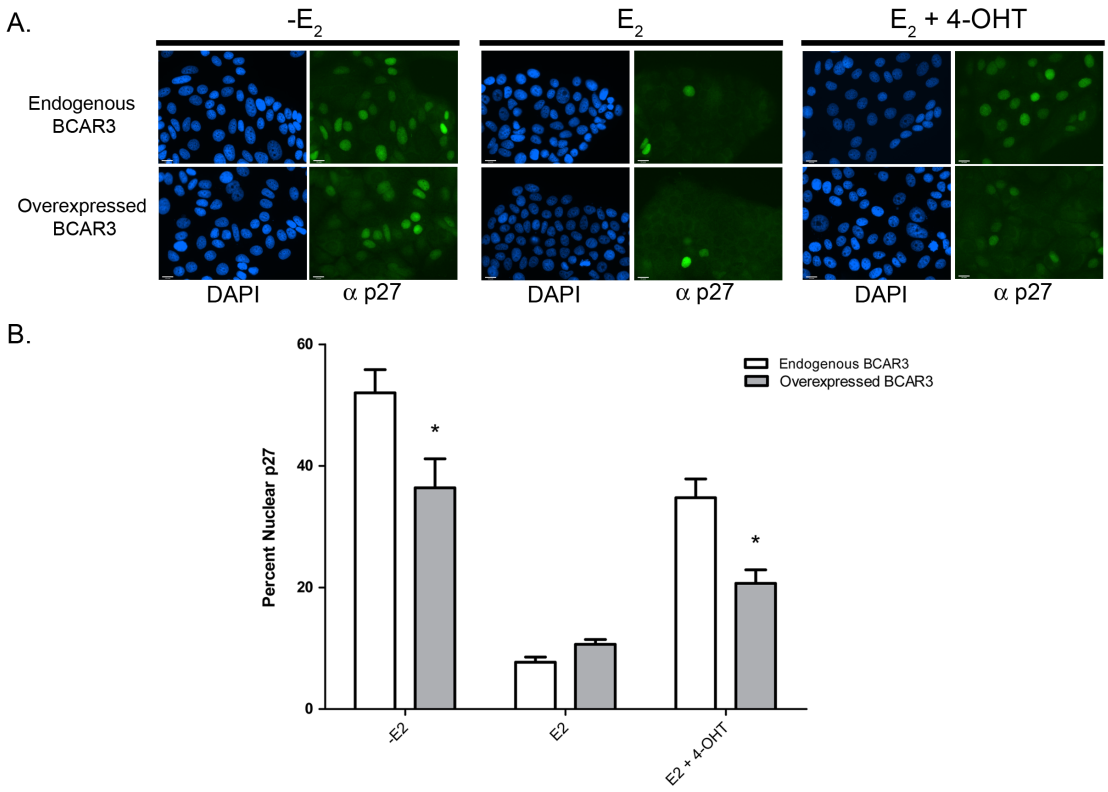


Figure A.3: BCAR3 overexpression reduces p27 nuclear localization following estrogen deprivation and tamoxifen treatment.

A. Immunofluorescence staining of p27. 72-hour growth assays were performed and coverslips from each condition were collected, fixed, permeabilized, stained with anti-p27 antibody, and mounted in Prolong Gold plus DAPI. Anti-p27 was used at a 1:500 dilution, and goat anti-mouse FITC-conjugated secondary used at 1:500. **B.** Quantitated data, n = 6.

A.3.2 – *The Effects of Doxycycline on Cell Proliferation*

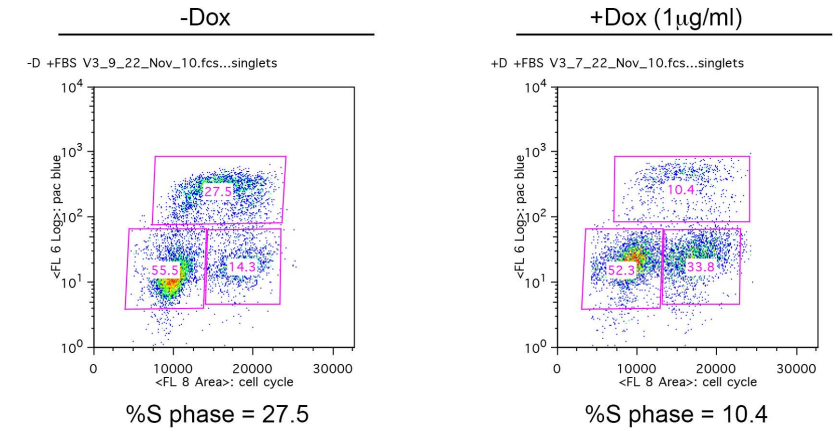
One major control lacking in the experiments described in the preceding section is the inclusion of samples to determine the potential effects of Dox alone on cell growth. To do so, MCF7 cells stably expressing empty vector should have been included for each condition of the growth assays described above. However, during work focused on the mechanism of Cas-mediated resistance to Adriamycin, a former graduate student, Brianne Ray, did include these empty vector-expressing cells in growth assays and found that concentrations of Dox as low as 0.25 μg/ml cause a dramatic reduction in cell proliferation (Fig. A.4). Briefly, vector MCF7 cells, V3S3, were cultured in the presence or absence of 1μg/ml or 0.25μg/ml Dox for 48 hours and incubated with the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU; 10μM) for the final 3 hours. Similar to BrdU, EdU allows for the detection of proliferating cells, and in this assay total DNA was dyed with CellCycle 633-red. Figure A.4 shows that treatment with Dox caused a 50 to 60% reduction in the percentage of cells in S phase following 0.25μg/ml and 1μg/ml Dox treatment, respectively.

As these results suggested that the positive effects on cell growth attributed to BCAR3 in the above section were likely to have been due to the negative effects of Dox, we next performed the growth assay using the MCF7 vector expressing clone, V3S2, as the control in place of Dox-treated cells. If the estrogen independence and tamoxifen resistance displayed by BCAR3 overexpressing cells is a genuine phenotype, than an increase in the percentage of cells in S phase should be observed in BCAR3 overexpressing cells compared

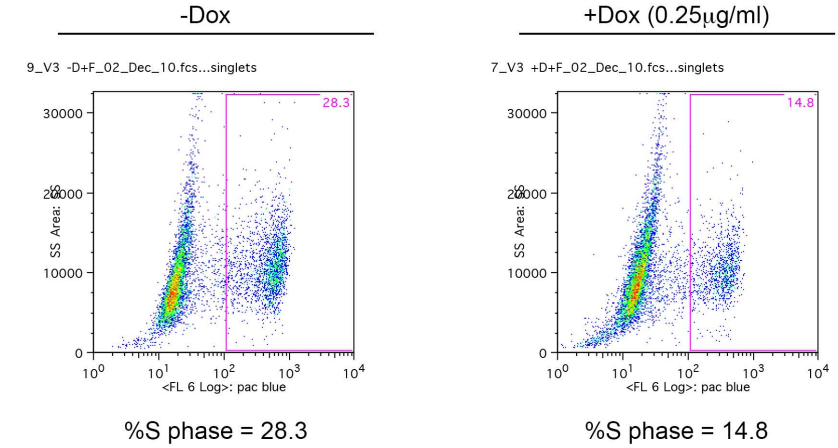
to the V3S2 controls in estrogen-deprived and tamoxifen-treated conditions. However, as shown in Figure A.5, no such result was found. This result, together with the observation that Dox causes a 2-fold reduction in cell proliferation, demonstrated that the BCAR3-mediated effects on cell growth shown in Figure A.1 were likely due to the deleterious effects of Dox. Given this, the decision to end this project was made.

Figure A.4

A.



B.



C.

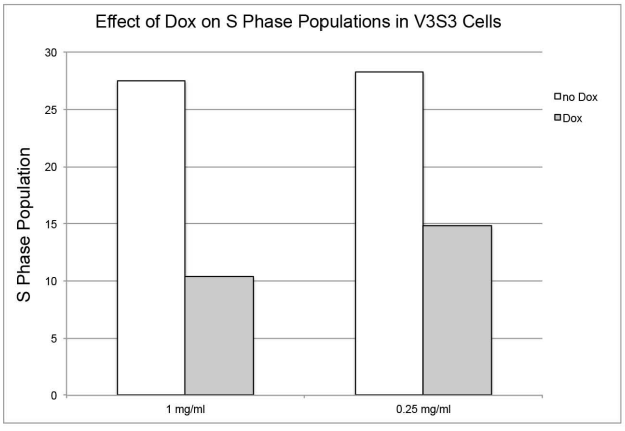
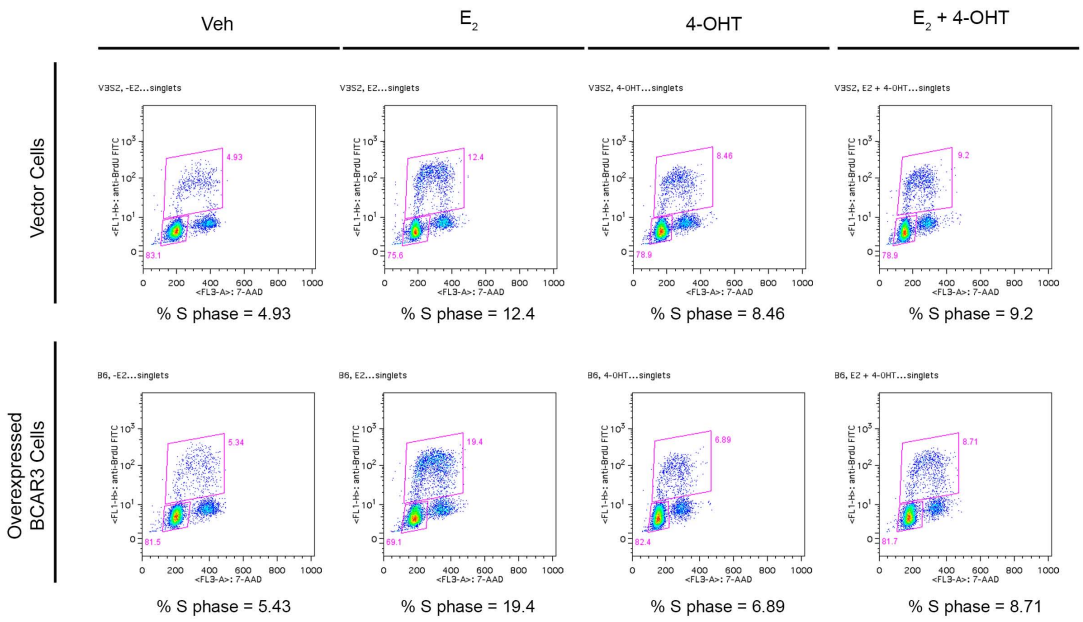


Figure A.4: The effects of doxycycline on proliferation.

A. 1μg/ml Dox treatment causes a 60% reduction in the population of S phase cells. Vector expressing V3S3 cells were cultured in the presence or absence of 1μg/ml Dox for 48 hours. EdU added to a final concentration of 10μM for 3 hours and cells were collected and stained with anti-EdU and the DNA dye CellCycle 633-red. Cell cycle profiles were analyzed by flow cytometry. **B.** 0.25μg/ml Dox treatment causes a 50% reduction in the population of S phase cells. Cells treated an analyzed as described in A. **C.** Graphical representation of data in A. and B. Experiments shown here are representative of experiments done by Brianne Ray.

Figure A.5

A.


Veh
E2
4-OHT
E2 + 4-OHT
Vector Cells
Overexpressed BCAR3 Cells
% S phase = 4.93
% S phase = 12.4
% S phase = 8.46
% S phase = 9.2
% S phase = 5.43
% S phase = 19.4
% S phase = 6.89
% S phase = 8.71

B.

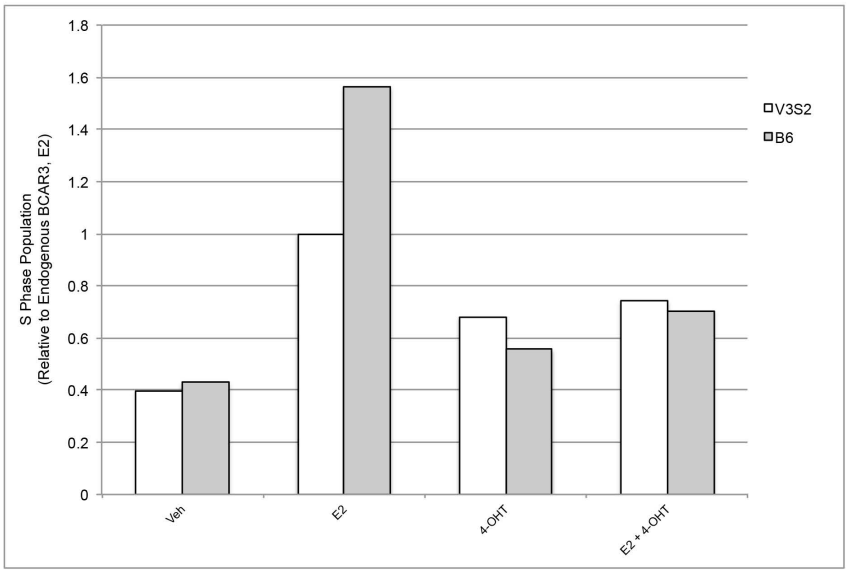

S Phase Population
(Relative to Endogenous BCAR3, E2)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
V3S2
B6
Veh
E2
4-OHT
E2 + 4-OHT

Figure A.5: BCAR3 overexpressing MCF7 cell clones are not estrogen independent or resistant to tamoxifen.

A. No differences in response to estrogen-deprivation (Veh), estrogen stimulation (E2), tamoxifen treatment (4-OHT), or treatment with estrogen plus tamoxifen (E2 + 4-OHT) were observed between vector expressing and BCAR3-overexpressing MCF7 cells. 72-hour growth assay was performed and analyzed as previously described. **B.** Graphical representation of data from A, n = 1.

A.4 – Conclusions and Perspectives

The findings presented in this appendix serve as an example of the importance of well-controlled experiments and a thorough knowledge of all reagents used, so as to be aware of any potential side effects. Surprisingly, despite its routine use in Tet-regulated systems, the anti-proliferative effects of Dox in breast cancer cell lines had been previously demonstrated almost 20 years ago (Fife and Sledge 1995).

As BCAR3-mediated antiestrogen resistance has been reported in several papers, using multiple cell lines, and by two independent groups (van Agthoven, van Agthoven et al. 1998, Cai, Iyer et al. 2003, Felekkis, Narsimhan et al. 2005), the inability of BCAR3 to induce resistance in our experiments is likely due to an issue with the cell lines used (MCF7 Tet-off) or to the specific clones isolated during the generation of stably transduced cells. Indeed, many of the vector clones display irregular growth patterns compared to parental cells (*lab communication*). Moreover, experiments demonstrating BCAR3-mediated resistance in the literature report such findings only after 9 to 12 days of culture in the presence of antiestrogens. While we did see significant increases in cell proliferation in our BCAR3 overexpressing cells following 6 days of tamoxifen treatment, these experiments were never followed up on after the anti-proliferative effects of Dox were revealed. It is very possible that BCAR3 confers a proliferative advantage that is only detectable following prolonged periods of challenged growth. Such that, at early time points following drug treatment these

cells arrest, yet are able to promote survival and then sustain proliferation while cells expressing endogenous levels of BCAR3 cannot.

If this project were to be begun again, the use of Tet-off cells is not advisable nor is the use of short-term (72 hour) growth assays. While it was demonstrated that 72 hours is sufficient to observe the effects of estrogen-deprivation and antiestrogen treatment in estrogen-dependent, ER-positive cell lines, it may not be optimal to detect the proliferative advantage conferred through BCAR3 overexpression. Lastly, the dependence on Src and the observed difference in p27 localization may be key elements in the mechanism of BCAR3-mediated resistance and therefore remain interesting questions to be addressed.

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