2008

# Characterization of SUDS3 as a BRMS1 Family member in Breast Cancer

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**A DISSERTATION**

**Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfilment of the requirements for the degree of Doctor of Philosophy**

**BIRMINGHAM, ALABAMA**

**2008**

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## ABSTRACT

BRMS1 and SUDS3 belong to a protein family characterized by the Sds3-like domain. These proteins are core members of SIN3-HDAC chromatin remodeling complexes and thus, regulate transcription.

BRMS1 decreases the expression of various metastasis-promoting proteins. This regulation contributes to BRMS1-mediated metastasis suppression through the inhibition of motility and by sensitizing cells to anoikis, as well as through other unidentified mechanisms related to growth at a secondary site.

Although BRMS1 and SUDS3 share high sequence similarity and are found in the same complexes, studies have yet to address whether these similarities result in overlapping functions. Additionally, it is unclear how BRMS1 and SUDS3 interact with each other and with SIN3-HDAC complexes to affect transcription. The studies presented here were undertaken to characterize BRMS1 and SUDS3 as family members and, moreover, to define their interaction.

We therefore sought to determine if SUDS3 over-expression could compensate for BRMS1 deficiency in breast cancer cells. We found SUDS3, unlike BRMS1, to be present in all breast cancer cell lines regardless of metastatic potential. Further, SUDS3

over-expression could not compensate for BRMS1 deficiency to effect metastasis suppression, decreased motility, increased anoikis, or decreased EGFR, OPN, and activated Akt.

We also sought to characterize the region of BRMS1 necessary for interaction with SUDS3. Mutational studies showed the first coiled-coil domain of BRMS1 to be necessary but not sufficient for interaction with SUDS3 and that a predicted imperfect leucine-zipper does not play a role in this interaction.

These studies increase the knowledge of the Sds3-like protein family by defining distinct, non-overlapping functions for BRMS1 and SUDS3 in breast cancer cells. Further, they contribute to the understanding of protein-protein interactions involved in the composition of SIN3-HDAC complexes by suggesting that BRMS1 and SUDS3 serve distinct functions within these complexes and by further defining how these two proteins interact. By understanding the role of this protein family and how these SIN3-HDAC complexes are assembled, we can begin to elucidate the normal roles of these proteins and complexes and further, how they are involved in pathological processes, especially metastasis.

## DEDICATION

This work is dedicated to my family - a rare model of strength, love, and compassion. To my father and mother, Manuel and Noemia Silveira, who have worked tirelessly to provide my sister and I with the best opportunities to advance our education and the best example of how to better the lives of those around us. To my sister, Andrea, who inspires me with her enthusiasm for all things beautiful and joyful. To my extended family, for their extraordinary involvement in my life and the caring and support they give. I am extraordinarily blessed and recognize that all these blessings come from God.

## ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor, Dr. Danny Welch. Dan has supported my scientific and personal growth these many years and provided an enriching work environment. Dan is an exemplary scientist and it was my honor to train with him.

Secondly, I would like to acknowledge the valuable contributions of all the Welch lab members, both past and present. I am especially thankful to Dr. Douglas Hurst who has shared his enthusiasm and special gift for science. Thanks also to Dr. Kedar Vaidya and Dr. Yi Xie who contributed their expertise and assistance on several projects. Finally, I would like to acknowledge all those who have bolstered me through difficult times and supported me with their friendship: Dr. Pushkar Phadke, Dr. Kevin Nash, Dr. Joe Stafford, Dr. Caroline Mills, Thomas Bodenstine, Mick Edmonds, Leah Cook, and Alka Mehta.

I am grateful to the students, faculty, staff, and members of the UAB Molecular and Cellular Pathology Division and additionally, the entire UAB scientific community, which has fostered learning and collaboration. I am especially grateful to Dr. Carrie Elzie and Dr. LaWanda Holland for their many insights and friendship.

I would also like to acknowledge the members of my foster family, Rachel Penton, Eva Clark, Nicole Brossier, Christine Sestero, Colin Farrell, Porter Lillis, and Melinda Merrell, who, as fellow scientists, have offered enthusiasm, constructive criticisms, and sympathetic ears.

Finally, I would like to thank the members of this committee, Dr. Andra Frost, Dr. Coral Lamartiniere, Dr. Robert Hardy, Dr. Joanne Murphy-Ullrich, and a former committee member, Dr. Jeffrey Kudlow, for their support, invaluable assistance, and guidance.

## TABLE OF CONTENTS

*Page*

## LIST OF FIGURES

*Figure* *Page*

# INTRODUCTION

## Breast Cancer Metastasis

In 2007, the American Cancer Society reported that approximately one in eight women in the United States will develop breast cancer and that approximately 40,000 women will die from this same disease (Jemal et al., 2007). The majority of these deaths will be due to metastasis, the formation of progressively growing tumors at sites discontinuous from the primary lesion (Stafford et al., 2008). In cases where breast cancer remains localized, the five year survival rate is 98%; however, when a breast cancer has metastasized the five year survival rate drops to 26% (Jemal et al., 2007). The statistics are clear indicators that the treatments that can successfully target a primary tumor are less effective at the treatment of metastases.

The multi-step process of metastasis is often referred to as "the metastatic cascade" because cells must sequentially complete every step in order to form a progressively growing secondary tumor (Chambers et al., 2002; Stafford et al., 2008). The steps required to complete the metastatic cascade are mostly the same for all carcinomas. The cascade begins with tumorigenesis: The genetic clonality model (Nowell, 1976) proposes that tumors are formed when a single cell is transformed through the accumulation of mutations including the dominant gain of function of oncogenes and the recessive loss of function of tumor suppressor genes (Fearon and Vogelstein, 1990; Vogelstein et al., 1988), an event known as loss of heterozygosity

(Donahue et al., 2006). The mutations disrupt cellular homeostasis and cell cycle regulation resulting in deregulated cellular proliferation and eventual tumor formation (Langley and Fidler, 2007). Deregulated cellular growth, division, and proliferation results in further chromosomal instability and mutations resulting in a heterogenous tumor cell population.

Contributing to tumor heterogeneity are selective pressures to which tumor cells are continuously subjected including immune cell response (Langley and Fidler, 2007), nutrient deprivation (Hanahan and Weinberg, 2000), and hypoxia (Harris, 2002). Cells that survive these selective pressures exhibit the following hallmarks: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, sustained angiogenesis, and invasion (Abraham et al., 2000).

Important to tumor growth and survival is the interaction with the surrounding stroma and immune cell infiltrate. Tumor cells are able to remodel the surrounding stroma through cell-stroma interaction and the secretion of enzymatic factors (Woodhouse et al., 1997). Stromal remodeling results in the release of growth factors that stimulate motility and invasion. These processes can be further stimulated by autocrine factors released by the tumor cells.

A cancer is defined as malignant once tumor cells have penetrated and invaded through a basement membrane. Once through the basement membrane, cells spread through the lymphatics and/or enter into the vasculature (Langley and Fidler, 2007). In the vasculature, cells must survive shear forces and evade immune detection. Cells circulate and finally arrest in the vasculature due to size restriction, extravasate from the

blood vessel, and enter the secondary tissue. There cells can die, become dormant, or proliferate to form micrometastases (Chambers et al., 2002). Once established, micrometastases can become progressively growing, vascularized tumors thereby completing the metastatic cascade.

Growth at these secondary sites is organ specific. For example, breast cancer metastasizes almost exclusively to lymph nodes, lung, brain, bone, and liver (Chambers et al., 2002). Evidence exists supporting several different theories regarding organ specificity. One such theory proposed by James Ewing is that site specificity is a function of circulation: Cells leaving a tumor will arrest in the first capillaries encountered. Another theory is Paget's seed and soil hypothesis, which states that the ability of a cell to grow at a secondary site is dependent on that site (Paget, 1889). There is evidence to support both theories and/or that mechanical factors and seed-soil interaction both play a role in organ specificity, nonetheless, the exact molecular basis for secondary site specificity is not well understood (Chambers et al., 2002).

A second aspect of organ specific metastasis is the ability of cancerous cells to survive, proliferate, and sustain angiogenesis in foreign tissues. At these sites, the cells must continue to evade immune detection, interact with the surrounding stroma, and proliferate in a foreign milieu of growth factors and matrix. The ability of the cells to successfully grow at the secondary site to form progressively growing secondary tumors is the defining step of metastasis (Chambers et al., 2002).

While much is known about the steps involved in the metastatic cascade, the complicated molecular mechanisms involved in metastasis have yet to be well-

characterized. Much of the knowledge derived from the study of primary tumor formation can be applied to the study of metastasis; however, there also exist unique pressures that metastasizing cells must survive. Unlike primary tumor cells, cells that are successfully metastatic must survive travel through the vasculature and then grow in a foreign microenvironment.

## Metastasis Suppressors

The metastatic process is highly inefficient (Luzzi et al., 1998; Liotta and Kohn, 2003). Within the heterogenous population of a tumor, only certain cells are able to acquire characteristics which allow them to successfully complete the metastatic process. The secondary tumors formed by these cells are molecularly distinguishable from primary tumors as demonstrated by several microarray studies (Vecchi et al., 2008). Functional studies using cell culture and xenograft models have identified genes that are causally important for metastasis promotion or suppression (Jacinto et al., 2007). Analogous to classical tumor suppressors and oncogenes, these metastasis-associated proteins act in concert to mediate the complicated process of metastasis (Eyol et al., 2008). The metastasis specific function of these genes suggests regulatory pathways that are unique to metastatic cells and distinguishable from pathways that are involved in tumor growth at both primary and secondary sites.

A practical means to the study of metastatic pathways and further, of metastasis suppression, is the study of metastasis suppressors. Metastasis supressors are genes that suppress metastasis without blocking primary tumor growth (Stafford et al., 2008). While

several candidate genes have been identified, only the following have been functionally validated as metastasis suppressors through experiments using *in vivo* models of metastasis: Cadherin-11, Caspase-8, CD44, Claudin-1, Claudin-4, CRSP3, Connective tissue growth factor, Gelsolin, E-cadherin, N-cadherin, KAI1, KISS1, RECK, RhoGD12, Src-suppressed C kinase substrate, JNKK1/MKK4, MKK6, MKK7, Nm23-H1, RKIP, BRMS1, Drg-1, and TXNIP (Rinker-Schaeffer et al., 2006; Stafford et al., 2008).

It is important to stress that the metastasis suppressing function of a gene can only be confirmed through *in vivo* models of metastasis as current *in vitro* assays do not sufficiently model the complexity of the process (Rinker-Schaeffer et al., 2006; Naito et al., 1987). Genes experimentally validated as metastasis suppressors have further been shown to be significantly reduced or missing in advanced cancers by immunohistochemical and RNA analysis of clinical samples (Stafford et al., 2008). Several mechanisms are implicated in the loss of metastasis suppressors including genetic mutations, chromosomal abnormalities, epigenetic silencing, and differential protein expression (Shevde and Welch, 2003).

The role of metastasis suppressors is context dependent: Suppressors differ in cancer type and mode of action. Several metastasis suppressors, including BRMS1, can suppress the metastasis of multiple types of cancer (Shevde and Welch, 2003). Metastasis suppressors also vary in that some inhibit at a single step of the metastatic cascade while others, like BRMS1, inhibit at several steps (Phadke et al., 2008). The complicated multi-step, cancer-type specific nature of metastasis suppressors adds to the complexity of defining specific mechanisms of action for these proteins.

In addition, as these metastasis suppressors have mostly been identified through functional studies in the pathological process of metastasis, there is limited knowledge concerning their normal role in the cell and the exact mechanism(s) by which they suppress metastasis. The list of known suppressors includes genes and gene families that are involved in signal transduction, intercellular communication, adhesion, cytoskeletal regulation, motility, and tissue remodeling (Rinker-Schaeffer et al., 2006). Increasing study, and cross-talk between several areas of research have increased the body of knowledge concerning the known functions of these suppressors.

## Breast Cancer Metastasis Suppressor 1

Breast Cancer Metastasis Suppressor 1 (BRMS1) was first identified by differential display comparing MDA-MB-435 breast cancer cells containing chromosome 11 to their parental, metastatic counterparts (Phillips et al., 1996; Seraj et al., 2000). Since then, BRMS1 has been experimentally validated as a metastasis suppressor in both human and murine breast cancer and melanoma, as well as in ovarian cancers (Meehan and Welch, 2003). Moreover, BRMS1 expression has been correlated with loss of metastatic competency in pheochromocytomas (Ohta et al., 2005), non-small cell lung carcinoma (NSCLC) (Liu et al., 2006), and a cell line model of bladder cancer progression (Seraj et al., 2001). The mechanism(s) by which BRMS1 suppresses metastasis has yet to be identified; however, BRMS1 has been shown to regulate the expression of several proteins associated with tumor progression and metastasis (Cicek et al., 2004; Rivera et al., 2007; Champine et al., 2007; Rivera et al., 2007). Further, experimental evidence suggests that

the ability of BRMS1 to interact with transcriptional regulating protein complexes might be involved in BRMS1-mediated metastasis suppression (Meehan et al., 2004).

### *Structure and Regulation of BRMS1*

*BRMS1* is located on the q arm of chromosome 11, a region that is lost in 40-65% of late stage breast cancers. Specifically, fluorescence *in situ* hybridization experiments mapped the BRMS1 gene to 11q13.1-q13.2. The gene is spread over 8.5 kb and is comprised of 10 exons the first of which is not translated (Seraj et al., 2000). Full-length BRMS1 is encoded by 738 nucleotides and recently mRNA splice variants have been identified, however, it is not known whether these variants are translated into protein (Hurst, DR, et al., in preparation).

BRMS1 protein is composed of 246 amino acids with a predicted molecular weight of 28.5 kDa, although the protein migrates at a molecular weight of approximately 35 kDa in denaturing polyacrylamide gel electrophoresis. Subcellular fractionation and immunofluorescence studies have demonstrated that BRMS1 is predominantly (>90%) nuclear (Samant et al., 2000b). Northern blotting and immunoblotting of tissue lysates show ubiquitous expression in all human tissues examined (Seraj et al., 2000). The protein is evolutionarily conserved: BRMS1 has orthologs or partial orthologs in mouse, rat, dog, and cow and homology to ESTs in *Droshophila* and pig. The mouse ortholog of BRMS1 is 95% homologous at the amino acid level and has also been shown to suppress metastasis in mouse models of breast cancer and melanoma (Samant et al., 2002; Samant et al., 2006).

The amino terminus of BRMS1 contains a glutamic acid-rich region of unknown function. Amino acid sequence analysis of BRMS1 predicts two nuclear localization sequences (NLS) at the carboxy terminus of the protein from amino acids 198-205 and 239-245 (Seraj et al., 2000). Deletion of the first nuclear localization sequence abrogates the ability of BRMS1 to localize to the nucleus as assessed by both subcellular fractionation and immunofluorescence studies (Hurst, DR, et al., in preparation). BRMS1 also contains multiple regions that are implicated in protein-protein interaction including two coiled-coil domains at amino acids 51-81 and 147-180 and several imperfect leucine zipper motifs at amino acids 67-88, 131-152, 138-159, 153-174 (Seraj et al., 2000). Yeast two-hybrid studies have demonstrated at least two proteins, SUDS3 and ARID4A (Meehan et al., 2004), that interact with BRMS1 in predicted protein-protein interaction domains (Hurst et al., 2008).

Amino acids 69-110 were initially described by the Sanger Institute as the Sds3-like region and encode a domain shared by SUDS3 and BRMS1, thereby establishing BRMS1 and SUDS3 as protein family members. This region represents a highly conserved domain of unknown function present among other members of the BRMS1 protein family including BRMS1, SUDS3, BRMS1-homolog protein (p40), their orthologs, and the yeast specific protein Dep1 (Finn et al., 2008). Recently, the definition of this protein family has been updated to define the region of BRMS1 from amino acids 52-223 as the Sds3-like domain, a change reflecting the fact that the entirety of the BRMS1 protein shares 23% identity and 49% similarity with SUDS3 (Meehan et al., 2004) (Figure 1).

Little is known about BRMS1 regulation; however, BRMS1 is stabilized by interaction with the chaperone protein, Hsp90, and is further regulated by proteasomal degradation (Hurst et al., 2006). BRMS1 co-immunoprecipitates with the two chaperone proteins Hsp70 and DANJB6, however the importance of these for BRMS1 regulation has yet to be investigated (Hurst et al., 2006). Loss of BRMS1 in breast cancer is suggested to be mediated by epigenetic silencing, specifically promoter methylation (Shevde et al., 2006).

***BRMS1 Metastasis Suppression***

BRMS1 was initially identified as a metastasis suppressor using the BRMS1 deficient human breast cancer cell lines MDA-MB-435 and MDA-MB-231 (Seraj et al., 2000). Stable ectopic expression of BRMS1 suppressed the ability of these cells to metastasize to the lung and lymph nodes in *in vivo* spontaneous and experimental models of metastasis respectively. Further studies used these cell lines in a cardiac injection model to find that BRMS1 can also suppress metastasis to bone, brain, pancreas, and adrenal tissues (Phadke et al., 2008).

Analogous studies were done using the murine form of BRMS1 in the murine mammary carcinoma cell lines 66cl4 and 4T1 (Samant et al., 2006). BRMS1 transfected cells were injected either orthopically or intravenously into syngeneic BALB/c mice and similarly showed reduced colonization of the lungs, thus demonstrating that BRMS1 can suppress metastasis in the presence of an intact immune system. BRMS1 also significantly suppressed metastasis of the melanoma cell lines MelJuSo and C8161.9


**Figure 1: Sds3-like Domain is Shared Among BRMS1 Family Members**

Alignment of the mammalian proteins BRMS1, SUDS3, and BRMS1-like (p40) shows high homology as defined by both identical and similar amino acids. The originally defined Sds3-like domain is high-lighted in dark gray. The newly defined Sds3-like domain encompasses a larger region of each protein and is highlighted in light gray.

(Shevde et al., 2002) as well as the ovarian cancer cell line HO-8910PM (Zhang et al., 2006a).

To date, a single study has comprehensively looked at levels of BRMS1 protein in breast cancer patient samples to find loss of BRMS1 in nearly 25% of 238 breast cancer cases examined. Further, loss of BRMS1 correlated with reduced disease-free survival when stratified by loss of estrogen receptor, progesterone receptor, or Her2 over-expression (Hicks et al., 2006). A second study examined a single tumor sample of each of the four different stages of human NSCLC to find reduced levels of BRMS1 protein and mRNA as compared to adjacent non-cancerous tissues suggesting that BRMS1 may also play a role in non-small cell lung carcinoma (Liu et al., 2006) however, BRMS1 has yet to be definitively shown as a NSCLC metastasis suppressor.

Studies looking exclusively at mRNA levels of BRMS1 in breast cancer samples are not as conclusive. This may be, in part, because BRMS1 is not only regulated at the RNA level but also at the protein level by interaction with Hsp90 and through the process of proteasomal degradation (Hurst et al., 2006). In one study, high levels of BRMS1 mRNA in primary breast carcinomas were associated with reduced disease-free survival (Lombardi et al., 2006); however, it is important to note that the RNA used was contaminated with stromal RNA. Conversely, two other studies demonstrated that decreased levels of BRMS1 correlated with reduced disease-free survival and cancer progression respectively (Hicks et al., 2006; Zhang et al., 2006b). Reduced levels of BRMS1 mRNA have also been shown in breast cancer derived brain metastases (Stark et al., 2004).

Reduced BRMS1 mRNA is linked to other cancers in clinical studies. BRMS1 RNA was significantly decreased in malignant pheochromocytoma as compared to benign pheochromocytoma (Ohta et al., 2005). Primary ovarian epithelial carcinomas had lower levels of BRMS1 compared to normal ovarian cells and benign tumors and this study further demonstrated an inverse link between BRMS1 levels and cancer progression (Zhang et al., 2006a).

### *Mechanistic Insights Into BRMS1 Metastasis Suppression*

In order to determine the step(s) in the metastatic cascade at which BRMS1 inhibits metastasis, several studies were undertaken using *in vitro* techniques that model the individual steps of the metastatic cascade including proliferation, evasion of apoptosis, motility, extracellular remodeling, invasion, adhesion, and cell-to-cell communication. While these *in vitro* assays cannot sufficiently model the complexities of metastasis, nor predict metastatic competency, they were undertaken in hopes of finding BRMS1-mediated phenotypes. BRMS1 transfected MDA-MB-435 and MDA-MB-231 cells showed no difference in *in vitro* morphology, growth rates, invasion, and adhesion to extracellular matrix components, nor in the expression and activation of matrix metalloproteinases -2 and -9 when compared to parental cells (Samant et al., 2001b). However, there was a 30-60% decrease in motility for both cell lines.

Studies done using the melanoma cell lines MelJuSo and C8161.9 similarly showed that *in vitro* morphology and growth rate for BRMS1 transfectants were unaffected; however, BRMS1 transfectants were less invasive in a collagen sandwich

assay (Shevde et al., 2002). Ectopic BRMS1 expression in the ovarian cancer cell line HO-8910PM decreased cell motility, invasion, and adhesion to the extracellular matrix components laminin and collagen IV (Zhang et al., 2006a). Taken together, these studies suggest that inhibition of motility and invasion may play roles in BRMS1-mediated metastasis suppression.

Several studies have also shown that BRMS1 plays a role in the induction of apoptosis. Over-expression of BRMS1 in both MDA-MB-435 breast cancer cells and H1299 human lung carcinoma cells resulted in suppressed growth in soft agar (Samant et al., 2001b; Liu et al., 2006). BRMS1 over-expression sensitized tumorigenic but non-metastatic Human Embryonic Kidney (HEK293T) and H1299NSCLC cells to tumor necrosis factor (TNF)-induced apoptosis and also increased sensitivity to anoikis (Prosser et al., 1990). Similarly, BRMS1 expressing MDA-MB-435 and MDA-MB-231 cells showed a greater susceptibility to anoikis when plated on poly-hydroxyethyl methacrylate cell culture dishes (Wallich et al., 1985).

To further assess at which step(s) BRMS1 inhibits metastasis *in vivo* and to confirm *in vitro* findings, MDA-MB-435 and MDA-MB-231 cells were stably transfected to generate cell lines that expressed enhanced green fluorescent protein and BRMS1 or a control vector. These cells were injected intracardiac into the left ventricle of the heart in mice. Assessment of the secondary tissues showed that BRMS1 inhibited both the number of cells that reached the secondary site and the growth of colonies from cells that were able to seed there (Phadke et al., 2008). This study established the ability of BRMS1 to suppress at multiple steps of the metastatic cascade following entry into the vasculature

and, moreover, confirmed *in vitro* findings that BRMS1 sensitizes cells to apoptosis. Further studies are needed to determine whether BRMS1 inhibits steps preceding the entry of tumor cells into the vasculature.

### *Molecular Mechanism(s) of BRMS1 Metastasis Suppression*

While the exact mechanism(s) of BRMS1 suppression has yet to be determined, several studies have examined the effects of BRMS1 expression on cancer-related proteins and cellular phenotypes. BRMS1 has been shown to restore homotypic gap junctional intercellular communication in MDA-MB-435 and MDA-MB-231 breast cancer cells and MelJuSo and C8161.9 melanoma cells by regulating the expression of connexins 43 and 32 (Samant et al., 2000a). A break down in gap junctional intercellular communication has been correlated with tumorigenesis (McLachlan et al., 2007). Further, ectopic expression of BRMS1 resulted in decreased expression of fascin (Zhang et al., 2006a), an actin-bundling protein associated with cell motility, and of urokinase-type plasminogen activator (UPA), a key mediator of extracellular remodeling (Cicek et al., 2005).

BRMS1 also regulates levels of osteopontin (OPN), a secreted phosphoglycoprotein that is strongly associated with tumor progression and aggressive cancers (Hedley et al., 2008; Samant et al., 2007). Ectopic expression of BRMS1 in MDA-MB-435 breast cancer cells decreased levels of OPN mRNA 90-95% and significantly decreased both intracellular and secreted protein (Hedley et al., 2008; Samant et al., 2007; Hurst et al., 2008). OPN was proven to play a part in BRMS1-mediated suppression of metastasis: Re-expression of OPN in MDA-MB-435-BRMS1 cells resulted

in increased incidence of spontaneous metastasis to lymph nodes and lungs in a mouse model of breast cancer (Hedley et al., 2008). Results from the same study suggested that BRMS1 down-regulation of OPN serves to sensitize cells to stress induced apoptosis including hypoxia-induced apoptosis and anoikis. BRMS1 further regulates OPN signaling by resulting in decreased surface expression of the integrins αvβ3 and αvβ5, both of which act as receptors for osteopontin.

Several key members of the EGFR-PI3K-Akt pathway cell survival pathway are down-regulated by BRMS1. The disregulation of this pathway has been well-characterized in several cancers (Vivanco and Sawyers, 2002). Ectopic expression of BRMS1 reduced phosphoinositide signaling, specifically levels of phosphatidylinositol-4,5)-bisphosphate, in BRMS1 transfected MDA-MB-435 and MDA-MB-231 cells resulting in decreased mobilization of intracellular calcium, a known regulator of metastasis, and decreased signalling through the PI3K-Akt pathway (DeWald et al., 2005). Further, over-expression of BRMS1 in both the MDA-MB-435 and MDA-MB-231 breast cancer cell lines resulted in a dramatic decrease in epidermal growth factor receptor (EGFR) (Vaidya et al., 2008) and decreased phosphorylation and activation of Akt as a result of decreased signalling. Finally, protein analysis of MDA-MB-435 cells stably expressing BRMS1 showed the down-regulation of annexin I, also known as lipocortin I, a known substrate of EGFR (Cicek et al., 2004).

It is important to note that OPN can also activate the PI3K-Akt pathway and further, the binding of OPN to αvβ3 can cause transactivation of EGFR (Bellahcene et al., 2008). BRMS1 down-regulation of multiple members of this pathway not only confirms

the importance of this pathway as a metastasis-promoting pathway but also suggests that BRMS1 can suppress metastasis by being a global regulator of this pathway: Studies are currently underway to determine which changes are necessary for BRMS1-mediated metastasis suppression.

The mechanism by which BRMS1 mediates these molecular changes is through the regulation of transcription. BRMS1 has been found to interact with several different proteins and protein complexes that regulate transcription, most notably with the transcription factor nuclear factor kappaB (NFκB) (Liu et al., 2006; Cicek et al., 2005; Samant et al., 2007; Vaidya et al., 2008) and with mammalian SWI-independent 3 (SIN3)-histone deacetylase (HDAC) complexes (Nikolaev et al., 2004; Meehan et al., 2004; Hurst et al., 2008).

### *BRMS1 Transcriptional Regulation via NFκB*

NFκB is a transcription factor that regulates the transcription of cytokines, adhesion molecules, chemokines, growth factors, and anti-apoptotic genes as well as other proteins involved in inflammation (Li and Verma, 2002). Aberrant or constitutively active NFκB has been detected in various malignant cancers, including breast carcinoma, and is associated with tumor invasion and metastasis (Rayet and Gelinas, 1999). An important role for NFκB is further demonstrated by studies where blocking NFκB activity resulted in suppressed tumor growth and metastasis *in vivo* (Fidler, 2002; Chambers et al., 2002; Huang et al., 2001; Huber et al., 2004).

There exist several members of NFκB transcription factor family that can bind

together to form homodimeric or heterdimeric protein complexes. The best studied and most common form is known as NFκB and is a heterodimer composed of the p65 (RelA) and p50 proteins (Rayet and Gelinas, 1999). In normal cells, NFκB is sequestered in the cytoplasm through its interaction with inhibitors of NFκB (IκB). A variety of signals can lead to the phosphorylation and subsequent proteasomal degradation of IκB resulting in the nuclear translocation of NFκB and subsequently NFκB-mediated transcription (Baeuerle et al., 1988; Beg et al., 1992; Thanos and Maniatis, 1995). Post-translational modifications are also important for NFκB functions: Acetylation of p65 is important for its transcriptional activity and DNA binding as well as for impairing its assembly with IκBα and nuclear exportation (Quivy and Van, 2004). Once in the nucleus, NFκB activity is regulated by such transcriptional coactivators as SRC-1, SRC-2 and SRC-3 and transcriptional corepressors that include HDAC1, HDAC2, HDAC3, SMRT, and NcoR (Gao et al., 2005).

BRMS1 function was initially linked to NFκB in studies that found an inverse correlation between BRMS1 expression and NFκB DNA binding activity in the MDA-MB-231 and C8161.9 cell lines, both of which have constitutive NFκB activity (Cicek et al., 2005). BRMS1 led to a reduction in NFκB translocation by inhibiting the phosphorylation and degradation of the NFκB inhibitor, IκBα. Moreover, BRMS1 expression was also shown to inversely correlate with both constitutive and TNF-induced activation of NFκB-dependent uPA expression.

A more direct involvement of BRMS1 in NFκB regulation was demonstrated through reciprocal co-immunoprecipitation studies that showed endogenous interaction of

BRMS1 with the RelA/p65 and p50 subunits of NFκB and direct binding of BRMS1 to the p65 subunit (Liu et al., 2006). BRMS1 interaction with p65 was demonstrated to decrease transactivation of NFκB to the nucleus, most likely due to decreased acetylation of NFκB lysine 310 regulated by the interaction of BRMS1 with HDAC1.

In these studies, BRMS1 was also found to further regulate NFκB transcription in the nucleus: Chromatin immunoprecipitation (ChIP) assays showed that chromatin bound BRMS1 facilitated HDAC1-mediated deacetylation of lysine K310 and subsequent inactivation of NFκB on the promoters of *CIAP-2* and *Bfl-1/A1* in both HEK293T and H1299 NSCLC cells.

To confirm that BRMS1 was indeed suppressing NFκB activity at these sites, real time quantitative analysis was performed in order to measure transcription of NFκB target genes. In both HEK293T and NSCLC cell lines, BRMS1 significantly reduced TNF-induced transcripts of the NFκB regulated anti-apoptotic genes *cIAP-2, Bfl-1/A1, Bcl-xL* and siRNA against BRMS1 restored expression of these genes. Interestingly, BRMS1 did not decrease *ICAM*, *VCAM*, and *E-selectin*, NFkB regulated genes that are also involved in metastasis. This stresses the specificity of BRMS1-containing co-repressor complexes.

While HDAC1 has already been confirmed as an NFκB co-repressor (Gao et al., 2005), these findings were the first to identify BRMS1 as a NFκB co-repressor. It has yet to be determined whether the HDAC complex member, SIN3, is involved in the BRMS1/HDAC1-mediated NF-κB repression within these cells; however, BRMS1 knock-down resulted in concomitant loss of SIN3A on the immunoprecipitated chromatin (Liu et al., 2006). It is interesting to note that knockdown of HDAC1 was unable to completely

abolish BRMS1-mediated RelA/p65 deacetylation or transcription in NFκB transcriptional activity assays suggesting that there are other HDACs that tether BRMS1. These findings indicate that multiple BRMS1/HDAC complexes might be involved in transcriptional regulation.

To date, the direct interaction of BRMS1 and NFκB has yet to be confirmed in breast cancer cells; however, in addition to previous studies, reporter assays have been used to demonstrate the specific BRMS1-mediated repression of NFκB. BRMS1 repressed transcription of a luciferase reporter under the regulation of OPN and EGFR promoters in breast cancer cell lines (Samant et al., 2007; Vaidya et al., 2008). Mutation of the NFκB binding sites within either of these promoters relieved BRMS1-mediated suppression. It has yet to be determined whether these effects were seen due to stabilization of IκBα, cytosolic deacetylation of NFκB, and/or the co-repressor activity of BRMS1 at NFκB binding sites within these promoters. However, data presented in this work suggest that in MDA-MB-231 cells, NFκB is not regulated by direct interaction with BRMS1, as the two proteins do not co-immunoprecipitate.

***BRMS1 Transcriptional Regulation via SIN3-HDAC Complexes***

Several studies show that BRMS1 associates with both Class I and Class II HDACs including HDAC1,-2,-4,-5, and -6 (Meehan et al., 2004; Hurst et al., 2006; Hurst et al., 2008; Nikolaev et al., 2004; Doyon et al., 2006; Le Guezennec et al., 2006; Shiio et al., 2006; Liu et al., 2006). Co-immunoprecipitation studies have further shown BRMS1 to be a core member of SIN3-HDAC chromatin remodeling, transcriptional repression

complexes in both human and murine cell lines (Meehan et al., 2004; Hurst et al., 2006; Hurst et al., 2008; Nikolaev et al., 2004; Doyon et al., 2006; Le Guezennec et al., 2006; Shiio et al., 2006; Liu et al., 2006). Specifically, immunoprecipitation with a BRMS1 specific antibody in mammalian cells showed the association of BRMS1 with the following members of SIN3-HDAC complexes: ARID4A, HDAC1/2, SAP30, SIN3A/B, RBBP4/7, and SUDS3 (Meehan et al., 2004). Moreover, in yeast two-hybrid genetic screens performed with breast, placenta, and prostate libraries, BRMS1 directly bound two of these proteins, SUDS3 and ARID4A (Meehan et al., 2004; Hurst et al., 2006; Hurst et al., 2008).

Of these interactions, the best characterized is that of BRMS1 with ARID4A. Yeast two-hybrid studies showed that the interaction of BRMS1 and ARID4A is mediated by the second coiled-coil domain of BMRS1 and can be abrogated by mutation of a single amino acid, leucine174 (L174), within this region (Hurst et al., 2008). Point mutation of this particular leucine to an aspartic acid ($BRMS1^{L174D}$) disrupted direct interaction of BRMS1 and ARID4A in yeast two-hybrid genetic screens; however, $BRMS1^{L174D}$ could still co-immunoprecipitate ARID4A in mammalian cells suggesting that BRMS1 still interacts with other members of the complex, possibly SUDS3.

Deletion of the first coiled-coil region of BRMS1 resulted in a mutant BRMS1 ($BRMS1^{\Delta CC1}$) that could not co-immunoprecipitate ARID4A in mammalian studies but that could interact with ARID4A in yeast two-hybrid studies. This deletion seems not to have considerably affected protein folding since BRMS1 and ARID4A were still able to interact in yeast, therefore, these studies suggest that the first coiled-coil region of BRMS1

mediates a protein-protein interaction that is critical for the association of BRMS1 and ARID4A.

Surprisingly, while abrogation of the interaction of ARID4A and BRMS1 relieved BRMS1-mediated transcriptional repression, we found that it did not alter the ability of BRMS1 to suppress metastasis, nor to down-regulate EGFR or OPN in both MDA-MB-435 and MDA-MB-231 cell lines. Similarly, deletion of the first coiled-coil region did not alter the ability of BRMS1 to suppress metastasis nor to down-regulate EGFR; however, this deletion did alter the ability of BRMS1 to down-regulate levels of secreted OPN. These data taken together suggest that the first coiled-coil region mediates critical protein-protein interactions that affect complex composition beyond the association of BRMS1 with ARID4A, and, moreover, that affecting complex composition in turn affects BRMS1-mediated phenotypes.

It is also important to stress, that while both BRMS1 mutants still suppressed metastasis, this does not eliminate the possibility that BRMS1-mediated metastasis suppression is through SIN/HDAC complexes. In order to determine the role of these complexes in BRMS1-mediated metastasis, further studies are needed to elucidate the composition of these complexes and the importance of individual protein-protein interactions within these complexes as well as how the complexes are involved in mediating specific BRMS1-associated phenotypes.

## Suppressor of Defective Silencing 3

### *Structure and Regulation of SUDS3*

Human Suppressor of Defective Silencing 3 (SUDS3, also known as Sin-3 Associated Protein 45, SAP45, and mammalian Suppressor of Defective Silencing, mSDS3) is a 328 amino acid protein encoded on chromosome 12q24.2. The protein is predominantly nuclear and migrates at 45 kDa in polyacrylamide gel electrophoresis under denaturing conditions (Alland et al., 2002). Expression arrays show SUDS3 mRNA to be expressed in breast and most other human tissues (Wheeler et al., 2005).

SUDS3 is evolutionarily conserved with orthologs in yeast, *Drosophila melanogaster*, and *mus musculus* (Alland et al., 2002). Human SUDS3 is 99.6% identical to its mouse ortholog; therefore, the results of mouse studies of this protein are expected to correlate with human SUDS3.

Amino acids 63-104 of SUDS3 were initially classified as the Sds3-like domain, a protein family domain that is shared by BRMS1 and BRMS1-like protein (p40) (Finn et al., 2008). Recently, the definition of this domain has been expanded to include amino acids 58-229 of SUDS3. SUDS3 further contains a coiled-coil domain from amino acids 66-171. Yeast two-hybrid genetic screens done using the mouse ortholog of SUDS3 showed residues 188-226 to be necessary for interaction with SIN3B suggesting that this region is the SIN3 interaction domain (SID) (Alland et al., 2002). Further, co-immunoprecipitation studies using the murine ortholog showed amino acids 72-108 to regulate homodimerization of SUDS3 (Li et al., 2004). Both the SID and homodimerization domains have been shown to play a role in SUDS3-mediated

transcriptional repression: Disruption of either the SID or homodimerization domains strongly diminishes the transcriptional repression activity of SUDS3 as measured by luciferase assays (Alland et al., 2002; Li et al., 2004).

Little is known about SUDS3 regulation; however it has been shown to directly interact with the protein p35 through amino acids 1-170 and through this interaction to associate with cyclin-dependent kinase 5 (cdk5) in brain tissue, resulting in phosphorylated SUDS3 (Li et al., 2004). This phosphorylation has been shown to affect transcriptional repression and homodimerization of SUDS3 but not the interaction of SUDS3 with SIN3A or SIN3B. This may be a tissue specific interaction as cdk5 is not ubiquitously expressed.

***SUDS3 Regulates Epigenetic Events in Development and Cellular Proliferation***

Germ-line deletion of SUDS3 showed SUDS3 to play an important part in per-implantation and the normal growth and division of mouse embryonic fibroblasts (David et al., 2003). Deletion of SUDS3 is embryonic lethal; intercrosses of $SUDS3^{+/-}$ mice failed to produce viable $SUDS3^{-/-}$ embryos after E6.5. $SUDS3^{-/-}$ blastocysts were isolated but failed to grow *in vitro* suggesting that SUDS3 plays an essential role in peri-implantation development.

Over time, cultured mouse embryonic fibroblasts (MEF) from $SUDS3^{-/-}$ mice exhibited dramatic growth arrest, diminished survival, a prominent increase in polyploidy, and a high proportion of polynucleated cells as compared to both wild-type and heterozygous MEFs. Fluorescent *in situ* hybridization using a centromere-specific probe

showed that chromosomes remained associated through their centromeric or pericentric regions but not throughout their arms in SUDS3 null cells due to deregulated pericentric-specific histone modifications and failure to recruit HP1α to pericentric domains (David et al., 2003). These data show that SUDS3 is necessary for pericentric heterochromatin formation and normal chromosomal segregation.

These fibroblasts showed the formation of micronuclei, a phenotype associated with transient pharmacological inhibition of histone deacetylases by Trichostatin A, suggesting that mammalian SUDS3 acts like its yeast ortholog to stabilize HDAC complexes. The introduction of a mutant SUDS3 that exhibited reduced SIN3A binding could not rescue cell growth and viability (Heppner, 1984). This suggests that the association of SUDS3 with SIN3A is necessary for the regulation of pericentric heterochromatin formation and normal chromosomal segregation by SUDS3.

***Haploinsufficiency of SUDS3 Associated With Cancer Progression***

Because SUDS3 deficiency results in embryonic lethality, the role of SUDS3 in cancer was investigated using heterozygous SUDS3 mice bred onto a p53 null background (David et al., 2006). These mice displayed an accelerated tumor onset and increased tumor burden. Cells from these tumors displayed marked aneuploidy and increased centromeric association, most likely associated with the involvement of SUDS3 in pericentric heterochromatin segregation. However, presence of a single copy of p53 suppressed accelerated tumorigenesis in these SUDS3 heterozygous mice. It is interesting to note that $SIN3A^{+/-}$ $p53^{-/-}$ mice did not display accelerated tumorigenesis. This suggests

that the effects of SUDS3 on chromosome segregation might be independent of its role in maintenance of the SIN3A complex or perhaps that SIN3B, a SIN3A family member, could compensate for SIN3A deficiency in heterozygous mice. Further, this demonstrates that the stoichiometry of the proteins involved in these SIN3-HDAC complexes regulates complex function.

### *SUDS3 Transcriptional Regulation via SIN3-HDAC Complexes*

The yeast ortholog of SUDS3, *Sds3p*, was first identified in *Saccharomyces cerevisiae* in genetic screens that restored silencing at the HMR mating-type locus in silencing defective mutants, thus implicating its role in transcriptional regulation (Vannier et al., 1996). Both Sin3p and Rpd3p, the yeast orthologs of SIN3A/B and HDAC1/2, also restored silencing at this same locus suggesting the involvement of these three proteins in the same genetic pathway. Later studies showed mutations in Sds3p to rescue LexA-Sin3 mediated transcriptional repression of promoters with LexA binding sites and to co-immunoprecipitate the Sin3p complex (Dorland et al., 2000). Furthermore, Sds3p was found to maintain the physical integrity of the Sin3p/Rpd3p complex and to be necessary for its activity (Lechner et al., 2000). The importance of Sds3p in the Sin3p/Rpd3p complex was confirmed in a study that showed *sds3p* yeast mutants to have the same phenotype as yeast that had both *sin3p* and *rpd3p* mutations (Vannier et al., 2001; Volk et al., 1984).

Similarly, studies have identified SUDS3 as a key component of mammalian SIN3 corepressor complexes. Using the mouse ortholog of SUDS3, it was demonstrated that

SUDS3 co-immunoprecipitates with SIN3B and HDAC1 in mouse brain, kidney, and liver (Alland et al., 2002). The same group found that immunoprecipitation of SUDS3 resulted in reproducible deacetylase activity above background levels showing that SUDS3 co-immunoprecipitates a functional HDAC complex. Co-immunoprecipitation of an active HDAC complex was abrogated by a mouse SUDS3 mutant that did not interact with SIN3B. Therefore, it is thought that, as in yeast, SUDS3 may contribute to the stability and/or activity of SIN3-HDAC complexes as reduced levels of SUDS3 result in decreased HDAC1 activity.

Studies done in human cells show that SIN3A co-immunopreciptiates SUDS3 (Fleischer et al., 2003). Reverse co-immunoprecipitation demonstrated that SUDS3 can co-immunoprecipitate SIN3A as well as other members of the SIN3A complex including HDAC1, HDAC2, SAP130, and SAP180. Moreover, these studies confirmed that SUDS3 can co-immunopreciptate catalytically active HDAC complexes in human cells (Ferguson and Cheng, 1989).

SUDS3 may also regulate transcription independently of SIN3-HDAC complexes. GAL-SUDS3 fusion proteins can repress transcription of a luciferase reporter in the presence of the HDAC inhibitor trichostatin A (Fleischer et al., 2003). This suggests that SUDS3 is able to recruit HDAC-independent co-repressor complexes. These complexes may contain SIN3, as it has been shown that SIN3 can recruit HDAC-independent co-repressor complexes (Yang et al., 2002), or this repression may be mediated by SIN3-independent complexes as fractionation studies and immunoblotting have shown SUDS3 to elute in the absence of SIN3A implicating the presence of SUDS3 in other protein

complexes (Fleischer et al., 2003).

## SIN3/HDAC Chromatin Remodeling Complexes

Sin3p was first identified in a genetic screen investigating mating-type switches in *Saccharomyces cerevisiae* (Sternberg et al., 1987). It was later identified as SDI1 in another mating-type switch screen that also identified SDI2, that would later be known as Rpd3p (Nasmyth et al., 1987). A histone deacetylase function for the Sin3p/Rpd3p complex was first shown by inactivation of the complex and subsequent acetylation of the HO endonuclease locus in yeast, a locus that is important for the mating-type switch. The mammalian homologs of Sin3p, SIN3A and SIN3B, and Rpd3p, HDAC1 and HDAC2, have been shown to also have transcriptional repressive abilities (Borges et al., 2000; Taunton et al., 1996; Yang et al., 1996). These and other interacting proteins assemble to form large protein complexes that regulate a variety of pathways and that are involved in cell cycle regulation, DNA replication, DNA repair, apoptosis, chromatin modifications, and mitochondrial metabolism (Dannenberg et al., 2005).

Reports vary regarding the many proteins involved in SIN3-HDAC complexes however several proteins have consistently been shown to co-immunoprecipitate with Sin3 complexes: HDAC1, HDAC2, Retinoblastoma Binding Proteins 7 and 4 (RBBP7/4, also known as RbAp46/48), 30 kDa SIN3-associated protein (SAP30), 18 kDA SIN3-associated protein (SAP18), AT rich interactive domain 4A (ARID4A, also known as retinoblastoma-binding protein 1, RBBP1), BRMS1, and SUDS3 (Tannenberg et al., 2005; Meehan et al., 2004; Hurst et al., 2006; Hurst et al., 2008; Nikolaev et al., 2004;

Doyon et al., 2006; Le Guezennec et al., 2006; Shiio et al., 2006; Liu et al., 2006). It is generally agreed that the composition of the complex dictates activity.

A key mediator of complex composition is SIN3. SIN3 has been shown to lack DNA binding ability and therefore functions as a co-repressor by interacting with DNA bound transcriptional repressor (Silver stein and Enwall, 2005). More recently, the role of SIN3 has been redefined as a global regulator of transcription as it can both positively and negatively regulate gene expression (Tannenberg et al., 2005). There are two SIN3 proteins, SIN3A and SIN3B (Bourges et al., 2000). Complex specificity is dependent on the ability of mammalian and yeast SIN3 to interact with sequence-specific transcriptional factors through its many protein-protein interaction domains. Among these yeast and mammalian factors that interact with SIN3 are Pf1, TIEG2, Opi1, Mad, Ume6 (Tannenberg et al., 2005). Moreover, SIN3A has been shown to associate with unliganded nuclear receptors by interaction with the co-repressor SMRT and N-CoR to repress basal transcriptional activity (Nagy et al., 1997). Additionally, SIN3 has been associated with N-acetylglucosamine transferase activity, DNA and histone methylation, and nucleosome remodeling (Tannenberg et al., 2005).

Other complex members serve important and distinct functions in the assembly of the complex and regulation of transcription. HDAC1 and HDAC2 both belong to the family of class I HDACs (HDAC 1, 2, 3, and 8) and are 82% identical (Taunton et al., 1996; Yang et al., 1996). As implied by the name, these enzymes deacetylate histones and other proteins. SAP30 confers specificity to the complex by interacting with other proteins (Sichtig et al., 2007). SAP18 has been shown to act as an adaptor molecule

(Sheeba et al., 2007). The highly homologous RBBP7 and RBBP4 are histone binding proteins that are necessary for the interaction between the nucleosome and the SIN3-HDAC complex. The roles for BRMS1, SUDS3, and ARID4A have yet to be fully identified. However, as mentioned, the yeast ortholog of SUDS3 serves to stabilize the complex (Lechner et al., 2000) and ARID4A has been shown to bind the retinoblastoma protein (Lai et al., 2001).

The role of SIN3-HDAC complexes and of the individual members that comprise these complexes in cancer and, more specifically, in cancer metastasis is still unclear. SIN3 association with the Max/Mad/Mxi family of transcription factors counteracts Myc-mediated cell transformation by binding to Myc/Mac consensus sites suggesting a role for the SIN3-HDAC complex in the halt of tumor progression (Bourges et al., 2000). A SIN3-HDAC complex has also been shown to interact with the tumor suppressor p33 (ING1) (Kuzmichev et al., 2002). Expression of RBBP7 has been linked with both tumor suppression and tumor promotion in human breast cancer (Zhang et al., 2003; Li et al., 2003). On the other hand, over-expression of HDAC1 has been linked to cancer progression (Kawai et al., 2003). The individual roles of these proteins in cancer progression further highlights the need to understand how complex composition affect function and how these proteins are coming together to affect complex composition.

## Summary

Since SUDS3 and BRMS1 are family members involved in the same chromatin remodeling complexes, we hypothesized that SUDS3 could compensate for BRMS1 loss

in metastastic breast cancer cells lines to inhibit metastasis or that SUDS3 might mediate key BRMS1 functions through transcriptional regulation via this complex. Thus, this work explores the role of SUDS3 in metastasis suppression.

I first tested whether or not over-expression of SUDS3 could compensate for BRMS1 deficiency/non-expression to suppress metastasis and mimic BRMS1-mediated regulation of metastasis-promoting proteins (Chapter 3). To begin to address the role of the interaction of these two proteins, I also attempted to map BRMS1-SUDS3 interaction using a systematic mutagenesis approach (Chapter 4).

# METHODS

## Materials and Methods

### *Cell Lines and Cell Culture*

MDA-MB-231 and MDA-MB-435 are human estrogen receptor- and progesterone receptor-negative cell lines derived from metastatic infiltrating ductal breast carcinomas (Natali et al., 1990; Cailleau et al., 1978). While there is some controversy as to the origin of MDA-MB-435 (Sellappan et al., 2004; Rae et al., 2006), the findings presented here are not dependent upon cellular origin. MDA-MB-436 and MDA-MB-468 cells are human mammary adenocarcinoma cells. These cells were cultured in a mixture (1:1, v/v) of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) with 5% fetal bovine serum, 2mM L-glutamine (Invitrogen, Carlsbad, CA), and 0.02 mM non-essential amino acids (Mediatech, Herndon, VA) without antibiotics or antimycotics. Cells were grown on 100-mm tissue culture dishes (Corning, Corning, NY) at 37°C with 5% $CO_2$ in a humidified atmosphere. Cultures were passaged upon reaching 80-90% confluency using a solution of 0.05% Trypsin/EDTA (Invitrogen) and were confirmed negative for *Mycoplasma spp*. infection using a PCR-based test (TaKaRa, Shiga, Japan).

MCF7 cells are tumorigenic human mammary cells. These cells were cultured in minimal essential medium (MEM) with L-glutamine and Earle's salts supplemented with 10% fetal bovine serum (Invitrogen), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate (Mediatech), and 10 mg/mL insulin (Sigma-Aldrich, St. Louis, MO).

The MCF10A cell line and those cells derived from it model cancer progression and originated from benign fibrocystic breast tissue (Soule et al., 1990). They include MCF10A (immortalized but nontumorigenic epithelial cells), MCF10AT (ras-expressing, premalignant, mildly tumorigenic epithelial cells), and MCF10CA1a.1 and MCF10CA1d.1α (malignant epithelial cells that form invasive orthotopic tumors that metastasize to lung and regional lymph nodes) (Pauley et al., 1993; Santner et al., 2001; Miller et al., 2000). These cell lines were cultured as described above with the substitution of 5% horse serum for fetal bovine serum. MCF10A and MCF10AT growth medium was supplemented with 10 ng/mL EGF, 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin (Sigma-Aldrich).

Stably transfected cells lines were selected using 500 μg/mL active G418 (Mediatech) and maintained in 100 μg/mL G418.

***Mammalian Constructs and Transfection***

pcDNA3.1-V5/His-SUDS3, pcDNA3.1-V5/His, and pcDNA3 (Invitrogen) plasmids were transfected into MDA-MB-435 and MDA-MB-231 cells using Lipofectamine 2000 (Invitrogen). Vector controls were kept as a mixed population while SUDS3 transfectants were single cell cloned and screened for expression of SUDS3 by immunoblotting.

***Antibodies, Co-Immunoprecipitation, and Immunoblots***

A polyclonal rabbit anti-SUDS3 antibody was previously described generated (Fleischer et al., 2003). The antibody was generated against a peptide corresponding to amino acids 83-328 of the SUDS3 protein. Other antibodies were purchased as indicated: mouse-anti-β-actin (A2228; Sigma-Aldrich), rabbit-anti-OPN (O7264; Sigma Aldrich), Akt-Ser473 (P4112, Sigma-Aldrich), Akt (P2482, Sigma-Aldrich), mouse-anti-V5 (R962-25; Invitrogen), anti-EGFR (2232; Cell Signaling Technology, Danvers, MA), mouse-mab-anti-GAPDH (ab9482; Abcam, Cambridge, MA), p65 (06-418, Upstate, Billerica, MA), anti-mouse secondary antibody conjugated to horseradish peroxidase (NXA931; Amersham-Pharmacia, Biotech, Buckinghamshire, UK), and anti-rabbit secondary antibody conjugated to horseradish peroxidase (NA934) (Amersham-Pharmacia).

For co-immunoprecipitation studies, cells were grown to 80-90% confluency and then lysed in native lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Igepal) supplemented with 1 μL/mL protease inhibitor cocktail (P8340; Sigma-Aldrich). Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). For each group, equal amounts of total protein were incubated with 0 or 5 μg of antibody for 2 hours at 4°C with shaking and then with 30 μL Protein A/G beads (Santa Cruz) at 4°C overnight with shaking.

Cells were grown to 80-90% confluency and then lysed in either RIPA Buffer (Millipore, Billerica, MA) or a 0.1% Triton X-100 lysis buffer as previously described (Phadke et al., 2008). Both lysis buffers were supplemented with 1 μL/mL protease

inhibitor cocktail. To evaluate OPN secretion, media were collected from cells that had been washed three times with ice-cold PBS and serum starved for 12 -24 hours in 5 mL of media/10-cm dish. Equal protein loading was determined by using the BCA assay for whole cell lysates or by cell count for media loading.

***In vitro Growth Assays***

Cells at 80–90% confluence were detached and seeded at a density of 50,000 cells per well in a 6-well tissue culture dish (Corning) in triplicate. The growth of cells was monitored for 13-15 days. Cell number and viability were determined using a hemacytometer.

***Anoikis Assay***

Cells at 70-90% were detached and seeded in triplicate at a density of 25,000 cells per well of a 24 well plate (Corning) in normal growth conditions or in wells coated with a solution of 120 mg/mL poly 2-hydroxyethyl methacrylate (Poly-Hema) (Sigma-Aldrich). Cells were grown in serum free media for 21 hours and then incubated with 100 uL CellTiter-Blue Cell Viability Assay Reagent (Promega, Madison, WI) for 3 hours. Cell fluorescence was read using the Cytofluor plate reader and software (Applied Biosystems, Foster City, CA) with an excitation of 580 nm and emission of 530 nm.

### *Wound Healing/Motility Assays*

Cells at 80–90% confluence were detached and seeded in triplicate at a density of 100,000 cells per well in a 6-well tissue culture dish. An octothorpe (#) was made using 10 uL pipette tips. Cells were kept in serum-free media to minimize effects of proliferation. Phase contrast photomicrographs were taken using a Nikon Eclipse inverted microscope (Nikon, Chiyoda-ku, Tokyo) equipped with the QICAM Mono capture device (Media Cybernetics, Inc., Bethesda, MD). Four images were taken, one from each intersection, at 0 and 8 hr for MDA-MB-231 cell lines. Initial and final distances were measured using QCapture Pro software (Media Cybernetics). Parent and vector control cell lines and SUDS3-transfected clones were compared using Dunn's comparison test. Calculations were performed using SigmaStat statistical analysis software (SPSS Inc., Chicago, IL). Statistical significance was defined as a probability $p \leq 0.05$.

### *Experimental Metastasis Assay*

Experimental metastasis assays were performed as previously described (Seraj et al., 2000; Samant et al., 2001a). Ten mice per experimental group were initially injected with 5 X $10^5$ cells. Animals were maintained under the guidelines of the National Institutes of Health and the University of Alabama at Birmingham. All protocols were approved by the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*. ANOVA calculations were performed using SigmaStat statistical analysis software (SPSS Inc., Chicago, IL). Statistical significance was defined as a probability $p \leq 0.05$.

***Treatments***

Cells were serum starved for 24 hours and treated with either PBS or epidermal growth factor (EGF) (50 ng/mL) for 15 minutes after which cell lysates were immediately collected.

***Yeast Two-hybrid Analysis***

*BRMS1* or *BRMS1* mutant cDNA and *SUDS3* were cloned in frame with the pENTR-SD/D-Topo vector (Invitrogen). Using the LR Clonase Enzyme (Invitrogen) as per manufacturer's instructions, recombination reactions were carried out to result in *BRMS1* and *BRMS1* mutants cloned in frame with the Gal4 DNA binding domain into the pDEST32 vector and *SUDS3* cDNA was cloned in frame with the Gal4 activation domain into the pDEST22 vector (Invitrogen). MAV203 cells (Invitrogen) were made competent, co-transformed with the combination of vectors and or/vector controls, and grown on SD/-Trp/-Leu plates at 30°C until colonies reached 1-3 mm in diameter. In this system, direct interaction resulted in transcription of β-galactosidase reporter. In order to assay β-galactosidase activity, representative colonies were streaked onto filter paper, incubated overnight, lysed in liquid $N_2$, and inverted onto another filter soaked with a solution composed of 100 µL 5-bromo-5-chloro-3-indolyl-β-D-galactosidase (X-gal) (Promega), 60 µL β-mercaptoethanol, and 10 mL Z buffer (8.5 g $Na_2HPO_4$, 4.8 g $NaH_2PO_4$, 0.75 g KCL, 0.12 g $MgSO_4$, in 1 L $ddH_2O$, adjusted to pH 7.0, and filter sterilized). β-galactosidase activity, as measured by conversion of the X-gal substrate, was observed by a blue color change over a twenty four hour period and visually measured against the

change seen in cells transformed with known interactors of varying interaction strengths.

# OVER-EXPRESSION OF THE BRMS1 FAMILY MEMBER SUDS3 DOES NOT SUPPRESS METASTASIS OF HUMAN CANCER CELLS

## Introduction

BRMS1 and SUDS3 are related members of SIN3-HDAC chromatin remodeling complexes. We hypothesized that they might have overlapping functions and that SUDS3 over-expression could compensate for BRMS1 deficiency. SUDS3 expression was ubiquitous in seven breast cell lines, regardless of metastatic potential. SUDS3 over-expression in BRMS1-non-expressing metastatic cells did not suppress metastasis, motility, osteopontin expression/secretion, EGF receptor expression, nor AKT phosphorylation, phenotypes associated with BRMS1-mediated metastasis suppression. This study demonstrates functional differences for BRMS1 family members and highlights how the composition of SIN3-HDAC (BRMS1/SUDS3) complexes uniquely affects protein expression and biological behaviors.

## Results

### *SUDS3 Expression Does Not Correlate With Metastatic Potential*

Previous studies have shown that BRMS1 re-expression in metastatic cells blocks metastasis without blocking orthotopic tumor growth (Hurst et al., 2008; Phadke et al., 2008; Seraj et al., 2000). Immunohistochemical analysis showed that loss of BRMS1 expression was correlated with reduced disease-free survival human breast cancers when

stratified by either loss of ER, PR, or HER2 over-expression (Hicks et al., 2006). Based primarily upon relatedness of BRMS1 and SUDS3, we hypothesized that SUDS3 shared metastasis suppressor function as well as other properties. Levels of SUDS3 were measured in multiple human breast cell lines using a polyclonal antibody generated specifically against SUDS3. SUDS3 (45 kDa) was present in all cell lines examined regardless of tumorigenicity or metastastic potential (Figure 2). In several, but not all, experiments a second band at 50 kDa was identified in the MCF10 series of cells but not in MDA-MB-231 or -435 cells (Figure 3). Although this band has yet to be characterized, the detection of this band with a SUDS3 specific antibody suggests that it is a modified version of SUDS3.

***Ectopic Expression of SUDS3 in MDA-MB-231 and MDA-MB-435 Cells Does Not Affect Proliferation***

To examine whether ectopic expression of SUDS3 affected cell growth or phenotypes associated with BRMS1 metastasis suppression, stable MDA-MB-231 and -435 breast cancer cell lines were generated to ectopically express a SUDS3-V5/His fusion protein. Several clones were isolated and SUDS3 expression was evaluated by immunoblot. Endogenous SUDS3 (45 kDa) and SUDS3-V5 (~ 50 kDa) were detected with anti-SUDS3 (Figure 4 A,B). The identity of the 50 kDa band was verified using an anti-V5 antibody (Figure 5). Ectopic SUDS3 expression did not affect *in vitro* growth rates or saturation densities (Figure 6 A,B). Similarly, there were no gross differences in morphology. Several clones of each cell line were selected to represent varying levels of

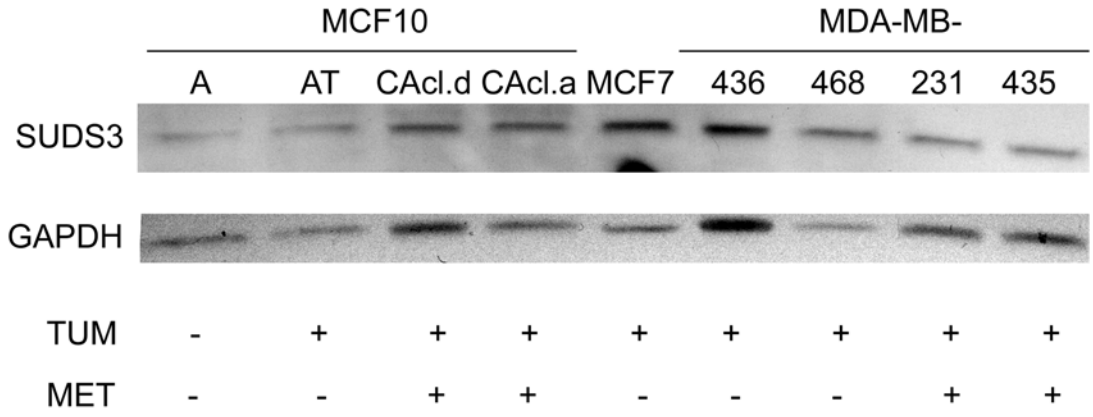



**Figure 2: SUDS3 Expression Does Not Correlate With Tumor Progression or Metastatic Potential**

A panel of human breast cell lines of varying tumorigenic (TUM) and metastastic (MET) potential in mouse models were probed with anti-SUDS3 antiserum. Endogenous SUDS3 was ubiquitously present. Levels varied from experiment to experiment, but did not correlate with metastatic potential. Blots were re-probed with GAPDH to verify equal loading.

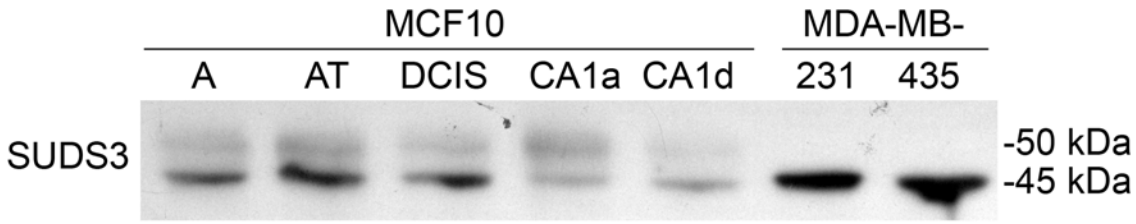



**Figure 3: SUDS3 Antibody Recognizes a Unique Band in MCF10 Series**

Probing whole cell lysate from several cell lines with the SUDS3 antibody showed two bands in the MCF10 series but only a single band in either the MDA-MB-231 or -435 cells. The unique band in the MCF10 series was detected at approximately 50 kDA.

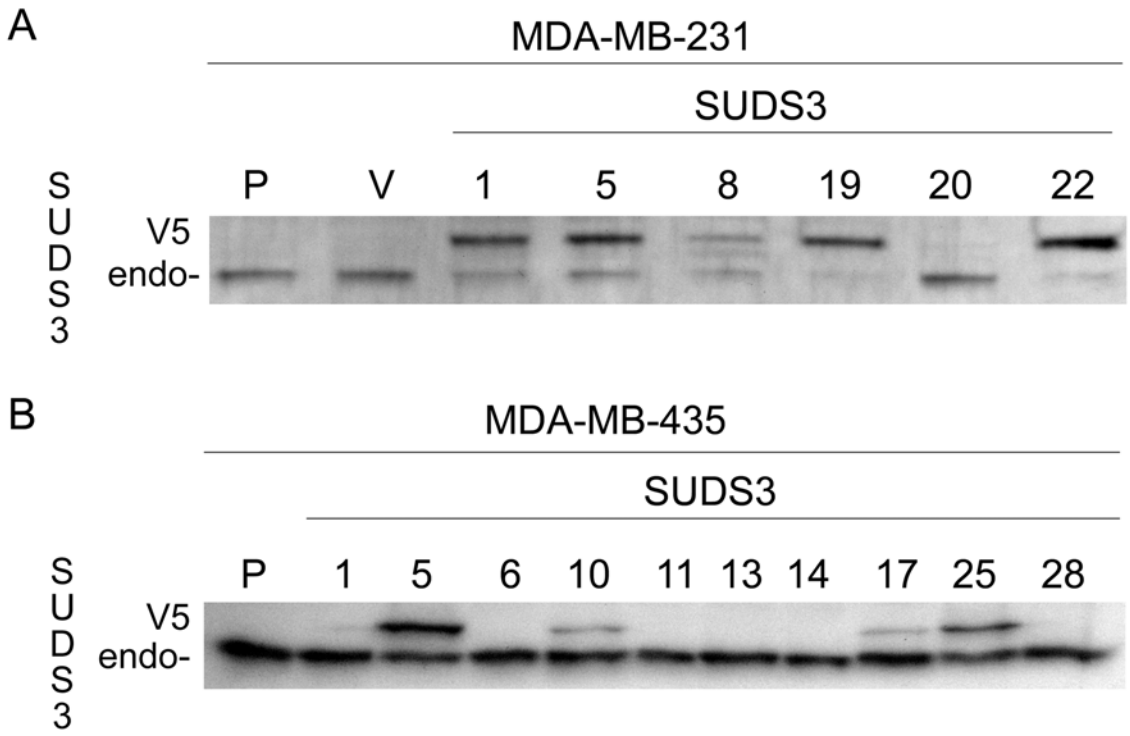



**Figure 4: Stable Expression of SUDS3 in MDA-MB-231 and MDA-MB-435 Cells**

MDA-MB-231 (A) and -435 (B) cells were stably transfected to express a SUDS3-V5 fusion protein. Single cells were isolated and from these clonal populations were derived and individual clones were assigned numbers. SUDS3 expression for individual clones was assessed using SUDS3 specific antiserum and showed an endogenous (endo-) 45 kDa band in all clones. A second band at 50 kDa corresponding to the fusion protein (V5) was also detected in some of the transfected cells.

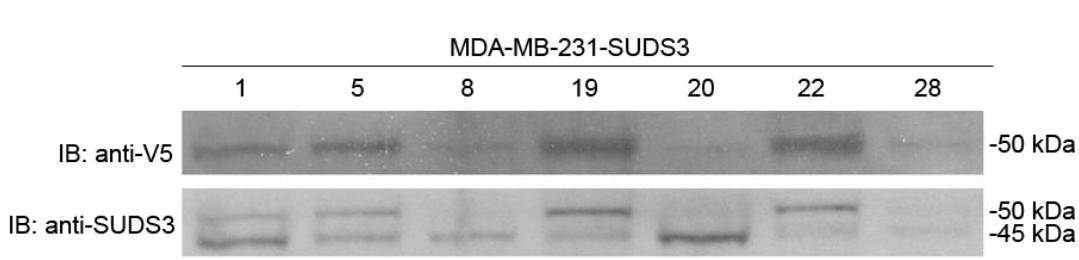



**Figure 5: Expression of SUDS3-V5/His Fusion Protein in MDA-MB-231-SUDS3 Stable Transfectants**

Expression of the SUDS3-V5/His fusion protein in MDA-MB-231-SUDS3 stable transfectants was verified using an anti-V5 antibody (IB: anti-V5) that detected a band at 50 kDa. The blot was stripped and reprobed with an anti-SUDS3 antibody (IB: anti-SUDS3) that similarly detected a 50 kDa band confirming expression of the fusion tagged protein.

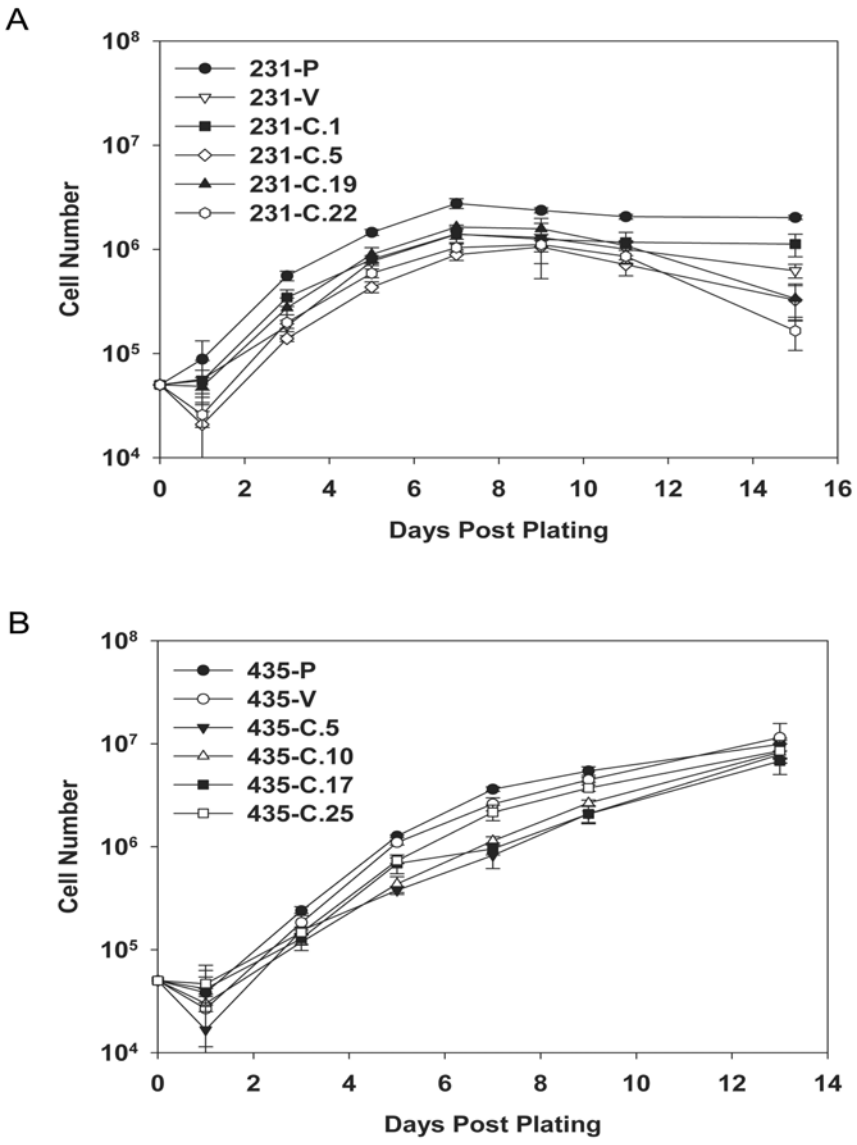



**Figure 6: Stable Transfection With SUDS3 Does Not Affect Growth Rates**

Cell lines were seeded at an initial density of 50,000 cells in triplicate. At each time point cells were detached and viable cells were counted with the aid of a hemacytometer to determine cell number. Ectopic over-expression of SUDS3 did not affect the *in vitro* growth rates of MDA-MB-231 cells (A) or -435 (B) cells when compared to parental and vector controls.

ectopic SUDS3 expression in MDA-MB-435 (clones 5, 10, 17, and 25) and -231 (clones 1, 5, and 22) cells.

***Ectopic Expression of SUDS3 Does Not Affect Anoikis of MDA-MB-231 Cells***

BRMS1 expression was found to significantly increase levels of anoikis *in vitro*. This might be (at least in part) responsible for the ability of BRMS1 to suppress metastasis. To investigate whether SUDS3 expression could similarly sensitize cells to anoikis, SUDS3 transfectants were grown on poly-hema, a substrate to which they cannot attach. Both groups of cells were incubated with a dye is converted in viable cells to fluoresce. Cellular viability of non-adherent cells was measured and compared to control cells plated under normal growth conditions. There was no significant difference in the percentage of non-viable cells between groups demonstrating that SUDS3 does not affect anoikis (Figure 7).

***Ectopic Expression of SUDS3 Does Not Affect Motility of MDA-MB-231 Cells***

Motility is required for tumor cell invasion and metastasis. $231^{BRMS1}$ cells showed a modest, but significant inhibition (~60%) of motility as measured using an *in vitro* wound healing assay (Samant et al., 2000a). To determine whether ectopic expression of SUDS3 affected *in vitro* motility in MDA-MB-231 cells, a similar *in vitro* wounding/motility assay was performed. SUDS3 did not alter motility compared to parental or vector controls (Figure 8).

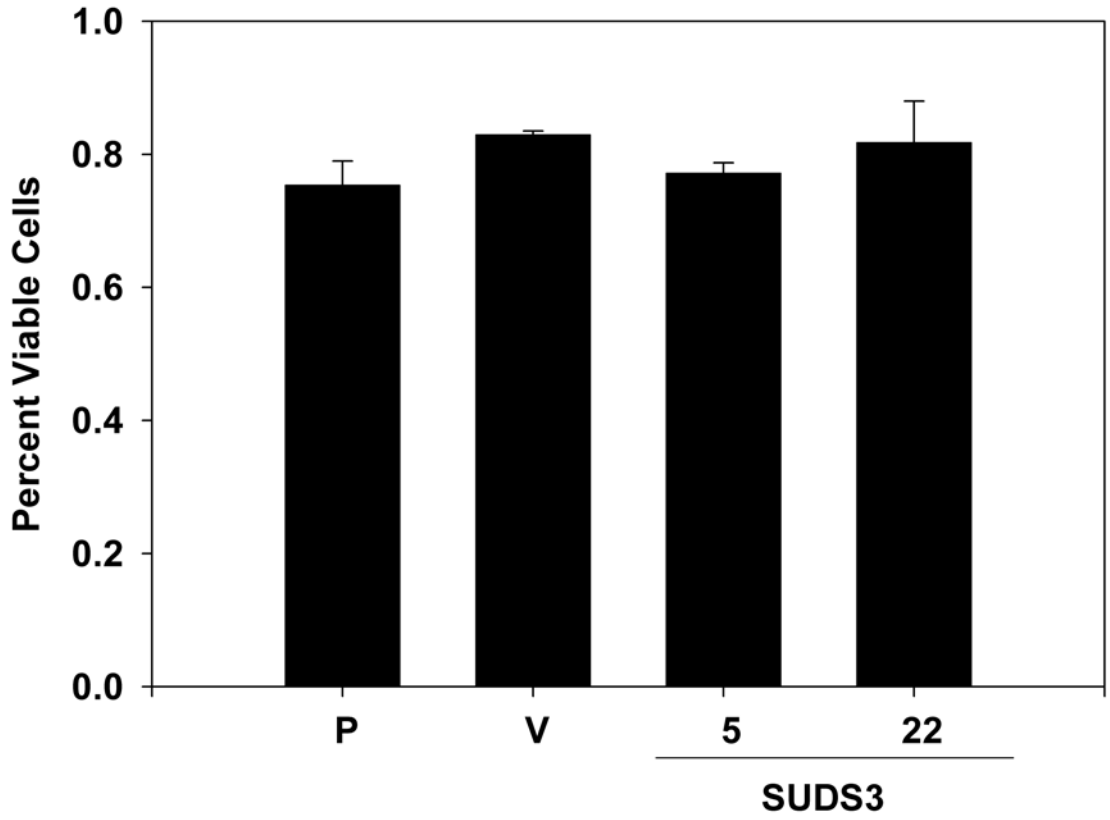



**Figure 7: SUDS3 Does Not Alter Anoikis**

MDA-MB-231 cells were grown up in either adherent or non-adherent (poly-hema coated wells) conditions in serum free medium and incubated with a redox dye that fluoresces upon conversion in viable cells. Fluorescence was measured in arbitrary units and the ration of non-adherent to adherent cells was used to calculate the percentage of viable cells. All cells lines responded similarly to serum starvation and lack of adhesion irrespective of SUDS3 expression.

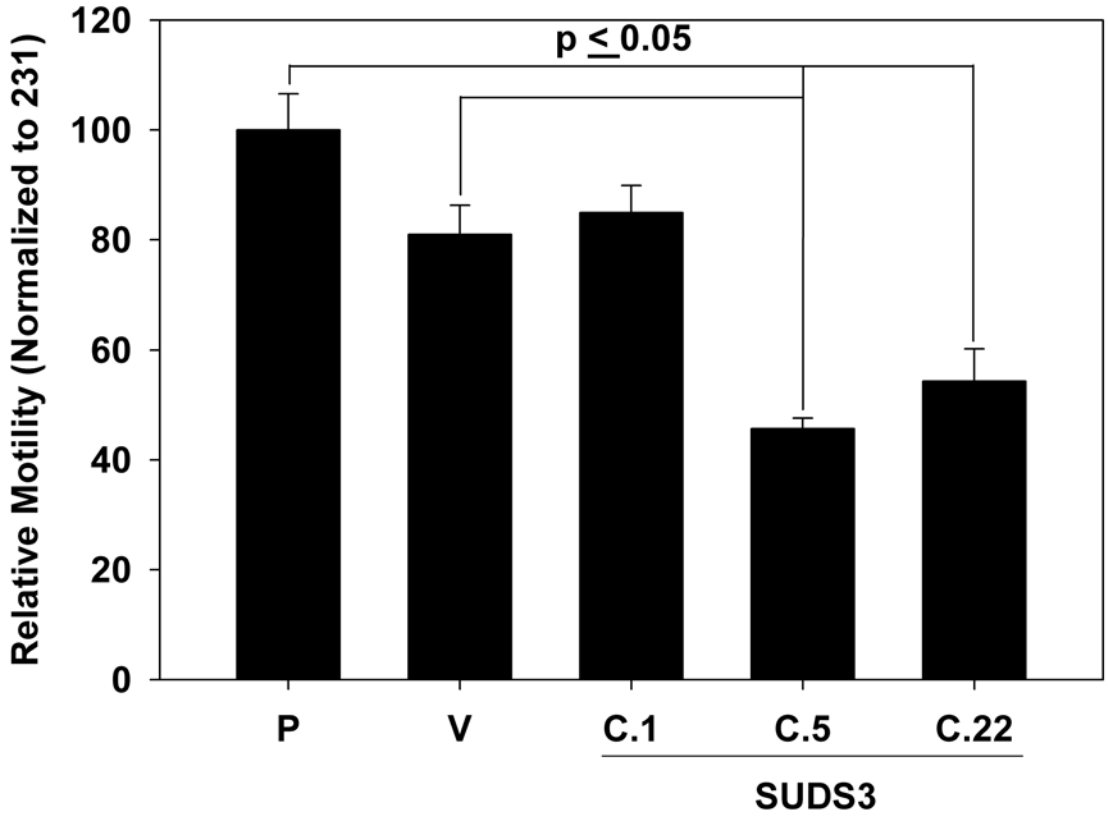



**Figure 8: Ectopic Expression of SUDS3 Does Not Affect Motility of MDA-MB-231 Cells**

Motility was measured using an in vitro scratch/wound healing assay. Confluent MDA-MB-231 monolayers were scratched and distances from edge to edge were measured at 0 and 8 hr. Relative motility is normalized to parental MDA-MB-231 (P) cells. Vectore control cells (V) and three selected 231 suds3 clones are shown. Data arecumulative for 3 independent experimetns with replicate wells. Differences among groups were determined using Holms-Sidak statistical analysis and represented as $p \leq 0.05$.

.

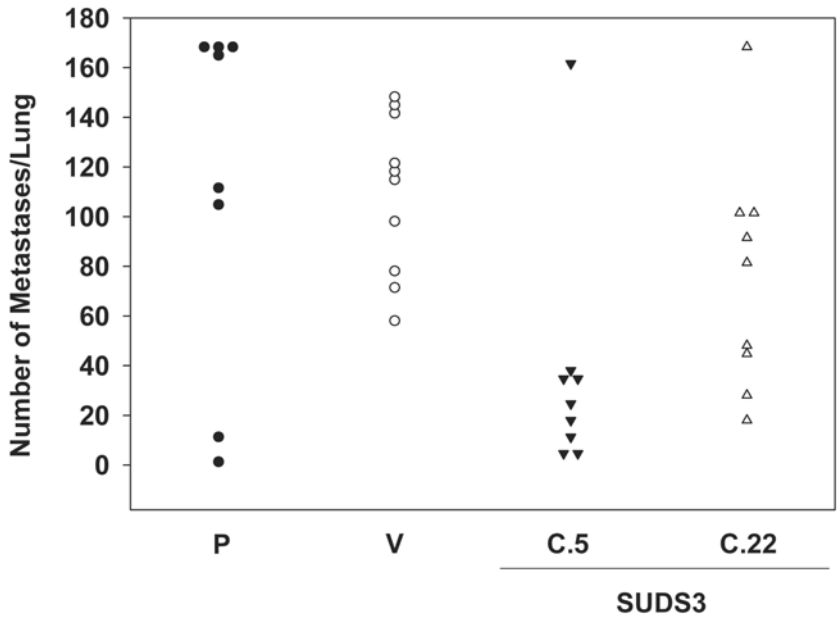
### *Ectopic Expression of SUDS3 Does Not Consistently Suppress Metastasis of MDA-MB-231 Cells*

To examine whether over-expression of SUDS3 could suppress metastasis, representative $231^{SUDS3}$ cells were injected into the lateral tail vein of athymic mice. Formation of macroscopic lung metastases was assessed as described previously (Hurst et al., 2008; Phadke et al., 2008; Seraj et al., 2000). $231^{SUDS3}$ clones 5 and 22 produced an average of 37 and 76 lung colonies/lung compared to 70-109 lung colonies in parental and vector controls (Figure 9 A,B). The size of the lung colonies were approximately equal for all of the cells. Although not exhaustive, the equivalence of clone 22 to parental and vector cells demonstrates that in this model over-expression of SUDS3 does not suppress metastasis.

### *Ectopic Expression of SUDS3 Does Not Reduce OPN or EGFR*

OPN is a secreted glycoprotein that can bind cell surface receptors to promote cell adhesion and migration. High expression of OPN generally correlates with aggressive tumor cell behavior and poor prognosis (Tuck et al., 2007; Denhardt and Guo, 1993). There exist various forms of endogenous osteopontin due to differential RNA splicing, protein modification, and susceptibility to proteases (Denhardt and Guo, 1993)}. Ectopic expression of BRMS1 in MDA-MB-435 cells decreased OPN mRNA and protein by 90-95% (Samant et al., 2007; Hurst et al., 2008; Hedley et al., 2008). OPN down-regulation is crucial to BRMS1 metastasis suppression since restoration of OPN in $435^{BRMS1}$ cells resulted in increased incidence of spontaneous metastasis to lymph nodes and lungs

A




B

| Cell Line | Lung Metastases | | |
|---|---|---|---|
| | Incidence | Mean ±SEM | p<0.05 |
| MDA-MB-231 | 8/8 | 70 ± 24 | |
| $231^{vector}$ | 10/10 | 109 ± 31 | |
| $231^{SUDS3}$-C.5 | 9/9 | 37 ± 48 | ** |
| $231^{SUDS3}$-C.22 | 9/9 | 76 ± 47 | |

**Figure 9: SUDS3 Does Not Suppress Metastasis in MDA-MB-231 Cells**

Lung colonization of MDA-MB-231 (P), vector control (V), and $231^{SUDS3}$ transfectants (C.5 and C.22) was measured (B) following i.v. of 5 X $10^5$ cells into athymic mice. Each symbol represents the number of surface lung metastases (maximum counted = 170) per mouse. Summary statistics of experimental metastasis for data represented in panel B (C). One-way analysis of variance with Dunn's post-test was used to determine differences among groups (** = $p<0.05$).

(Hedley et al., 2008).

To assess whether OPN was similarly affected by SUDS3, $435^{SUDS3}$ cell-conditioned media and whole cell lysate was collected from serum-starved cells. Immunoblots with anti-OPN identified a single band at 50 kDa in the whole cell lysate and two bands between 40 and 70 kDa in conditioned media as previously described (Hedley et al., 2008). Ectopic expression of SUDS3 did not decrease either intracellular or secreted levels of OPN (Figure 10).

The EGF receptor tyrosine kinase activates a variety of signaling transduction pathways that affect cell proliferation, differentiation, adhesion, migration, and apoptosis. EGFR is expressed in patients with breast cancer and activated EGFR is often associated with poor patient survival in invasive breast cancer (Ayer et al., 1995). BRMS1 significantly decreased EGFR in MDA-MB-231 and -435 cells, (50-100%) respectively (Vaidya et al., 2008).

Ectopic over-expression of SUDS3, however, did not change EGFR when comparing parental, vector, and SUDS3 overexpressing MDA-MB-231 and -435 cells (Figure 11).

***Ectopic Expression of SUDS3 Does Not Affect Phosphorylation of Akt***

BRMS1 expression regulates the levels of various proteins and molecules involved in signaling through the PI3K-Akt pathway: BRMS1 down-regulates EGFR expression (Vaidya et al., 2008), decreases levels of phosphatidylinositol-(4,5)-bisphosphate (DeWald et al., 2005), and down-regulates OPN (Samant et al., 2007; Hurst et al., 2008;

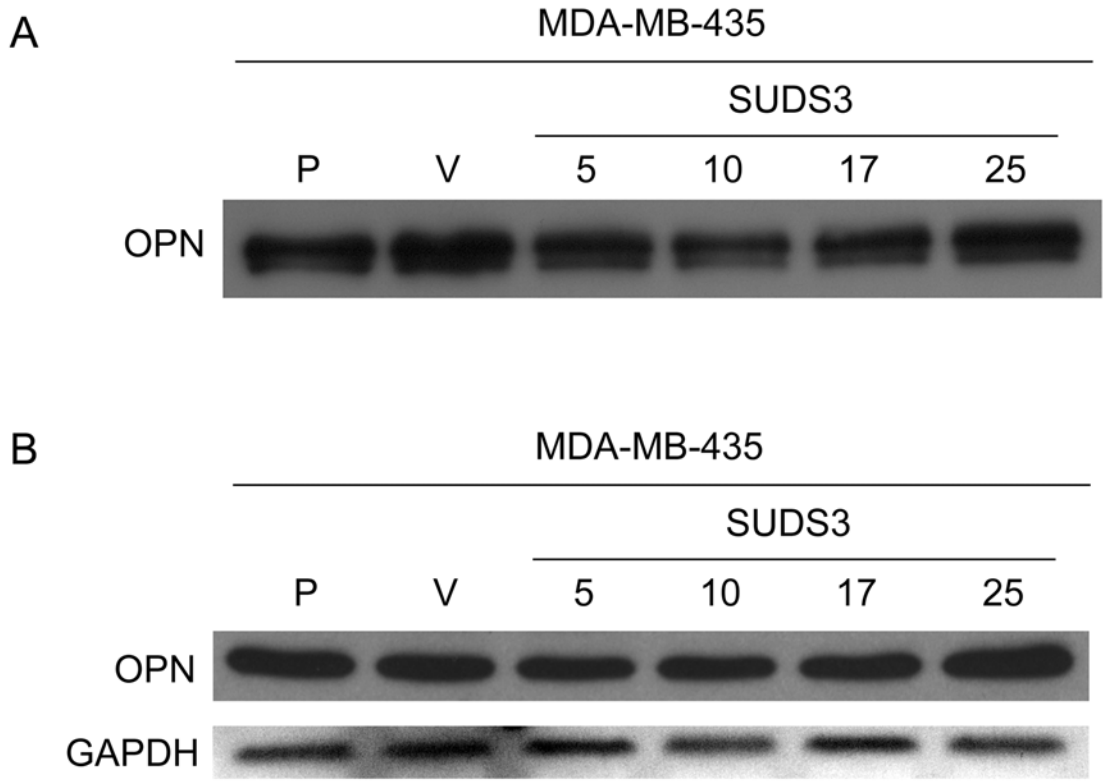



**Figure 10: SUDS Does Not Affect Levels of Secreted or Intracellular OPN**.

An anti-OPN antibody was used to detect levels of OPN in the secreted media (A) and whole cell lysate (B) of SUDS3 transfected MDA-MB-435 cells as compared to parental and vector controls. All cell lines displayed similar levels of both secreted and intracellular OPN. GAPDH was evaluated as a control for equal loading (B), while secreted OPN measurements were normalized by cell number (A).

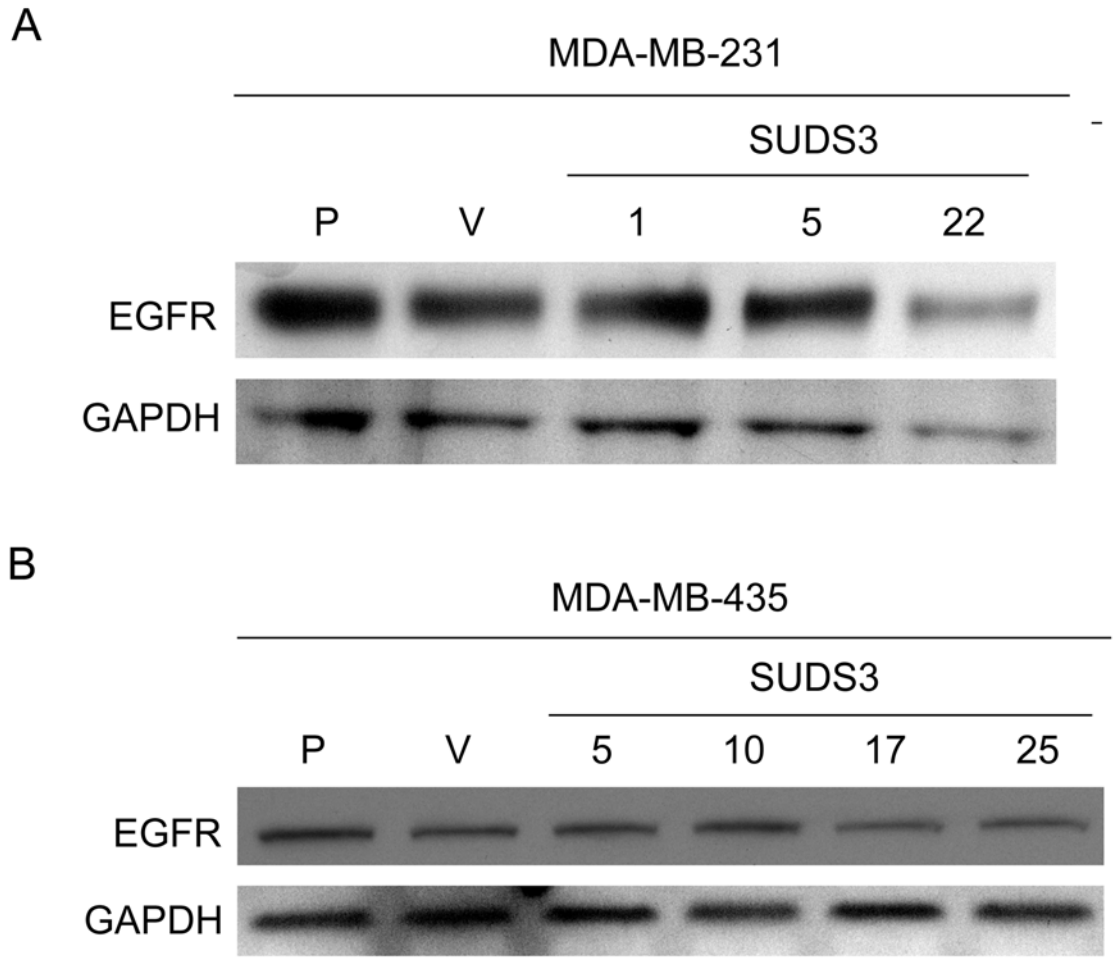



**Figure 11: SUDS3 Does Not Change Levels of EGFR**

An anti-EGFR antibody was used to detect levels of EGFR in SUDS3 transfected MDA-MB-231 (A) and -435 (B) cells as compared to parental and vector controls. All cell lines displayed similar levels of EGFR. GAPDH was evaluated as a control for equal loading.

Hedley et al., 2008), a molecule whose signaling can transactivate EGFR. Further, these effects have downstream consequences leading to reduced phosphorylation, and thus activation, of Akt (Vaidya et al., 2008). To examine if ectopic expression of SUDS3 affected other molecules and phenotypes leading to down-regulation of this signaling cascade, levels of phosphorylated Akt were examined to find no consistent difference in levels of phosphorylated Akt in response to treatment with epidermal growth factor in the MDA-MB -231 cells (Figure 12).

### *BRMS1 and SUDS3 Do Not Co-Immunoprecipitate with p65 in MDA-MB-231 Cells*

BRMS1 has been shown to interact with p65 in NSCLC cells by both co-immunoprecipitation studies and in studies looking at direct interaction. This interaction has not been demonstrated to take place in other cells types, although BRMS1 has been shown to regulate the activity of NFκB via the regulation of IKK. Because BRMS1 and SUDS3 are contained within the same SIN3-HDAC complexes in breast cancer cells, we sought to determine if they were similarly involved in the same complex with p65. Preliminary data (n=1) shows that a p65 specific antibody was not able to co-immunoprecipitate BRMS1 nor SUDS3 (Figure 13).

## Summary

Immunoblotting of several breast cell lines shows that SUDS3 is present in all cell lines regardless of metastatic potential or expression of its family member, BRMS1. Identification of an MCF10 specific band at 50 kDa by a SUDS3 specific antibody


**Figure 12: SUDS3 Does Not Affect Levels of Phosphorylated Akt**

Serum starved MDA-MB-231 cells were treated with either PBS (P) or 50 ng/mL EGF (E) for 15 minutes. Cell lysate was immunoblotted using anti-p-Akt, anti-total Akt, and anti-SUDS3 antibodies to show that levels of phosphorylated (p-Akt) and total Akt (t-Akt) were similar for all MDA-MB-231-SUDS3 transfectants and parental and vector cells with the exception of C.5 where levels of p-Akt were noticeably reduced.

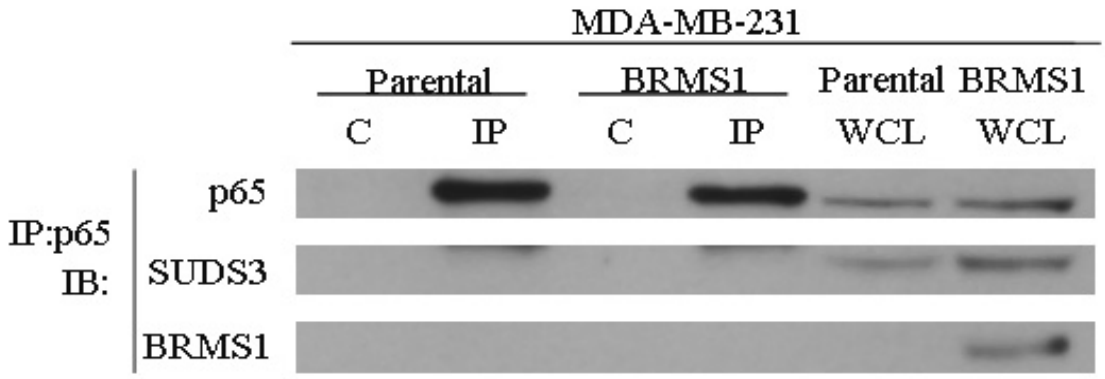



**Figure 13: BRMS1 and SUDS3 Do Not Co-Immunoprecipitate With p65 in MDA-MB-231 Cells**

Cells were lysed in native lysis buffer and immunoprecipitated with an antibody against p65. Western blot analysis showed p65 to be specifically immunoprecipitated (IP) when compared to beads alone (C). Whole cell lysate (WCL) input for the co-immunoprecipitation studies was also blotted to ensure presence of proteins of interest. The antibody against p65 did not co-immunoprecipitate SUDS3 or BRMS1.

suggests that a modified form or splice variant of SUDS3 may exist in the MCF10 series of cell lines but not in either MDA-MB-231 or -435 cells.

Further, these studies demonstrate that over-expression of SUDS3 cannot compensate for BRMS1 deficiency in the phenotypes tested for MDA-MB-231 and -435 cells. Specifically, we found that in MDA-MB-231 cells, over-expression of SUDS3 could not compensate for BRMS1 to suppress metastasis, decrease motility, decrease expression of EGFR, or down-regulate the phosphorylation of Akt. Similarly, in MDA-MB-435 cells, over-expression of SUDS3 could not compensate for BRMS1 to decrease levels of intracellular and secreted OPN nor to decrease expression of EGFR. Taken together, these studies suggest that BRMS1 and SUDS3 have distinct, non-overlapping functions in the cell lines examined.

## THE FIRST COILED-COIL REGION OF BRMS1 IS NECESSARY BUT NOT SUFFICIENT FOR BRMS1-SUDS3 INTERACTION

### Introduction

BRMS1 has been shown to directly bind the proteins ARID4A and SUDS3 by yeast two-hybrid studies (Meehan et al., 2004; Hurst et al., 2008). Moreover, all of these proteins have been shown to be a part of SIN3-HDAC complexes. The specificity and function of these repressor complexes are determined by the protein composition of the complexes which is, in turn, determined by the interactions of these proteins (Dannenberg et al., 2005). It is therefore important to map sites of interaction in order to determine how the complex is organized around individual proteins and moreover, how proteins interact to form these complexes.

Sequence analysis has shown that BRMS1 contains several domains that regulate protein-protein interaction including two coiled-coil domains and several imperfect leucine zippers. Coiled-coil domains are stable protein structures resulting from the interaction of hydrophobic side chains of amino acids that are organized in alpha-helices. These domains are well-characterized as regions of protein-protein interaction. Sequence analysis of BRMS1 predicts two coiled-coil domains, amino acids 51-81 and 147-180. As previously published (Hurst et al., 2008), BRMS1 mutants were generated to analyze the role of these domains in the interaction of BRMS1 with ARID4A. It was found that the second coiled-coil domain is both necessary and sufficient for BRMS1-ARID4A

interaction, and further, that mutation of leucine 174 to an aspartic acid ($BRMS1^{L174D}$) abrogates the interaction of BRMS1 and ARID4A in a yeast two-hybrid screen. Co-immunoprecipitation of $BRMS1^{L174D}$ in mammalian cells showed that it could still interact with ARID4A and, moreover, that it could still suppress metastasis and decrease levels of EGFR and OPN.

Similarly, in order to characterize the domains of BRMS1 necessary for SUDS3 interaction, previously described BRMS1 mutants (Hurst et al., 2008) were screened for interaction with SUDS3 in yeast two-hybrid studies. It was found that deletion of the first coiled-coil region disrupted interaction with SUDS3, therefore further mutations were made within this and surrounding domains in order to characterize the nature of the protein-protein interaction. These studies led to the conclusions that the imperfect leucine zipper contained within the first coiled-coil region does not mediate BRMS1-SUDS3 interaction and further, that while the first coiled-coil region is necessary, it is not sufficient for interaction.

## Results

### *Deletion of Either Coiled-coil Domain of BRMS1 Disrupts Interaction With SUDS3*

To determine whether these coiled-coil regions are involved in the interaction between BRMS1 and SUDS3, a yeast two-hybrid assay was performed using the mutants previously described. Interaction was assayed by comparing intensity of color change to

BRMS1-SUDS3 control (Figure 14, left panel). A schematic representing the results of the yeast two-hybrid (Figure 14, right panel) demonstrates that deletion of either domain disrupts interaction with SUDS3; however, SUDS3 can still interact with BRMS1$^{L174D}$, a point mutant that disrupted interaction with ARID4A.

### *Deletions Within the First Coiled-coil Domain of BRMS1 Disrupt Interaction with SUDS3*

Deletion of the first coiled-coil region disrupted the interaction of BRMS1 with SUDS3 but not with ARID4A (Hurst et al., 2008); therefore, our studies focused on the first coiled-coil region in order to distinguish the role of these interactions. Amino acids 61-92 were deleted in the BRMS1$^{\Delta CC1}$ mutant, a region containing both the majority of the first coiled-coil region of BRMS1, and further, a portion of the Sds3-like domain that does not overlap with the first coiled-coil region of this protein. To address which amino acids were involved in mediating the protein-protein interaction, BRMS1 mutants were generated to span this region. Small, five amino acid mutations were made in preparation for studies to follow where disruption would be focused on the mutation of a single amino acid. The following deletions were made: Δ61-65, Δ66-70, Δ71-75, Δ76-80. Δ81-85, Δ86-88, Δ89-92. With the exception of Δ66-70 and Δ71-75, mutation of these small regions disrupted BRMS1 interaction with SUDS3 in yeast two-hybrid (Figure 15).

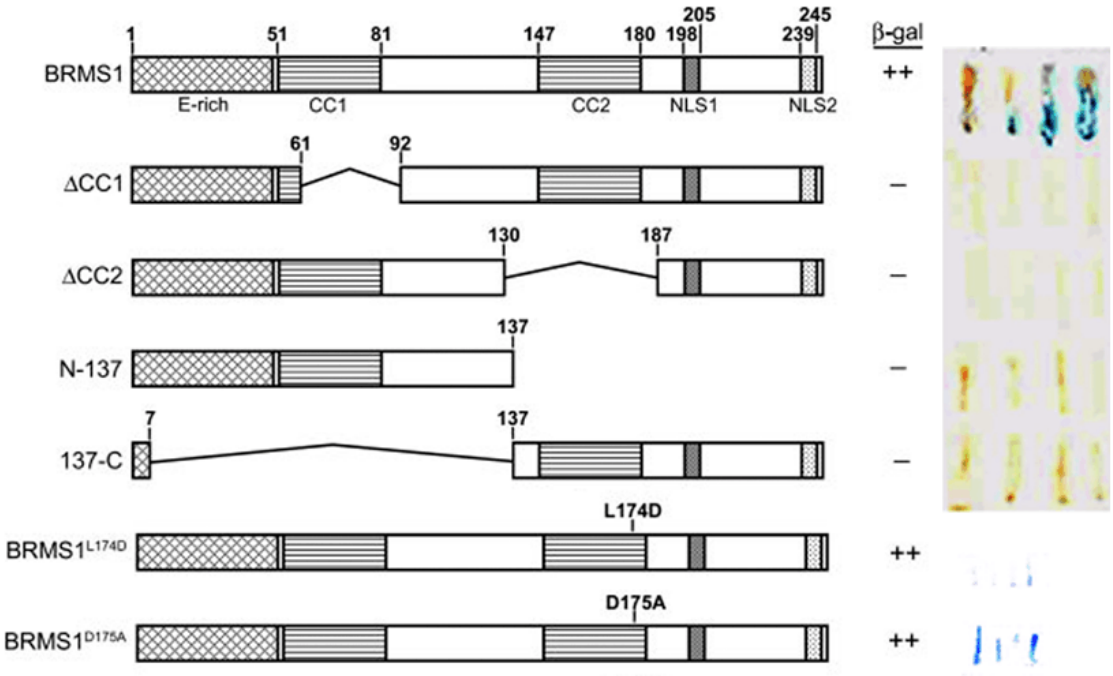



**Figure 14: Deletion of Either Coiled-Coil Region Disrupts the Interaction of BRMS1 with SUDS3 in Yeast Two-Hybrid**

Several regions of BRMS1 were deleted to generate mutants that either lacked coiled-coil domain 1 (CC1), coiled-coil domain 2 (CC2), or either the N-terminus (N-137) or C-terminus (137-C) of the protein. These mutants were fused to the GAL4-DNA binding domain and transfected with a SUDS3-GAL4-AD fusion protein in yeast to test interaction. Deletion of these regions resulted in disrupted interaction as assayed by a lack of color change as compared to control BRMS1. Point mutation of BRMS1 at L174D or D175A did not disrupt interaction of the two proteins. Original figure adapted from Hurst, et. al., 2008.

### *Mutation of Imperfect Leucine-zipper Within the First Coiled-Coil Domain of BRMS1 Does Not Disrupt Interaction With SUDS3*

Leucine zipper motifs are formed by the interaction of two alpha-helices, via the hydrophobic side chains of leucine residues, to form short coiled-coil domains. The common leucine zipper motif is characterized by repeated leucine residues separated by six amino acid spacers. These motifs are involved in protein-protein interaction, specifically homodimerization, and DNA binding. Sequence analysis of BRMS1 showed several imperfect leucine zipper motifs at amino acids 67-88, 131-152, 138-159, 153-174 (Seraj et al., 2000). The motif at amino acids 67-88 coincides with the region deleted in BRMS1 $^{\Delta CC1}$ , the deletion of which resulted in disrupted interaction with SUDS3. In order to address the role of this leucine zipper in mediating BRMS1-SUDS3 interaction, several conservative single amino acid substitutions were made within these regions to create BRMS1 mutants. Additionally, several amino acids were substituted with an alanine within regions where deletion of five or less amino acids resulted in disruption of the BRMS1-SUDS3 interaction in order to assess whether a single amino acid substitution within this region could disrupt interaction of BRMS1 with SUDS3. The following amino acids were substituted with an alanine L78, L83, R89, L90, E91. All of the point mutants were still able to interact with SUDS3 in yeast two-hybrid genetic screens (Figure 16).

### *The First Coiled-coil Domain of BRMS1 is Not Sufficient for Interaction with SUDS3*

Point mutation of several hydrophobic amino acids in areas where deletion had disrupted interaction did not disrupt interaction. This suggested that perhaps a point

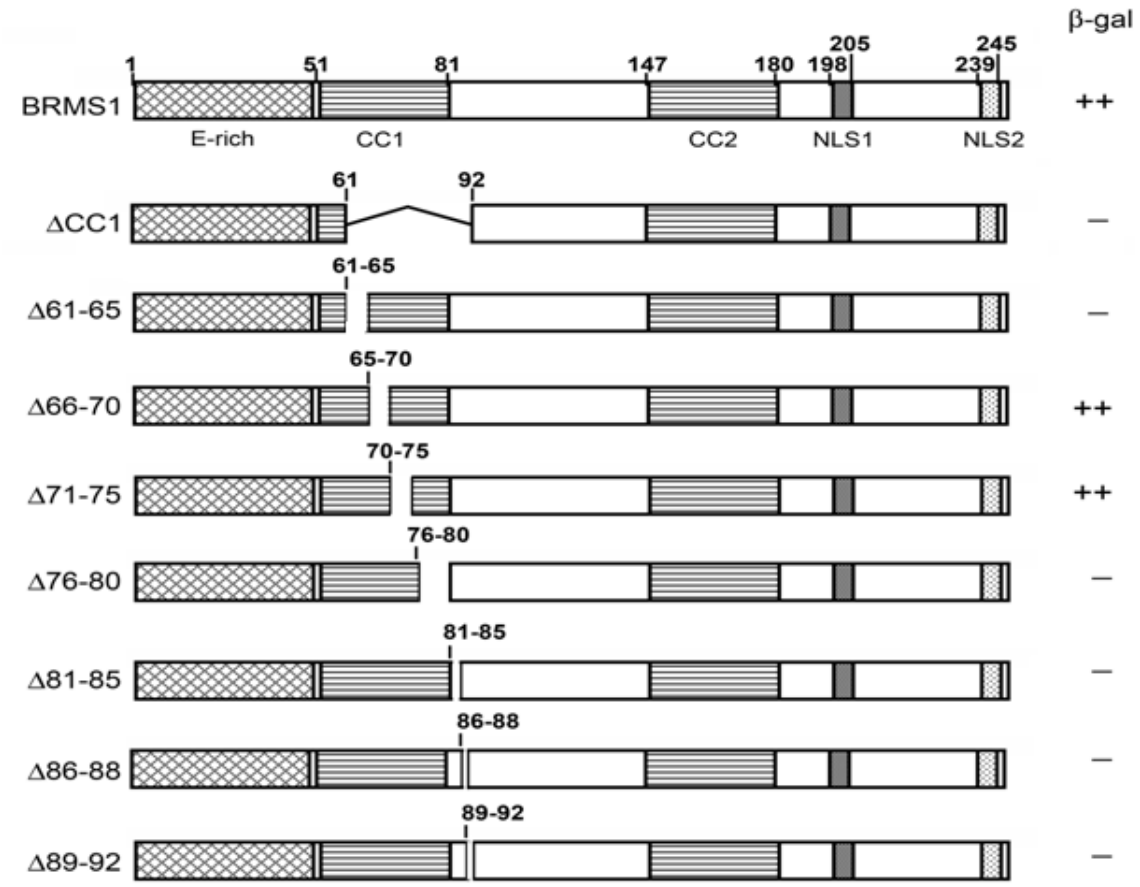



**Figure 15: Deletions Within the First Coiled-coil Region of BRMS1 Disrupts Interaction With SUDS3 in Yeast Two-Hybrid**

Deletions of 5 amino acids or less were made to span the region between amino acids 61-92, containing the first coiled-coil region of BRMS1. With the exception of Δ66-70 and Δ71-75, all mutations with this region disrupted interaction with SUDS3.

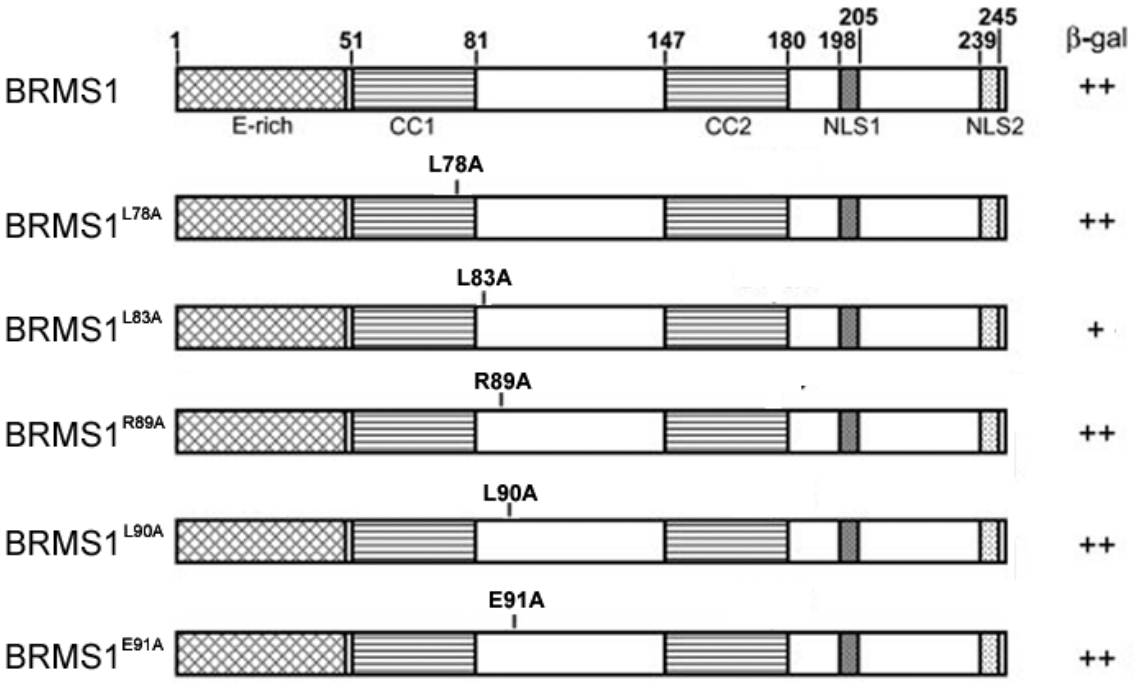



**Figure 16: Point Mutations Do Not Disrupt BRMS1-SUDS3 Interaction**

Several point mutations were generated within a region whose deletion disrupted BRMS1 interaction with SUDS3. Each of these mutants is represented by the amino acid mutated, the location of the residue within the protein, and the amino acid to which it was mutated, in all cases an alanine. In all cases, point mutation did not disrupt the interaction of BRMS1 with SUDS3 in yeast two-hybrid studies.

mutation was insufficient to alter interaction and led us to assess the overall importance of the various coiled-coil domains both alone and together. In order to assess the ability of these domains to mediated interactions, mutants were generated to contain the first seven amino acids of BRMS1 protein followed by either the first coiled-coil region (amino acids 51-81), the second coiled-coil region (amino acids 147-180), or a combination of both the first and second coiled-coil regions with the intervening sequence (amino acids 51-180), and transformed into yeast with either SUDS3 or a control plasmid (Figure 17). While the combination of the first and second coiled-coil domain was able to interact with SUDS3, the first coiled-coil domain alone was insufficient to interact with SUDS3. The second coiled-coil domain, fused with the Gal4 DNA binding domain, resulted in a self-activating mutant, where self-activation is the induction of the β-galactosidase gene without the binding to a second fusion protein containing an activation domain. Therefore, we could not assess the ability of the second coiled-coil domain to interact with SUDS3.

## Summary

These yeast two-hybrid studies demonstrate that both gross and more refined deletions of the first coiled-coil region and of a small adjacent region of BRMS1 can disrupt the interaction of BRMS1 with SUDS3 highlighting the importance of this domain for this protein-protein interaction. Further it was found that within this domain, point mutations resulting in the disruption of an imperfect leucine zipper did not affect the interaction of BRMS1 and SUDS3, thereby illustrating that this is not an important motif

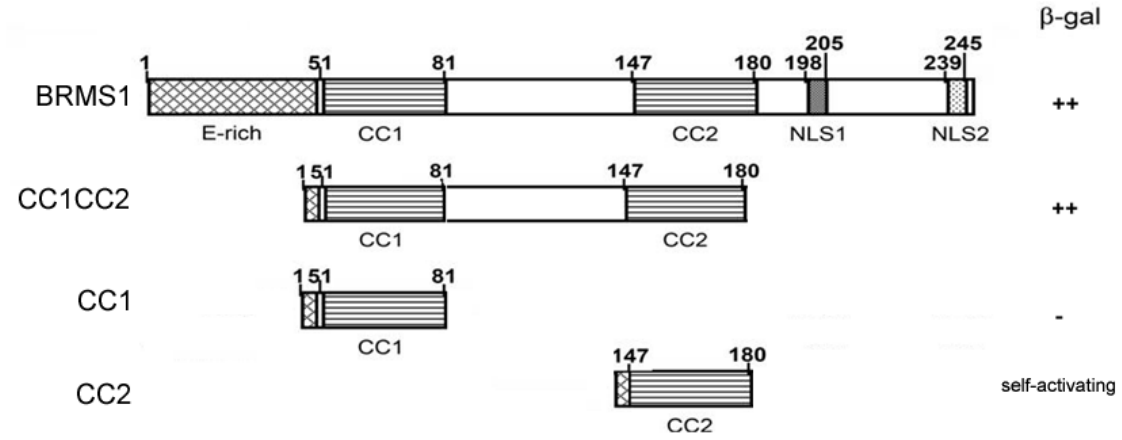



**Figure 17: CC1 Region of BRMS1 is Not Sufficient for Interaction With SUDS3**

Several BRMS1 mutants were generated to contain either the first and second coiled-coil regions (CC1CC2), the first coiled-coil region alone (CC1), or the second coiled-coil region alone (CC2). In yeast two-hybrid screens, like full-length BRMS1 CC1CC2 interacted with SUDS3 as measured by β-galactosidase activity. However, CC1 did not interact with SUDS3. The interaction of CC2 and SUDS3 could not be assessed in this model system as CC2 was shown to promote transcription of the β-galactosidase reporter in the presence of a control vector.

for interaction. Finally, studies using specific domains of BRMS1 showed that the first coiled-coil region of BRMS1 is necessary but not sufficient for interaction with SUDS3. These studies further define the regions of BRMS1 necessary for protein-protein interaction and distinguish areas that mediate specific interactions with SUDS3 and ARID4A.

## DISCUSSION

Functional and transcriptional compensation among protein families is a well-characterized phenomenon, as demonstrated by the Rb family of proteins (Donovan et al., 2006). The studies reported here were undertaken, in part, because of the strong homology between SUDS3 and BRMS1 and the involvement of both proteins in SIN3-HDAC chromatin remodeling complexes. These facts taken together suggested that SUDS3 and BRMS1 share some functional redundancy and that SUDS3 could compensate for BRMS1 deficiency. Not only did SUDS3 not suppress metastasis like BRMS1, but their apparent functions and/or regulation of chromatin remodeling-based transcriptional regulation differed as well. Nonetheless, this study clearly shows that BRMS1 and SUDS3, while sharing the Sds3-like domain and participating in similar (and sometimes even the same) SIN3-HDAC complexes, have distinct functions in the breast cancer cell lines examined.

Among the many proteins that comprise the SIN3-HDAC core complexes, there are several that have high sequence similarity for which both overlapping and distinct functions have already been, and continually are being, discovered. These proteins include SIN3A/B, HDAC1/2, ARID4A/B, and Rbbp4/7 (Silverstein and Ekwall, 2005). Therefore, it is possible that while BRMS1 and SUDS3 function did not overlap for the phenotypes examined, there may be other characteristics for which both proteins overlap.

While SUDS3 is necessary for normal somatic cell survival (David et al., 2003), there have been no reports to date on levels of endogenous SUDS3 in breast cell lines.

SUDS3 is expressed in all of the breast cell lines examined. In this study SUDS3 expression did not appear to change to compensate for loss of BRMS1 expression in the MDA-MB-231 and -435 cell lines. Interestingly, we frequently observed a second SUDS3 band in MCF10-derived cells. Thus, modified variants may exist within these cells, however, additional experimentation will be required in order to characterize this band. It is important to note that a post-translation modification for SUDS3 has indeed been identified; SUDS3 has been shown to be phosphorylated in brain tissue via interaction with the protein p35. However, p35 and phosphorylated SUDS3, have not been identified in breast tissues. Because SUDS3 acts to stabilize SIN3-HDAC complexes in yeast, and most likely in mammalian cells, its modification may be integral in the regulation and composition of these complexes and/or in other SUDS3 functions. If this second band is indeed verified to be SUDS3, the existence of an MCF10 specific variant might explain functional differences of BRMS1 in MCF10 and non-MCF10 cell lines. Further, this suggests that post-translational modifications of proteins and protein variants most likely play a key role in SIN3-HDAC complex composition and regulation.

It is not uncommon for protein isoforms and post-translational modifications to vary among cell lines and further, among cell types. Similarly, SIN-HDAC complex composition varies among cell types and cell lines. In fact, it is well known that these complexes play an important role in differentiation and maintenance of cell-type specific transcription. The cell type specificity of BRMS1 protein-protein interactions is demonstrated by the preliminary studies presented in this work that show that BRMS1 does not co-immunoprecipitate with p65 in MDA-MB-231 cells, unlike co-immunoprecipitation

studies done in 293T cells, nonmetastatic human embryonic kidney. A caveat to this study is that it was undertaken using non-stimulated MDA-MB-231 cells that, although containing an activated form of NFκB, might require additional stimulation in order to prompt p65-BRMS1 association. This adds to the complexity of defining the role of BRMS1 and SUDS as family members within these complexes, and moreover, in defining the function of these complexes.

To avoid the question of cell type specific complex formation and subsequent differences in functions, our studies focus on distinct roles for SUDS3 and BRMS1 within the same cell lines. Having discounted cell line variation, a possible explanation for the apparent differences between SUDS3 and BRMS1 is related to protein-protein interactions. Previous studies have begun to define the role of BRMS1 protein-protein interactions. In mammalian cells, deletion of the first coiled-coil region resulted in a mutant BRMS1 ($BRMS1^{\Delta CC1}$) that, while still able to interact with ARID4A, could not co-immunoprecipitate ARID4A in mammalian cells (Hurst et al., 2008). Further, this mutant was not able to decrease levels of phosphatidylinositol-(4,5)-bisphosphate or OPN, although it did decrease levels of EGFR. This suggests that this domain, individually or as a part of the larger Sds3-like domain, could mediate interaction with an additional protein(s) that in turn regulates the interaction of BRMS1 and ARID4A, and further, that regulates the ability of BRMS1 to mediate certain metastasis-associated phenotypes. The studies presented here show that deletion of this first coiled-coil domain disrupted BRMS1 interaction with SUDS3 in yeast two-hybrid studies. Since SUDS3 is known to play a role in maintaining SIN3-HDAC chromatin remodeling complexes, this suggests that SUDS3 is

a key mediator of BRMS1-ARID4A interaction, perhaps by serving a scaffolding or recruiting function. Additionally, this reinforces the idea that the protein composition of SIN3-HDAC regulates specificity and function. In order to address this question, further studies need to be done to determine if the direct interaction of BRMS1 and SUDS3 is disrupted by the deletion of this first coiled-coil region in mammalian cells.

More specifically, defining the means/domains by which these proteins interact is vital to understanding how these and perhaps other members of this protein family might interact or take part in SIN3-HDAC domains; the studies presented in this work began to address how these BRMS1 and SUDS3 interact through the mutation of BRMS1. The first coiled-coil domain was found to be necessary but not sufficient. Since mutation/deletion of a large portion of a protein might result in improper folding and disruption of the tertiary structure, we generated more specific mutations that in turn led to the discovery that at least one of the imperfect leucine zippers identified by sequence analysis of BRMS1 is not important for BRMS1-SUDS3 interaction.

Deletion of either coiled-coil domain disrupted interaction of BRMS1 and SUDS3, therefore, because both coiled-coil domains lie within the Sds-like domain, these studies further suggest that the interaction of these two proteins may be in part due to an interaction between their shared Sds3-like domains. It is interesting to note that a part of the originally defined Sds3-like domain has been shown in separate studies to be necessary for the homodimerization of murine SUDS3 (Alland et al., 2002). It is possible that other protein family members can directly interact with BRMS1 or SUDS3 via this domain.

Although the data presented here do not support our original hypothesis - that

SUDS3 is a metastasis suppressor - the collective findings further define the understanding of the protein family containing the Sds3-like domain by distinguishing functions for BRMS1 and SUDS3 and by suggesting that the Sds3-like domain mediates protein-protein interaction between family members. Further, by addressing how the members of this protein family interact, these studies also suggest means by which they might interact with the larger protein complexes as scaffolding partners.

Nonetheless, further studies are necessary to characterize the importance of the BRMS1-SUDS3 interaction for BRMS1-mediated transcriptional repression and moreover, in the larger context of metastasis suppression. The studies described in chapter 4 of this dissertation serve as a starting point for further mutation of BRMS1 in hopes of generating a point mutant that specifically disrupts the interaction of SUDS3 and BRMS1. Similarly, SUDS3 must be mutated to disrupt interaction with BRMS1. Studies done using yeast two-hybrid will be able to satisfy the question of whether or not a single point mutation can disrupt interaction of these two proteins. In the case that it cannot, then a mutant will be used that minimally disrupts protein folding while still disrupting BRMS1-SUDS3 interaction.

The study of mutated BRMS1 is facilitated through the use of the MDA-MB-231 and -435 cells lines which are BRMS1 deficient. Introduction of mutated BRMS1 into these cell lines allows us to use antibodies directed against BRMS1 to co-immunoprecipitate interactors and to determine how the SIN3-HDAC complex composition changes upon disruption of BRMS1-SUDS3. This further allows us to test if there are resultant functional differences.

Reciprocal studies done using a mutated SUDS3 are complicated by the fact that SUDS3 is present in all breast cell lines examined. A mutated and tagged version of SUDS3 may be co-immunoprecipitated to study how mutation disrupts formation of the complex. However, the effect of endogenous SUDS3 might interfere with functional analyses. Therefore, siRNA directed against SUDS3 must be used to look at function. These studies will be performed using the MDA-MB-231 and -435 cell lines in order to avoid the issue of cell line variation and the effect that it may have on the regulation of SIN3-HDAC complexes.

In addition to defining the role that SUDS3 plays in BRMS1 function, these studies will provide insights into how BRMS1 might play a role in SUDS3 functions, for example pericentric heterchromatin segregation. These studies may further lead to a better understanding of the role of this family of proteins in normal and pathological conditions.

In a broader context, these studies will aid in further defining the binding partners and functions of the myriad proteins in the larger SIN3-HDAC complexes. This in turn will contribute the understanding of how complex composition dictates specificity and function, and, moreover, what role these complexes play in BRMS1-mediated metastasis suppression and in the larger context of metastasis. The studies described in this work and the suggested future studies have the potential to not only increase our understanding of how metastasis is regulated and the role of SIN3-HDAC complexes in metastasis, but may also further lead to the development of targeted therapies that can be used to prevent and treat metastasis.

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# APPENDIX

## IACUC APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

*Institutional Animal Care and Use Committee (IACUC)*

**MEMORANDUM**

**DATE:** July 24, 2008

**TO:** Danny Welch, Ph.D.
VH, G038 0019
FAX: 975-1126

**FROM:** Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

**SUBJECT:** **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

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The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on July 24, 2008.

Title of Application: Molecular Regulation of Breast Cancer Metastasis
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

**Institutional Animal Care and Use Committee**
B10 Volker Hall
1670 University Boulevard
205.934.7692
FAX 205.934.1188

Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

*Institutional Animal Care and Use Committee (IACUC)*

**NOTICE OF APPROVAL**

**DATE:** July 24, 2008

**TO:** Danny Welch, Ph.D.
VH, G038 0019
FAX: 975-1126

**FROM:** Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

**SUBJECT:** Title: Molecular Regulation of Breast Cancer Metastasis
Sponsor: NIH
Animal Project Number: 080706777

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On July 24, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

| Species | Use Category | Number in Category |
|---|---|---|
| Mice | C | 5000 |

Animal use is scheduled for review one year from July 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

**Please keep this record for your files, and forward the attached letter to the appropriate granting agency.**

Refer to Animal Protocol Number (APN) 080706777 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

**Institutional Animal Care and Use Committee**
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